Patrizia Restani *Editor*

Food Supplements Containing Botanicals: Benefits, Side Effects and Regulatory Aspects

The Scientific Inheritance of the EU Project PlantLIBRA

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Introduction

Plant food supplements (or food supplements containing botanicals) have high acceptance by European consumers. Potentially, they can deliver significant health benefits, safely, and at relatively low costs. However, concerns about safety, quality, and efficacy of these products remain, and bottlenecks in risk and benefit assessments need to be solved.

In fact, although botanicals have been used for decades for their health-promoting effects, there is still a lack of data, information, and tools to ensure their safety and reap the benefits that they can convey to consumers.

The development of plant food supplements (PFS) in Europe could bring substantial market growth. However, the scientific and regulatory situation for PFS poses barriers to such growth. To protect consumers and exploit the market opportunities, a new integrated approach is needed in research. Since the European PFS industry does not operate in isolation, this integrated approach requires international cooperation.

Relatively recent changes in policy at the EU level suggest that more research is warranted. EFSA (the European Food Safety Authority) started working in this area, which should be complemented in parallel. In producing its guidance EFSA identified bottlenecks that ought to be addressed.

Several EC-funded research projects defined methodologies and developed tools that could but had not been applied to PFS: EuroFir, MoniQa, Beneris/Qalibra, and Eurreca.

In this international scenario, the European Project **Plant Food Supplements: Level of Intake, Benefit and Risk Assessment**s PlantLIBRA (no. 245199), was financed in the seventh framework program with the aims of supporting science-based decision-making and safe use of plant food supplements.

PlantLIBRA involved 4 continents and 25 partners (Fig. 1 and Table 1), comprising leading academics, small- and medium-sized enterprises (SME), industry, and nonprofit organizations. The period of activity was June 2010 to May 2014, but due to the numerous activities started, partners are still publishing results and concluding the research activities.

Fig. 1 Distribution of PlantLIBRA partners in the world

Supporting Science-Based Decision-Making

Several points were considered to reach the goal of supporting science-based decision:

1. Although the evidence on PFS is incomplete and complex to evaluate, PFS are doubtless associated with biological effects, with both benefits and risks;
2. An as yet undetermined number of plant species can currently be used in the EU, mainly based on the history of use. Compared with such a vast group of plants, very little, up-to-date scientific information on risks and benefits is available. Hence, decision-making, which needs to be made on a daily basis by authorities and food chain operators, may not be fully science based;
3. To respond to such a huge and frequent need for data, adequate methodologies and vast, sustainable, immediately accessible databanks need to be made available to the authorities making decisions.

Ensuring Safe Use of Food Supplements

Critical aspects in evaluating the risk associated with the consumption of food supplements containing botanicals were as follows:

Table 1 List of participants to the EU Project PlantLIBRA

Beneficiary number	Beneficiary name	Beneficiary short name	Country
1 (coordinator)	Università degli Studi di Milano	UMIL	Italy
2	BioDetection Systems B.V.	BDS	The Netherlands
3	Council for Scientific and Industrial Research	CSIR	South Africa
4	European Advisory Services	EAS	Belgium
5	European Botanical Forum	EBF	Belgium
6	Evira	Evira	Finland
7	Fundación para la Investigación Nutricional	FIN	Spain
8	Hylobates Consulting Srl	HYLO	Italy
9	International Association for Cereal Science and Technology	ICC	Austria
10	Institute of Food Research	IFR	United Kingdom
11	Institute of Medicinal Plant Development	IMPLAD	China
12	Istituto Superiore di Sanità	ISS	Italy
13	Phytolab GmbH & Co. KG	PLFIN	Germany
14	Società Italiana Scienze e Tecniche Erboristiche	SISTE	Italy
15	Swiss Toxicological Information Center	STIC	Switzerland
16	Terveyden ja Hyvinvoinnin Laitos	THL	Finland
17	Hospital de Clinicas “José de San Martín”, University of Buenos Aires	UBA	Argentina
18	University of Surrey	UNIS	United Kingdom
19	University of Leeds	UoL	United Kingdom
20	Universidade de São Paulo	USP	Brazil
21	Universitatea Transilvania DIN Brasov	UTBV	Romania
22	Universität Wien	VUW-Bot	Austria
23	Wageningen University	WUR	The Netherlands
24	European Food Information Resource Network AISBL	EuroFIR	Belgium
25	Department for Environment, Food and Rural Affairs	Defra	United Kingdom

1. Safe use requires valid and adequate risk and safety assessment of plants, of raw materials, and of food supplements by competent authorities or private sector risk assessors;
2. Safe use also implies awareness of risks and benefits by consumers, citizens, the private sector, and authorities, thanks to a user-friendly and in-language information. Critical is the contribution to consumer understanding in this area.

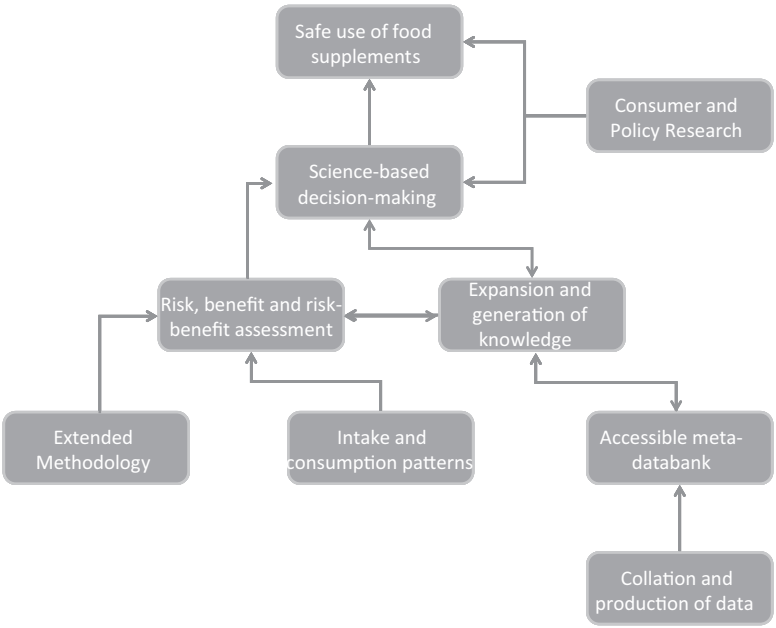

Fig. 2 The conceptual map of PlantLIBRA

On these bases, the conceptual map of PlantLIBRA project is illustrated in Fig. 2.

Safe use of PFS is the result of science-based decision-making, when informed by policy and consumer understanding. Science-based decision-making requires methodologies and data for assessment.

The Regulatory Context

PlantLIBRA operated considering that:

1. Legally, food supplements are unambiguously food. Nevertheless, they possess unique characteristics and specific legislation (EC Directive 2002/46);
2. The regulatory context affects the risk and benefit assessment;
3. Benefits of PFS can be communicated to European consumers by food chain operators only if they are proven under the EC Regulation 1924/2006 (Health claims regulation). This regulation provides an unavoidable reference for PFS benefit assessment.

Intake and Consumption Patterns

Whereas in other areas collation and review of existing data may be the most appropriate approach, for intake and consumption of PFS such steps need to be followed by development of methodologies and by the generation of actual, novel, crucially missing survey data.

Health Promoting Properties/Health Claims/Benefit Assessment

While EFSA has started the evaluation of claims related to PFS under art. 13 of Reg. 1924/2006 and published general guidance for applicants, there is still considerable uncertainty on the grading of evidence for benefits.

Risk Assessment and Safety Assessment

Risk assessment of botanicals, PFS, is necessary to ensure safety. One of the major data gaps in risk assessment of botanicals, as identified by EFSA, is the extreme paucity of information on intake and consumption patterns across Europe, which had become a major focus of PlantLIBRA. However, adverse events also remained a critical, but understudied source of information regarding safety.

More generally, PlantLIBRA, starting by the EFSA guidance, developed further steps in this area, with the application of new concepts, such as mode of action, margin of exposure, and threshold of toxicological concern, to overcome the major bottlenecks in the current methodologies. One of the main concerns was also interactions, or matrix effects, and methods for their evaluation.

International Cooperation and Dissemination

Most of the raw materials imported in the EU are sourced from China or other Third Countries. Concerns about the quality of the raw materials have been voiced and documented, also by industry. It was therefore necessary to work with exporting industry from those countries and with scientists there, to facilitate capacity building, and to retrieve information and intelligence on the probable contamination. PlantLIBRA disseminated the results obtained in 4 continents: Europe, Africa, Asia, and South America in specific meetings involving scientists, industries, and consumers.

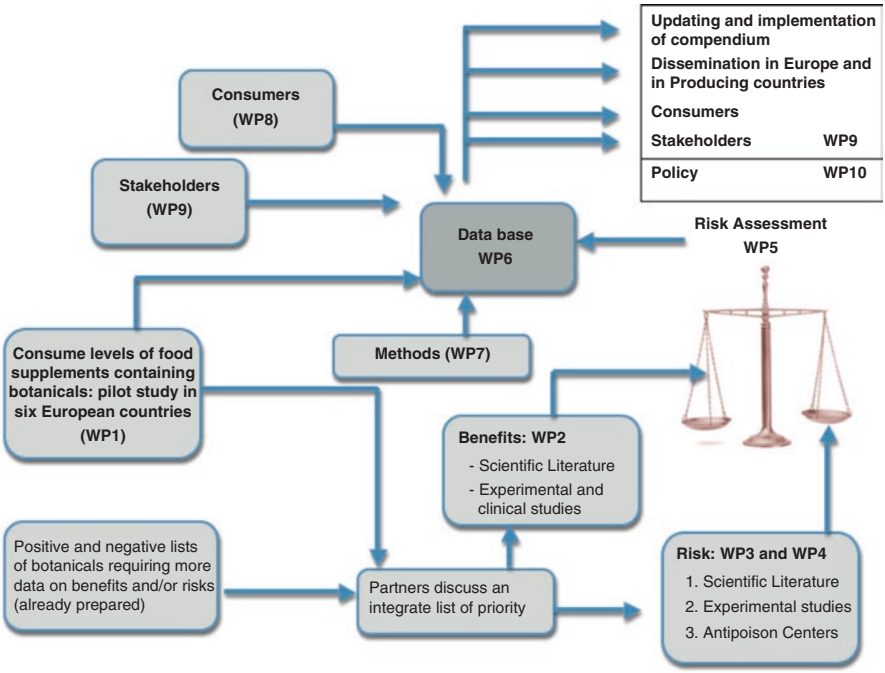

Fig. 3 The general structure of the EU Project PlantLIBRA, based on 11 workpackages

The research plan of PlantLIBRA project, as developed in the 4 years of activity, is illustrated in Fig. 3. Workpackage 11 is not reported in the plan since it corresponds to management.

The Book

This book collects most documents and scientific results produced during the PlantLIBRA project. Even though numerous papers were published during and at the end of the project, several documents, organized as Deliverables and Milestones, were still unpublished and are here made available to researchers, public institutions, food industries, and regulators.

The content is organized in three parts.

Part I: Classification and Regulatory Aspects of the Products Containing Botanicals

The first two chapters introduce the general aspects of the science of "botanicals," starting from the definition, an aspect particularly complex due to the different scientific positions and legislations between countries both in Europe and in other

continents. According to the authors: *"Botanicals" is the term now commonly used to describe plant materials when used in foods and food supplements, thereby differentiating them from plant materials used in herbal medicinal products, which are more usually described as "herbs."* A clear classification of the products present in the market is listed in a table with the relative legislative framework; these indications are very useful to introduce the reader to the topic. The traditional use of botanicals in the centuries is at the basis of the present legislations even though, as said above, with significant differences between countries and continents. The publication of positive lists of plants and guidelines, decided by some countries, is definitely a novel approach to guarantee the safety of consumers. Due to the objective of this book, more details are reported on food/dietary supplements, including the European rules to obtain "claims." It is a big honor to remember that the BELFRIT List originated from the first meeting of PlantLIBRA PAB (Policy Advisory Board).

Chapter 3 is an overview of how food supplements containing botanicals (or PFS) are being assessed in survey and epidemiological research. It describes the market structure, and the methods and administration techniques used to assess individual food consumption. The methodology designed for data collection on PFS within the PlantLIBRA project is here used as an example. Finally data published in the area of PFS used for gastrointestinal discomforts are reviewed and organized according to the body of evidences.

Chapter 4 introduces the concept of benefits and describes the experimental approaches useful to assess mechanisms responsible for the physiological effects of PFS. Human studies are considered with critical comments on their positive aspects and relative concerns.

Chapter 5 faces the problem of adverse effects in humans. This was one of the most relevant activities of PlantLIBRA due to the limited body of evidences in this area. The chapter summarizes all data produced during PlantLIBRA project, which have been published in peer-reviewed papers. They are integrated with new data, thanks to the collaboration with the French Agency ANSES and the collection of data from the FDA website. New information have been organized and compared with the previous results.

Part II: The Quality Control for the Safety of Consumers

The topic of quality control was originally distributed in two chapters but the authors collected so many documents and considerations that a change in the book structure was necessary. The second part is now organized in eight chapters with the objective to help researchers and analysts involved in food supplement analysis. Food supplements containing botanicals are complex products, where the analytical methods validated on other matrices could fail. Chapters in this part are a sort of guidelines for dealing with any kind of problems associated with the quality control of PFS: authentication of raw material as such or in commercial products; characterization of the botanical chemical profile; quantification of active or toxic molecules; control of adulteration or counterfeits; control of treatments, which are not allowed internationally or locally.

Part III: The Risk and Benefit Assessment for Consumers' Safety

The last part of the book describes two activities, which are included among the main goals of the project:

1. The risk and benefits assessment of PFS, with the development of a new scientific approach, which was validated using a certain number of botanicals known for both their positive and negative aspects;
2. The consumers' perception of risk and benefits, starting from the common belief that "natural (and then PFS) is always safe."

The Editor of this book, being the coordinator of the Project PlantLIBRA, hopes that this new collaborative work could represent a useful guide for all people working in the field of botanicals. Moreover, the Editor thanks the Springer Publisher for the opportunity to disseminate as much as possible the results obtained by the 25 partners and hundreds of researchers, who worked with passion during the four years of the project. To all of them, my deepest thanks.

Acknowledgements The research leading to the results presented in all chapters of this book has received funding from the European Community's Seventh Framework Program (FP7/2007-2013) under grant agreement no. 245199. It has been carried out within the PlantLIBRA project. This book does not necessarily reflect the Commission's views or its future policy on this area.

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Part I
The State of the Art of Products Containing Botanicals

Chapter 1
Botanical Products: General Aspects

Marinella Trovato and Cinzia Ballabio

Abstract Since ancient times, the use of plants is related to health effects and the correlation between their use, the maintenance of a good health, and the prevention of certain risk factors is well recognized. Food supplements, intended as a well-defined class belonging to a specific regulatory framework, have bound tradition and empiricism of observational data to a scientific reality, today represented by a conspicuous literature and a high number of scientific researches indicating that plants and their derivatives have become objects of privileged interest.

The definition of food supplement was introduced in the European Union with the Directive 2002/46/EC, that represented a first step in the harmonisation process of these products in the EU, albeit this concept of "harmonisation" is still far away from becoming reality, particularly as regards the use of plants, that differs from country to country in relation to different cultural approach and tradition.

In this chapter, aspects regarding the history of botanicals food supplements in EU and the different approach and relative legislation of the Member States for the use of plants in food supplements will be dealt. Some general data on consumption of these products worldwide are further supplied.

Keywords Botanicals • Plant Food Supplements • Positive list • Health benefits • National botanicals regulation

1.1 Definition of Botanicals

Plants and their derivatives are widely used in various products for human health and wellness nowadays. This renewed interest in natural derivatives is related to a request coming directly from the market, heavily influenced by consumers more aware of their health on holistic terms and looking for answers to the new concerns of the modern age in natural products.

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Hence the increased use of plants as active ingredients and raw materials by such companies that just a few decades ago based their business only on active ingredients and substances of chemical origin, despising plants and their derivatives because plants were considered of little economic interest and not as safe or because of their unclear regulatory definition.

In recent years, some rules have been enacted in the European Union (EU) to regulate products of plant origin, trying to bring some sort of order to a market that has exploded without any precise direction.

The legislator, however, have defined different products using rigid labels (e.g. traditional herbal medicines, dietary supplements based on plant extracts) not duly considering the fact that all these products, different from a regulatory point of view, have the same common denominator: plants.

According to the European Food Safety Authority (EFSA), the term "botanicals" includes all botanical materials (e.g. whole, fragmented or cut plants, plant parts, algae, fungi and lichens) (EFSA 2009). The term "botanical preparations" means all preparations obtained from botanicals by various processes (e.g. pressing, squeezing, extraction, fractionation, distillation, concentration, drying up and fermentation).

In the term "botanicals" are included "medicinal, aromatic and cosmetic plants" used as such in the preparation of infusions and decoctions, as spices for culinary use, or in processed form as ingredients of food, food supplements, cosmetic, drugs, medical devices, animal feed and products for animal husbandry, products for textile dyeing and tanning industry, pesticides and household products.

In Italy, the term used to indicate "medicinal, aromatic and cosmetic plants" is "*piante officinali*". This term, that is a unique meaning of the Italian language, derives from the Latin word "*opificina*" to highlight the aspect of transformation and processing that plants undergo before they can be used for other purposes. Botanicals should be considered "primary products" that, according to art. 2, paragraph 1, letter *b)* of Regulation (EC) No 852/2004 (EU Regulation 2004a), means "products of primary production including products of the soil…"

"Botanicals" is also the term now commonly used to describe plant materials when used in foods and food supplements, thereby differentiating them from plant materials used in herbal medicinal products, which are more usually described as "herbs".

1.2 Botanicals in Products for Human Health

Man's relationship with botanicals is very long-standing. In times of our ancestors, herbal infusions were well-known home remedies present in most households. They were used as remedies to cure all kind of common ailments like a cold or an upset stomach. But with the times, the uses of the products changed and due to their pleasant aromatic flavor, a lot of them were appreciated as foodstuffs. Therefore, some plants still have a double function. They may either be used as a health remedy or as a foodstuff. A well-known example for this double function is chamomile (*Matricaria chamomilla* L.) known for its use both as an infusion and herbal medicine.

Nowadays, the botanicals and products thereof are used in a wide range of products such as foods, food supplements, medicines, cosmetics, animal feed and veterinary medicines, medical devices, household products, etc., each of which fall under a specific regulatory framework.

1.3 Botanicals in the History from Ancient Times to Our Day

The beneficial effects of foods, beyond their nutrient function have been recognized since ancient times. In the fifth century BC, the Greek healer Hippocrates advocated "*Let food be your medicine, and your medicine your food*".

An immense heritage of the traditional uses of the plants is still preserved in many abbeys and monasteries that rose throughout Europe in the Middle Ages. These religious houses had herb-gardens where many botanicals still well-known and used today were grown. Among them feverfew (*Tanacetum parthenium* (L.) Sch.Bip.), used for a variety of indications in relation to inflammation; lavender (*Lavandula officinalis* Chaix), sage (*Salvia officinalis* L.) and peppermint (*Mentha x piperita* L.) for the digestion, and dandelion (*Taraxacum officinale* (L.) Weber ex F.H.Wigg.) for its beneficial effects on the urinary tract.

Interest in the health properties of botanicals and their uses continued and become more formalised in Europe in the sixteenth and seventeenth centuries, during the renaissance, when European universities teaching botany and herbalism planted "Botanical gardens", where a wide variety of species were grown. Many such gardens still exist today in university towns throughout Europe, providing a living history of the health benefits of botanicals.

In the twentieth century, during World Wars, herbs were used to treat soldiers wounded on the battlefield: garlic (*Allium sativum* L.) for its antiseptic properties and bilberry (*Vaccinium myrtillus* L.) to help English soldiers to see better during night-time bombardments. Still today, bilberry is used in botanical food supplements for the maintenance of eye-sight.

Many botanicals are today used for both medicinal and physiological purposes. Among them rosemary, sage, thyme, and mint which are regularly used as culinary aromatic herbs to flavor foods, cinnamon, caraway, nutmeg, cloves, and pepper as food spices. The historical use of these botanicals to help maintain health can be traced through the centuries (the ethnobotanical investigations on the domestic use of plants in many rural regions of Europe bear witness), and the current use for this purpose is demonstrated by the many herbal teas and infusions prepared from these plants that are still commonly consumed in the European Union for their digestive properties.

In more recent years, advances in research and technology, which have allowed to better preserve plants and to purify and concentrate the constituents holding health benefits, have meant that the health-promoting benefits of botanicals can be presented in a convenient form that can be made widely available. The development of botanical food supplements has meant that an ever-growing number of consumers in our urbanised society can safely and easily use botanicals to both maintain and optimize their health.

1.4 Classification of Botanicals Products

As mentioned, the botanicals are used in a wide range of products such as foods, food supplements, medicines, cosmetics, animal feed and veterinary medicines, medical devices, household products, etc.

Examples of the different uses of botanicals in the European Union are represented by herbal and fruit infusions that can be marketed both as foodstuffs and food supplements. Essential oils (e.g. rosemary e.o.) are used as flavoring ingredients in and on foods, as ingredients of food supplements, cosmetics and household products (e.g. detergents). Botanicals and products thereof (e.g. aqueous or hydro-alcoholic extracts, tinctures, etc.) can be used both as an active ingredient of medicinal products and as an ingredient of dietary supplements.

The botanicals, as such, do not fall in a specific regulatory framework by virtue of their structure, composition or properties; as depicted above they can be used as ingredients of diverse products if the use of the same is compatible with the intended use. However, every product underlies a specific rule for labelling. For instance, when an essential oil is used as ingredient in a dietary supplement, it must comply with Regulation (EU) No 1169/2011 (EU Regulation 2011) on the provision of food information to consumers and with Directive 2002/46/EC (EU Directive 2002) relating to food supplements, but when it enters the formulation of a cosmetic product, Regulation (EC) No 1223/2009 (EU Regulation 2009a) is applied.

Table 1.1 reports a classification and the legal definition of the main products on the EU market, with the relevant legislative framework when established, in which the botanicals and products thereof are used.

1.5 Botanicals Food Supplements in EU: History

The use of botanicals to maintain health has been the popular habit throughout Europe for many centuries. The consumption of teas, infusions, juices, elixirs, and extracts prepared from botanicals and used for health maintenance purposes has become part of European cultural heritage. The beneficial effects of plants on human have been gathered through experience, and the knowledge has been passed from generation to generation.

Botanical food supplements are a modern-day extension of this process. Dose forms such as capsules, pastilles, tablets, and pills (see legal definition of food supplements in Table 1.1) represent a convenient way of supplying consumer benefits from traditional practices, with the added advantages for the consumer that the processing which botanicals undergoes in the manufacturing process for dietary supplements guarantees the absence of any potentially harmful substances and allows to concentrate the beneficial components of the plant, and to increase the stability of the final product.

Today, the EU market offers the consumer a wide range of food supplements containing botanicals in comminuted or powdered form, or obtained by traditional techniques such as extraction, distillation, expression, fractionation, purification, concentration or fermentation.

Table 1.1 Classification of the main products based on botanicals and products thereof available on the UE market

Product	Definition	Legislation	Examples of products based on plant or derivatives
Medicinal products	**Medicinal product:** • Any substance or combination of substances presented as having properties for treating or preventing disease in human beings; or • Any substance or combination of substances which may be used in or administered to human beings either with a view to restoring, correcting or modifying physiological functions by exerting a pharmacological, immunological or metabolic action, or to making a medical diagnosis.	**Directive 2001/83/EC (as amended by Directive 2004/27/EC)** (EU Directive 2001; EU Directive 2004)	Traditional herbal medicinal products (THPM[a]) and herbal medicinal products based on whole, fragmented or cut plants, plant parts, algae, fungi, lichen in an unprocessed, usually dried, form, but sometimes fresh, and preparations thereof (e.g. comminuted or powdered herbal substances, tinctures, extracts, essential oils, expressed juices and processed exudates).
Food	**Food (or foodstuff)**: any substance or product, whether processed, partially processed or unprocessed, intended to be, or reasonably expected to be ingested by humans. "Food" includes drink, chewing gum and any substance, including water, intentionally incorporated into the food during its manufacture, preparation or treatment.	**Regulation (EC) No 178/2002** (EC Regulation 2002)	• Tea blends, herbal and fruit infusions, loose or in sachets, made from plant parts obtained from a single plant, e.g. peppermint, as well as blends of different herbs and/or fruits. • Dried aromatic herbs (sage, rosemary, thyme, marjoram, oregano, etc.) and spices (pepper, chilli, caraway, cinnamon, turmeric, etc.) used as flavorings to enhance the flavor of meat, fish, sauces, salads, etc. • Essential oils used as flavourings. • Vegetable oils (rice oil, wheat germ oil, etc.) used as salad dressing or as seasoning of various dishes.

(continued)

Table 1.1 (continued)

Product	Definition	Legislation	Examples of products based on plant or derivatives
Food supplement	**Food supplements**: foodstuffs the purpose of which is to supplement the normal diet and which are concentrated sources of nutrients or other substances with a nutritional or physiological effect (not limited to, vitamins, minerals, amino acids, essential fatty acids, fibre and **various plants and herbal extracts**), alone or in combination, marketed in dose form, namely forms such as capsules, pastilles, tablets, pills and other similar forms, sachets of powder, ampoules of liquids, drop dispensing bottles, and other similar forms of liquids and powders designed to be taken in measured small unit quantities.	**Directive 2002/46/EC** (EU Directive 2002)	Products based on dried aqueous or hydro-alcoholic extracts of plants, essential oils, vegetable oils, herbal infusions, concentrated juice, tinctures, glycerine macerate, etc., made from different parts of plants.
Cosmetic	**Cosmetic product**: any substance or mixture intended to be placed in contact with the external parts of the human body (epidermis, hair system, nails, lips and external genital organs) or with the teeth and the mucous membranes of the oral cavity with a view exclusively or mainly to cleaning them, perfuming them, changing their appearance, protecting them, keeping them in good condition or correcting body odours.	**Regulation (EC) No 1223/2009** (EC Regulation 2009a)	Products based on extracts, powders, essential oils, oils, juices, etc., made from different parts of plants.
Medical device	**Medical device:** any instrument, apparatus, appliance, software, material or other article, whether used alone or in combination, to be used specifically for diagnostic and/or therapeutic purposes and which does not achieve its principal intended action in or on the human body by pharmacological, immunological or metabolic means, but which may be assisted in its function by such means.	**Council directive 93/42/EEC** (Council Directive 1993)	Medical devices based on substances and preparation with as example mallow (*Malva sylvestris* L.) and althea (*Althaea officinalis* L.), plants rich in mucilage.

Household product	**Detergent**: any substance or preparation containing soaps and/or other surfactants intended for washing and cleaning processes. Detergents may be in any form (liquid, powder, paste, bar, cake, moulded piece, shape, etc.) and marketed for or used in household, or institutional or industrial purposes. Detergents also include: • **Auxiliary washing preparation**, intended for soaking (pre-washing), rinsing or bleaching clothes, household linen, etc.; • **Laundry fabric-softener**, intended to modify the feel of fabrics in processes which are to complement the washing of fabrics; • **Cleaning preparation**, intended for domestic all purposes cleaners and/or other cleaning of surfaces (e.g.: materials, products, machinery, mechanical appliances, means of transport and associated equipment, instruments, apparatus, etc.); • **Other cleaning and washing preparations**, intended for any other washing and cleaning processes.	**Regulation (EC) No 648/2004** (EC Regulation 2004b)	Detergents with fragrance of lavender, sandalwood, bergamot, aloe, orange, peppermint, eucalyptus, etc.
	• **Air fresheners:** a legal definition and labelling have not been established in European Union.	**Not regulated**	Perfume spray for the environment, oil diffusers sticks marketed in various fragrances.
Pesticides	**Pesticides:** products, in the form in which they are supplied to the user, consisting of or containing active substances (chemical elements and their compounds, as they occur naturally or by manufacture, including any impurity inevitably resulting from the manufacturing process) intended to protect plants or plant products against all harmful organisms or prevent the action of such organisms.	**Regulation (EC) No 1107/2009** (EC Regulation 2009b)	Plant active substance approved: Salix spp. cortex, garlic extract, orange oil, pepper, Plant oils/Clove oil, Plant oils/Spear mint oil, Plant oils/Rapeseed oil.

(continued)

Table 1.1 (continued)

Product	Definition	Legislation	Examples of products based on plant or derivatives
Biocidal products	**Biocidal product:** • Any substance or mixture, in the form in which it is supplied to the user, consisting of, containing or generating one or more active substances, with the intention of destroying, deterring, rendering harmless, preventing the action of, or otherwise exerting a controlling effect on, any harmful organism by any means other than mere physical or mechanical action. • Any substance or mixture, generated from substances or mixtures which do not themselves fall under the first indent, to be used with the intention of destroying, deterring, rendering harmless, preventing the action of, or otherwise exerting a controlling effect on, any harmful organism by any means other than mere physical or mechanical action.	**Regulation (EU) No 528/2012** (EU Regulation 2012)	E.g.: Mosquito repellent based on citronella and essential oils of basil, eucalyptus geranium, etc.

[a]*THMP* Medicinal product used throughout a period of at least 30 years preceding the date of the law application, including at least 15 years within the European Union

The concept of food supplement did not exist before 2002 in the European Union when Directive 2002/46/EC entered into force. This directive represented a first step in the harmonisation process of these products in the EU as it lays down specific rules for vitamins and minerals used as ingredients of food supplements. For the range of other substances used in dietary supplements, the Commission should have to submit, not later than 12 July 2007, to the European Parliament and the Council a report on the advisability of establishing specific rules, including, where appropriate, positive lists of categories of nutrients or of substances with a nutritional or physiological effect, such as botanicals, accompanied by any proposals for amendment to this directive which the Commission deems necessary (Coppens et al. 2006b).

To date, none of this has been done. Therefore, in order to fulfill the shortcomings at European level, several EU Member States, in the national implementation of Directive 2002/46/EC, have established a positive list of botanicals and related parts of plants to be used in food supplements; among them: Belgium, France, Italy, and Romania. Belgium, with the Royal Decree of 29 August 1997, was the first EU Member State to adopt a positive list of plants to be used in food supplements.

In recent years, Belgium, France and Italy developed a joint project (the so-called BELFRIT Project) in order to establish, on the basis of scientific evidence, harmonized rules regarding the use of botanicals in food supplements. Within this project a common list of admitted plants has been prepared in order to ensure their harmonization in at least these three countries with the hope that other countries join the project in the future (Klaus and Gherardini 2014).

In many EU Member States, such as Belgium, France, Germany, Ireland, Italy, Romania and Spain, food supplements are subject to a compulsory notification procedure before placed on the market. This means that the person responsible for the first placing on the market of a food supplement must inform the competent authorities of the marketing of the product by forwarding a copy of the product's label. Vice versa, in Austria, Sweden and the UK there is no requirement for food supplements to be registered or authorised before sale. In order to simplify, speed up and make more transparent the notification procedure both for companies and the regulatory authorities, Belgium and France, and shortly also Italy, have introduced the digital notification system of the product label.

In some EU Member States, such as Belgium, France, Germany, Italy and Spain, the companies that produce, package or place food supplements on the market in their territory should be registered, authorised or approved by national or local competent authorities.

To guarantee food safety according to Regulation (EC) 178/2002, some Member States, such as Belgium, France and Italy, published guidelines to support companies in preparing dossier for all their plant food supplements on the market, providing indications on all the necessary documentation and analytical controls to be carried out to ensure the safe use of botanicals and products thereof with reference to their quality (botanical information, preparation and processing methods of botanicals, titration in active ingredients) and on the final product (recommended daily intake, warnings and specific contraindications for certain

groups of consumers or people taking drugs, rationale underlying the botanical preparation, post-marketing surveillance). In Belgium, for food supplements containing essential oils of plants, additional toxicological data to guarantee their safe use are requested to be annexed to the notification dossier.

Belgium implemented guidelines, which address the specific needs of the food supplement industry in relation to Good Manufacturing Practice (GMP), with special attention paid to the requirements of EU food legislation. It covers the complete cycle of production and quality control of a food supplement, from the acquisition of all materials through all stages of subsequent processing, packaging and storage to the distribution or release of the finished product. In the wake of Belgium, also Italy is developing guidelines for Good Manufacturing Practice (GMP) to ensures that food supplements are consistently produced and controlled to the quality standards appropriate to their intended use and as required by the product specification.

Some Member States (e.g. Belgium, Italy) include food supplements approved into an electronic register and assigned a notification number.

Table 1.2 gives a brief overview of the Member States that, in the absence of harmonized EU legislation in the area of botanicals, have set their own legislation on the use of plants and plant extracts in food supplements. Some Member States such as Belgium, Germany and Romania, in addition to having introduced positive lists of botanicals, have developed negative lists of plants that cannot be used in food supplements. Other Member States such as Lithuania has notified the European Commission through the 2015/1535 notification (TRIS) procedure a negative list of ingredients of plant origin prohibited in food supplements. Among them there are plants, such as *Centella asiatica* L. and *Hypericum perforatum* L., which use in food supplements is authorized in other Member States, including Italy, Belgium and Romania.

In the EU, botanicals have a long tradition of use for their health effects. Botanical products in pre-dosed forms have been used for decades for their health-promoting and therapeutic properties. Many countries regulate the use of these products on their own territory, both as medicinal products and as food supplements. Medicinal products containing botanicals can be registered as traditional herbal medicinal products (THMP) in the EU if bibliographical or expert evidence is available that the product has been in medicinal use throughout a period of at least 30 years, including at least 15 years within the Community (Directive 2004/24/EC) (Anton et al. 2014). In this case, the THMP are registered under a simplified registration procedure, without the necessity of providing proof of efficacy with clinical trial.

Botanical food supplements can be defined as food supplements under the Directive 2002/46/EC, consisting of or containing botanical ingredients and that may make a health claim on the relationship that exists between the botanical ingredient and health. Such botanical ingredients may be whole botanicals in comminuted or powdered form, or obtained by traditional techniques such as extraction, distillation, expression, fractionation, purification, concentration or fermentation. Such products, which are presented in pre-dosed forms, may at first glance look very similar to medicinal products, but the intended use is quite different. While medicinal products are intended to prevent or treat a disease or modify the way in which the body functions, food supplements are intended to complement the diet with substances possessing health-maintenance or promoting properties (Coppens et al. 2006a).

Table 1.2 Examples of different regulation of plants and products thereof in food supplements in the European Union

Member states	Notification	List of plants	Legislation
Belgium	YES	**List 1**: dangerous plants that must not be used as or in foods; **List 2**: edible mushrooms; **List 3**: plants subject to notification if in pre-dosed form	**Royal Decree** amending the Royal Decree of 29 August 1997 on the manufacture and marketing of foods composed of plants or containing plant preparations (last amendment: 24 January 2017)
France	YES	**List 1**: plants that can be used in food supplements	**French Decree of 24 June 2014** establishing the list of plants, other than mushrooms, allowed to be added into food supplements, and their conditions of use
Germany	YES	**List A**: plants and plant parts not recommended for use as food or food ingredient due to known risks. **List B**: plants and plant parts for which restricted use in foods is recommended **List C**: plants and plant parts which cannot yet be completely assessed due to lack of sufficient data (generally are substances that have so far been used exclusively in food supplements but are otherwise novel foods)	–
Ireland	YES	The Irish Medicines Board in April 2011 published a draft list of Herbal Substances that may be acceptable for inclusion in food supplements. Some herbs are considered medicines (Schedule I of the Medicinal Products – SI no. 540 of 2003)	**S.I. No. 506 of 2007**—European communities (food supplements) regulations 2007
Italy	YES	**Annex 1**: provides the Italian positive list of botanicals **Annex 1.bis**: includes the BELFRIT list	**Ministerial Decree of 9 July 2012**, as amended by the Decree of 27 March 2014, regarding the use of botanicals in food supplements.
Lithuania	YES	Neither a positive nor a negative official list regarding the use of plants in food supplements A draft legislation amending the Hygiene Norm HN 17:2010 on Food Supplements was prepared and notified to the European Commission in 2014 through Directive 98/34/EC. The document establishes a list of botanicals that are prohibited in food supplements	**Order** amending order No V-432 of the Minister for Health of the Republic of Lithuania of 13 May 2010 approving the Lithuanian hygiene norm HN 17:2010—food supplements

(continued)

Table 1.2 (continued)

Member states	Notification	List of plants	Legislation
Romania	YES	**Annex 1**: Genera and species of plants whose parts or derivatives are not permitted to be used in food supplements **Annex 2**: Species of algae, lichens and fungi which are permitted to be used in food supplements **Annex 3**: Species of plants which are permitted to be used in food supplements, if subject to pre-dosing	**Order** of the Ministry of Agriculture and Rural Development and the Ministry of Health with regard to the preparation, processing and marketing of medicinal and aromatic plants used on an "as is" basis, which are partially processed or processed as pre-dosed food supplements
Slovenia	YES	**List H**: herbs permitted for use in foods, including food supplements, provided that non medicinal claims are made **List Z**: herbs permitted for use in over-the-counter medicines **List ZR**: herbs permitted for use in prescription only medicines **List ND**: herbs prohibited from use in all types of food and medicinal products	**Decree 103/2008** on the classification of medicinal herbs
Spain	YES	**No list of plants** Products that are placed onto market for the first time in Spain can only contain vitamins and minerals. Products containing substances other than vitamins and minerals (e.g. plants and product thereof) can only be marketed if they have been lawfully placed on the market in another EU Member State (mutual recognition principle)	**Royal Decree 1487/2009** of 26 September 2009 on food supplements
Sweden	NO	List of plants "unsuitable" in foods. The list is based on toxicological evaluations carried out by the National Food Agency after receiving questions about various plants. The list is not exhaustive and is no legal document but serves as guidelines for food operators and food inspectors	–
United Kingdom	NO	The Medicines and Healthcare products Regulatory Agency has created a list of herbal ingredients and their reported uses. The list is for information only and has no legal status	–

For food use, the effects observed must be proven under the Regulation (EC) No 1924/2006 on nutrition and health claims made on foods. The Regulation provides that all health claims, including those on plants and their preparations used in food, should be assessed on the basis of scientific evidence at "the highest possible standard".

The use of plants is different from country to country in Europe, in relation to different cultural approach and tradition and availability of plant species. There are about 1900 botanical species inventoried in Europe. Many of these, for instance, are allowed in the production of food supplements in some countries, while in others are forbidden for reasons related to a discretionary procedure not codified and shared at European level (e.g. while Belgium, France and Italy inserted *Peumus boldus* Molina and *Plantago major* L. in the positive list of plants to be used in dietary supplements, Germany listed them in the substances not recommended for use in foods, allowing the use only in medicinal products).

Under the current EU rules, it is possible that a Member State classifies a botanical product as food or as medicine on a case-by-case basis. In other words, as EU law stands, it is possible that the same product is classified as a foodstuff in one Member State and as a medicinal product in another.

In the absence of clear borderlines and reference lists, the food industry may be overtaken by the pharmaceutical one because of the concept expressed by Directive 2004/27/EC (amending Directive 2001/83/EC on the Community code relating to medicinal products for human use), that in art. 2, paragraph 2, provides that "*in cases of doubt, where, taking into account all its characteristics, a product may fall within the definition of a "medicinal product" and within the definition of a product covered by other Community legislation, the provisions of this Directive shall apply*", that is the pharmaceutical one.

This being understood that directive on traditional herbal medicinal products (Directive 2004/24/EC recital No. 12) "*allows non-medicinal herbal products, fulfilling the criteria of food legislation, to be regulated under food legislation in the Community*".

The lack of a unique list of harmonized European plants of potential use in food supplements, as opposed to what is established for example for minerals and vitamins, is a source of continuous dispute and creates a great confusion about these products in the European market.

The issues on how to clarify the differentiation between the use of botanicals for medicinal and health-promoting purposes on a scientific basis, to ensure the safety and quality of botanicals used in food supplements and to substantiate claims for botanical health products will be investigated in more depth in Chap. 9.

1.6 Botanicals Food Supplements in the Extra EU Countries

The classification of dietary supplements in the extra EU countries is sometimes conceptually very far from the definition of food supplement applied by the Directive 2002/46/EC in the European Union. In China, for instance, nutritional

supplements, defined as single substance vitamin and mineral products as well as vitamin and mineral complexes, are included in health foods ("foods useful for specific consumers, designated to regulate bodily functions, without having therapeutic effects") (O'Brien 2015). Due to the deeply entrenched appreciation of the Chinese culture for the health benefits of foods, the latter fall within the complex system of traditional Chinese medicine (TMC), that aims to promote health and enhance the quality of life, with therapeutic strategies for treatment of specific diseases or symptoms in holistic fashion. These medical practices are based on accumulated anecdotal evidence, clinical observations, millennia of practice and ultimately founded on methodologies which diametrically oppose the scientific foundations on which the West's functional food regulatory frameworks are built (O'Brien 2015). Some foods and ingredients from natural sources of plants are used in China as TCM.

Major reforms of the regulatory schemes governing health foods and nutrient supplements in China are in progress. These have been implemented with the promulgation of China's second Food Safety Law on 1 October 2015.

In the United States of America (USA) food supplements are regulated by the FDA (U.S. Food and Drug Administration) under the Dietary Supplement Health and Education Act of 1994 (DSHEA), hence under a different set of regulations than those covering "conventional" foods and drug products.

As stated by the Directive 2002/46/EC in the European Union, according to the DSHEA the term "dietary supplement" means "*a product (other than tobacco) intended to supplement the diet that bears or contains one or more of the following dietary ingredients: vitamin, mineral, herb or other botanical, amino acid, enzyme, dietary substance for use by man to supplement the diet by increasing the total dietary intake, or a concentrate, metabolite, constituent, extract, or combination of the preceding substances*". Food supplements are marketed in forms such as tablets, capsules, powders, softgels, gelcaps, or liquids.

The use of specific ingredients in dietary supplements is defined in the Federal Food, Drug, and Cosmetic Act (as Amended Through P.L. 107–377, Dec. 19, 2002). The manufacturer, packer, or distributor who wish to market a dietary supplement in US should notify FDA regarding the statement on the label or in the labeling of its product, pursuant to section 403(r)(6) of the Act.

Manufacturers and distributors of dietary supplements and dietary ingredients are prohibited from marketing products that are adulterated or misbranded. This means that these firms are responsible for evaluating the safety and labeling of their products before marketing to ensure that they meet all the requirements of DSHEA and FDA regulations.

China and USA are two examples of how the Western and Eastern world, on the basis of their own culture, apply a different regulatory approach to plant food supplements. In Chap. 9, it will be deepened the different lmhegal framework for botanicals in some selected non-EU countries.

1.7 General Data on Consumption

The market of food supplements is growing significantly both in Europe and the USA. In 2005, the total size of the EU food supplement market was estimated to be around five billion euros (retail selling prices). This figure is divided between food supplements containing vitamins and minerals that have a market share of 50%, and products containing other substances with a market share of 43% equivalent to 2.15 billion euros. The 75% the latter value (75%) refers to products sold in Germany, Italy, France and the United Kingdom (EAS 2007). Between 1997 and 2005, the growth of the market for food supplements containing other substances ranged between 20% in the United Kingdom to 219% in Poland (Commission of the European Communities 2008). The market of food supplements in Europe in the last few years is steadily growing, as also shown by the results of the fourteenth barometer on selfcare products presented by Afipa (French Federation of the Pharmaceutical Industry for Responsible Self-Medication), that underline a rising trend of the sales of food supplements in 2015 *versus* 2014 (+9.6%; +59.1 million euros). Consumption of dietary supplements in Germany and other European countries ranged between 17.9% and 60% of the population (Willers et al. 2015).

In Italy, between 2012 and 2014, the sales of food supplements have been increasing by '+7.4%' with a turnover of 2.4 million euros) (Source: IMS Health multichannel). According to this research, in 2014 botanicals covered 46% in value and 43% in volume of the market of food supplements in Italy.

This data and the outcomes of the PlantLIBRA PFS Consumer Survey, a research conducted as part of the PlantLIBRA[1] project aiming to provide an overview of the characteristics and usage patterns of PFS consumers in six European countries (Finland, Germany, Italy, Romania, Spain and the United Kingdom), underline that botanical food supplements receive great acceptance by European consumers (Garcia-Alvarez et al. 2014). The survey, conducted in 2011–2012, involved 2359 adult volunteers (aged 18–59 and > 60 years), who had taken at least a plant food supplement in the last 12 months, in an appropriate dose form and for a consecutive or non-consecutive period of at least 2–4 weeks.

A total of 1288 products across the six countries with 491 different botanical ingredients were reported by respondents (the maximum number of different botanicals contained in a German product was 46). The United Kingdom differed from the other countries both as the products reported contained a lower number of botanical ingredients (maximum 8) and for the number of plant food supplements reported, approximately half that of the other countries.

Figure 1.1 depicts the differences across countries in the type of products consumed. In the six countries, the most consumed products are those based on a single botanical ingredient (52%) with values ranging from 21% (Finland) to 85% (United Kingdom), followed by products containing two or more plants or products thereof (32%).

[1] PlantLIBRA project has received funding from the European Community's Seventh Framework Programme (FP7 2007–2013) under grant agreement no. 245199).

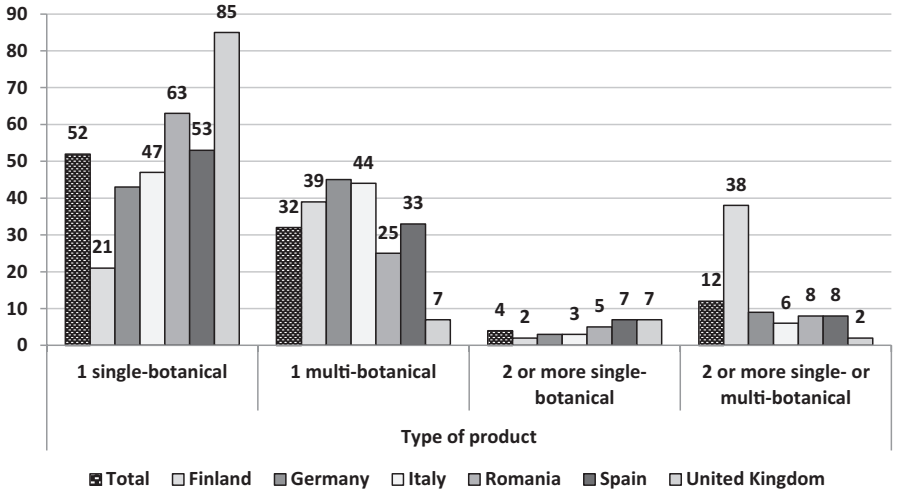

Fig. 1.1 Type of products taken (%), by country

The habit of taking two or more single-botanical products was less widespread in all countries, as was the usage of two or more single- and multi-botanical products. Finland was an exception to the latter, with 38% of respondents taking multiple products.

Based on the survey results, the top ten of the most frequently used botanicals (numbers of consumers ranging from 194 to 100) in descending order are *Ginkgo biloba* L. (ginkgo), *Oenothera biennis* L. (evening primrose), *Cynara scolymus* L. (artichoke), *Panax ginseng* C.A. Meyer (ginseng), *Aloe vera* L. (aloe), *Foeniculum vulgare* Mill. (fennel), *Valeriana officinalis* L. (valerian), *Glycine max* (L.) Merr. (soybean), *Melissa officinalis* L. (lemon balm), and *Echinacea purpurea* Moench (echinacea).

Evident differences in the most used plant food supplements emerge when the overall top-40 botanicals more frequently present in these products are stratified by age groups. In the group of 18–59 year-olds, the general ranking remains largely unchanged with a few changes (evening primrose being the most frequently used botanical in place of ginkgo). In the group of 60+ year-old ginkgo is still the most reported botanical, but other plants such as *Harpagophytum procumbens* DC. (devil's claw), *Vaccinium myrtillus* L. (blueberry) and *Allium sativum* L. (garlic) are within the most frequently reported botanicals, whereas *Glycine max* (L.) Merr., *Melissa officinalis* L. and *Echinacea purpurea* Moench do not appear in the top ten ranking.

Products based on gingko and garlic were the most widely used herbal supplements in the elderly as also evidenced in a review of 16 studies that evaluated the use of plant food supplements in subjects over 65 years old (the average age in the review ranged from 71 to 80 years), with a number of patients per study ranging from 69 to 5860 (de Souza Silva et al. 2014). Most of the research included in this review originated in the United States, two studies were conducted in Europe and one study in Asia. *Gingko*

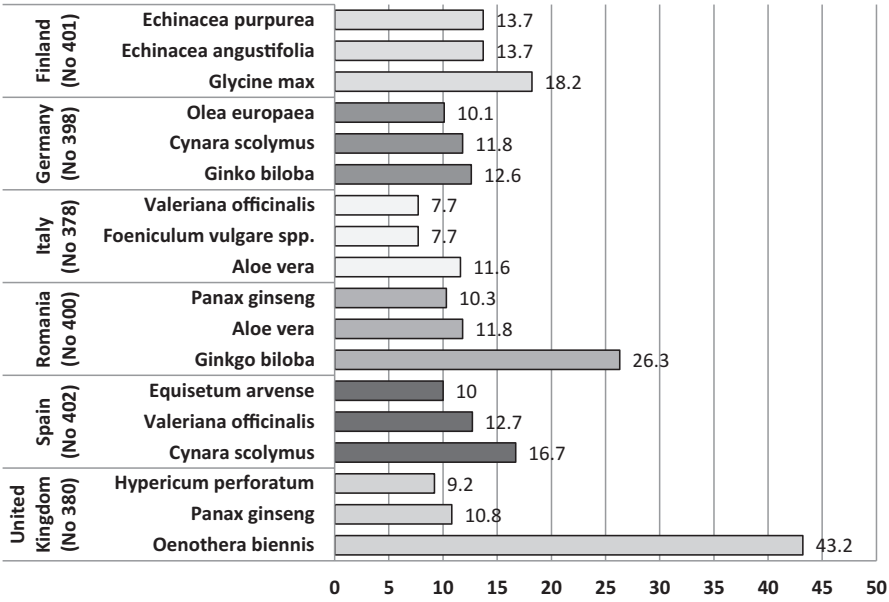

Fig. 1.2 Consumer distribution of the three most used PFS-contained botanicals (%), per country

biloba is widely used for its reported beneficial effects on memory, concentration, and treatment of cognitive dysfunction, whereas garlic is more commonly used for its antilipemic, antihypertensive, and antiatherosclerotic effects.

Although the use of herbal supplements is relatively common among the elderly, the high health risk due to the concomitant intake of drugs for chronic diseases and herbal products (e.g. the use of *Gingko biloba* with antiplatelet drugs and/or anticoagulants may increase the risk of bleeding complications, because both gingko and these drugs decrease the blood's ability to clot) should be seriously considered.

In the PlantLIBRA PFS Consumer Survey, cross-country differences appeared, when considering the overall top-40 botanicals more frequently present in PFS products in each of the six countries. As shown in Fig. 1.2 Finnish consumers use products mostly based on soybean, followed by those containing *Echinacea angustifolia* DC. and *E. purpurea* Moench. German consumers reported *Ginkgo biloba* L., *Cynara scolymus* L. and *Olea europaea* L. as the most; ginkgo was also the ingredient most frequently indicated in Romania, followed by *Aloe vera* L. (aloe) and *Panax ginseng* C.A. Meyer (ginseng). Amongst Italian consumers, aloe was the most recurrently used botanical, followed by fennel and valerian. In Spain, products containing artichoke were the most frequently used products, followed by those based on valerian and *Equisetum arvense* L. (horsetail). In the United Kingdom, *Oenothera biennis* L. (evening primrose) was the most frequently reported botanical ingredient, followed by *Panax ginseng* C.A. Meyer (ginseng) and *Hypericum perforatum* L. (St. John's wort) (Garcia-Alvarez et al. 2014).

The results of PlantLIBRA survey reflect the data published in a study carried out by the European Advisory Services (EAS) on behalf of the European Commission, that reports that ginkgo, echinacea, garlic and ginseng are the four most commercially important botanicals in the combined markets of 17 EU Member States, although echinacea and gingko are part of the composition of products registered as medicines (EAS 2007).

The intake of botanical food supplements is common also in childhood, although the pediatric use of herbs or plant food supplements has raised particular concern for children health. Infants may be more susceptible to adverse effects than adults because of differences in physiology, metabolism, and dose per body weight.

In 2013, the National Center for Health Statistics (NCHS) published the results of the first National Health Interview Survey (NHIS) conducted in 2007 on the prevalence of herb and dietary supplement use among children and adolescents in the United States (Wu et al. 2013). The representative sample consisted of 72,654 children aged 4–17; among them 2850 (3.9%) reported to use herbs or dietary supplements in the past 12 months, compared to 69,804 (96.1%) who did not use herbs or food supplements. Echinacea and "combination herb pills" were the most commonly herbal supplements taken by children and adolescents in the United States, followed by those containing flaxseed oil (from *Linum usitatissimum* L.), goldenseal (*Hydrastis Canadensis* L.), garlic (*Allium sativum* L.), cranberry (*Vaccinium macrocarpon* Aiton), ginkgo (*Ginkgo biloba* L.), ginseng (*Panax ginseng* C.A. Meyer) and soybean (*Glycine max* (L.) Merr.). The use of herb and food supplements was more common among older age groups (age 13–17) and among non-Hispanic whites. Socioeconomic measures, such as parental education and household income, were positively correlated with the herb and supplement use. Children with chronic health conditions, long-term prescription use, or relatively frequent use of physician services were also more likely to use these preparations (Wu et al. 2013).

Other interesting data about the use of plant food supplements and teas among infants were drawn by the Infant Feeding Practices Study II, a longitudinal survey of women aged 18 years and older studied from late pregnancy through their infant's first year of life, conducted by the US Food and Drug Administration (FDA) in collaboration with the Centers for Disease Control and Prevention between 2005 and 2007 (Zhang et al. 2011). Overall, 5.7% of mothers in the sample ($n = 2.653$) reported giving botanical food supplements and teas to their infants at least once during the first 12 months and 3.6% reported "more than once", compared to 90.7% of mothers who have never given their babies any type of botanical preparation. The mothers were more likely to give their infants plant food supplements and teas (include also products that are not herbal supplements), if they had used these products themselves, were primiparous, older (≥25 years), Hispanic, had higher education or higher income, and were married and longer breastfed. The percentage of infants given any plant food supplements and teas varies only slightly by infant age, from 2.4% in month 1 to 4.4% in months 4–6, till 3.4% in months 10–12. The most frequently supplements given to children in their first year of life were "gripe water" containing ginger (*Zingiber officinalis* Rosc.) and fennel (*Foeniculum vulgare* Mill.)—the ingredients may vary by brand -, chamomile

(*Matricaria chamomilla* L.) and teething tablets based on chamomile and other ingredients depending on brand. The most common reasons that mothers fed botanical preparations to their infants were to help with fussiness (e.g. chamomile), digestion (e.g. fennel, ginger), colic (e.g. fennel, ginger), and relaxation (e.g. chamomile). The most commonly reported sources of information about plant food supplements and teas were friends and relatives (30%), the media (28%), and the health care professionals (27%) (Zhang et al. 2011). This survey found that about 9% of infants were fed dietary botanical supplements and herbal teas in their first year of life, a data much higher than the percentages (from 0.8% to 5%) detected in previous surveys. This figure may be overestimated because it included unspecified tea, which may have been ordinary tea and not an herbal supplement.

No prevalence estimates exist for use of plant food supplements and herbal teas in infants and children in Europe or other parts of the world.

The results of this study cannot be generalized to the overall US population of mothers or infants due to several limitations. The respondents who participated in the study over-represented non-Hispanic white, older mothers of higher socioeconomic status, and no specific data were collected for subgroups such as Asians and certain immigrant groups who are frequent users of botanical preparations and tend to use plant food supplements and teas for their infant care because of culture and tradition (Zhang et al. 2011).

The use of food supplements by pregnant women has been investigated in a very few surveys. A study conducted in the USA in 2001 evaluated the usage patterns of dietary supplements (as defined by the FDA in the Dietary Supplement Health and Education Act of 1994 (DSHEA)–see par. 5), containing hence all kinds of ingredients from vitamin or mineral to herb or amino acid, during pregnancy in women followed at the Obstetrics and Gynecology Clinic of the University of California, San Francisco (Tsui et al. 2001). To the survey, conducted from November 1999 through March 2000, responded 150 pregnant women (nearly 24% of patients to whom the survey was distributed), who were in their first through third trimesters of pregnancy.

Overall, the use of dietary supplements among pregnant women was low. Of the 150 patients surveyed, 104 patients (70%) declared not to use any dietary supplements, 26 patients (17%) reported using a food supplement before their pregnancy, and 20 patients (13%) reported using a dietary supplement during pregnancy. Among the latter, 40% (8/20) initiated use before the pregnancy and continued it throughout, whereas 60% (12/20) began use because of the pregnancy. Among the women using dietary supplements during pregnancy, 32 different products were being used. Most women took more than one product (45 total) or an average of 2.24 products per person. The most common dietary supplements used were based on plant: echinacea (4/45, 8.9%), pregnancy tea[2] (4/45, 8.9%), and ginger (3/45, 6.7%).

[2] **Pregnancy tea**: a combination product that contains a blend of herbs such as spearmint (*Mentha spicata* L.) leaf, raspberry (*Rubus idaeus* L.) leaf, strawberry (*Fragraria vesca* L.) leaf, nettle (*Urtica dioica* L.) leaf, rose hip (*Rosa canina* L.), fennel (*Foeniculum vulgare* Mill.) seed, lemongrass (C*ymbopogon schoenanthus* (L.) Spreng. var. Motia) leaf, alfalfa (*Medicago sativa* L.) leaf, and lemon verbena (*Lippia citriodora* Kunth) leaf. This product claims to support a healthy pregnancy and states on the packaging that it is used to "tone the uterine muscles and prepare the womb for childbirth" and is to be used "throughout pregnancy and for a few weeks postpartum."

Table 1.3 Types of dietary supplements used in pregnant women

Type of products	Total products (N = 45)
Echinacea	4
Pregnancy tea	4
Ginger	3
Vitamin B6	2
Vitamin C	2
Multivitamin with herbs	2
Raspberry leaf	2
Enzymes	2
Other[a]	24

[a]Include one of each of the following: evening primrose oil, pregnancy tincture, goldenseal, proanthenol, chamomile, garlic, herbs (not specified), elderberry, phytoestrogens, zinc, fatty acid supplement, omega-3 fish oil, coenzyme Q-10, black currant oil, reishi mushroom tea, yin chiao, alpha sun, omega sun, acidophilus, bifidus, sea-fish supplement, L-lysine, super blue algae, and nettle

Echinacea was the most common herb initiated before pregnancy and continued throughout, whereas pregnancy tea and ginger were initiated for the childbearing. The total types of dietary supplements declared to be used in pregnant women are depicted in Table 1.3 (Tsui et al. 2001).

Ginger was commonly used for its antiemetic effects, although to date, there is no consensus regarding the use of ginger during pregnancy. Herbal products based on echinacea are commonly used to alleviate symptoms associated with the common cold, albeit the outcomes on their efficacy are conflicting. The use of this plant during pregnancy has been poorly documented, but a recent study did not observed an increased risk of malformations in women who used echinacea preparations from their first to third trimesters of pregnancy (Gallo et al. 2000).

From the data collected emerged that the most common reason for beginning to use a dietary supplement during pregnancy was to relieve nausea and vomiting (5/20, 25%), whereas the most common cause for discontinuing use of a dietary supplement was to avoid potential harm to the fetus (25%). Most patients reported no side effects (17/20, 85%), with the exception of nausea and stomach discomfort in one patient taking elderberry, taste disturbance in one patient taking echinacea, and intestinal gas in one patient using borage seed oil.

The most commonly reported sources of information about food supplements for pregnant women were equally distributed between themselves, friends or family members, the media, physicians or nurse practitioners, and alternative health care providers (including midwives, chiropractors, or naturopaths). Most patients informed their primary care provider of their use of dietary supplements (15/20, 75%). In Figs. 1.3 and 1.4 are reported, respectively, the most popular places where pregnant women purchase their dietary supplements and whether or not they inform primary care providers about their use.

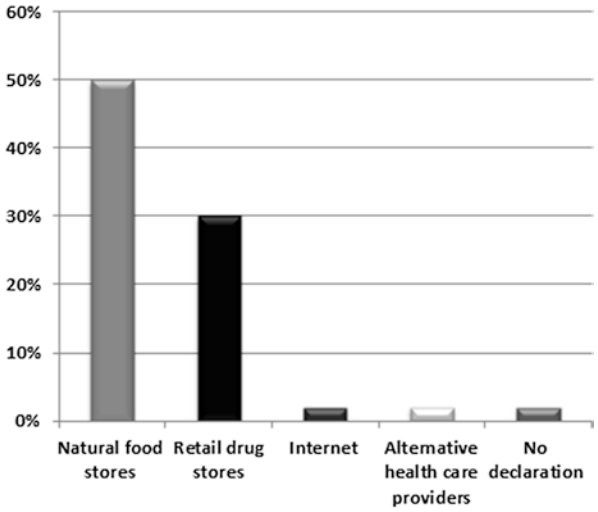

Fig. 1.3 Places of purchase of food supplements

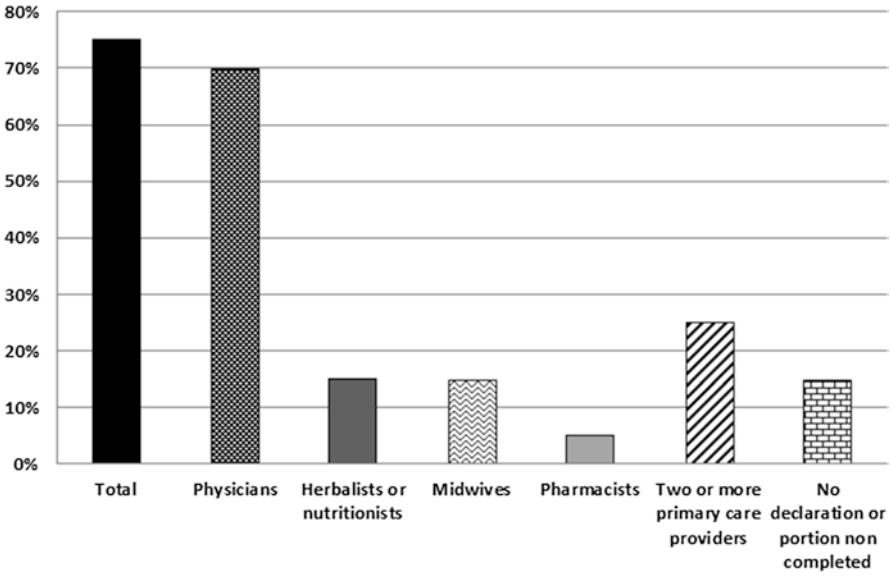

Fig. 1.4 Frequency (%) to inform primary care providers about the use of dietary supplements during pregnancy

This study did not evaluate the impact of potential socioeconomic, educational, or cultural variables on survey results. The use of many dietary supplements during pregnancy remains poorly documented and controlled clinical trials are needed to evaluate the efficacy and safety of these products both for the mother and the fetus.

1.8 Conclusions

Plants and their derivatives are used in a wide range of products intended for human health and wellness, such as foods and food supplements.

The use of plants in food supplements is regulated by Directive 2002/46/EC in EU.

There is a wide range of ingredients that might be present in food supplements including, but not limited to, vitamins, minerals, amino acids, essential fatty acids, fibre and various plants and herbal extracts. As a first stage, Directive 2002/46/EC established specific rules for vitamins and minerals used as ingredients of food supplements. Specific rules concerning other substances with a nutritional or physiological effect as plants and vegetable extracts used as ingredients of food supplements should be laid down at a later stage, provided that adequate and appropriate scientific data about them become available. Until such specific Community rules of harmonization are adopted, national rules concerning other substances with nutritional or physiological effect used as ingredients of food supplements, for which no Community specific rules have been adopted, may be applicable.

In the absence of a common approach at European level on plants that can be used in food supplements, each country has adopted its own rules, with lists of plants and their parts permitted or prohibited, lists that often are very different each other raising many problems in terms of free movement of goods in the UE.

In this context it is worth mentioning the BELFRIT initiative concerning Italy, Belgium and France, which aim has been to create a positive list shared by these three countries. Belfrit list is the only example, until now, of work shared between Member States to create a common list of accepted plants, but could be a good starting point for the European Commission when it will decide to proceed to an harmonization at European level.

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Chapter 2
The Regulatory Situation in Europe and Other Continents

Patrick Coppens and Simon Pettman

Abstract The regulations on botanical food supplements differ substantially between countries world-wide, both in terms of safety and benefit assessment. In the European Union, significant differences exist between the Member States as to what botanicals are allowed and what conditions of use apply. Also the legal status of botanicals differs (medicinal vs. food) and products lawfully marketed in one Member State are often not allowed in other Member States. Also in non-European countries such differences exist. This paper explores these differences on the basis of work carried out by the Policy Advisory Board of the EU funded PlantLIBRA project, which ran from 2010 to 2014. It provides an overview of the various regulations that apply in the EU Member States and a selection of countries at global level and provides insights into how aspects of safety and benefit have been addressed.

Keywords Food supplements • Plants • Plant preparations • Botanicals • Regulation • European Union • Food safety • Health benefit

2.1 Introduction

In the European Union (EU) and many other jurisdictions, food supplements (FS) are regulated under food law. These products are defined by Directive 2002/46 as: "*Foodstuffs the purpose of which is to supplement the normal diet and which are concentrated sources of nutrients or other substances with a nutritional or physiological effect, alone or in combination, marketed in dose form, namely forms such as capsules, pastilles, tablets, pills and other similar forms, sachets of powder, ampoules of liquids, drop dispensing bottles, and other similar forms of liquids and powders designed to be taken in measured small unit quantities*".[1]

[1] Directive 2002/46/EC of the European Parliament and of the Council of 10 June 2002 on the approximation of the laws of the Member States relating to food supplements. Official Journal of the European Union: L136/85, 12 July 2002.

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Botanical Food Supplements (BFS), also called Plant Food Supplements, are not legally defined in the EU but can be considered as food supplements that contain plants as such or plant ingredients (extracts, isolates, etc.), with or without other substances, such as vitamins, minerals or other bioactive compounds.

This paper presents the regulatory environment of such products in the EU and other jurisdictions. It presents information on the extensive but complex regulatory situation of BFS addressing safety, labelling and health benefits.

The information in this paper is largely collected in the framework of the PlantLIBRA project, a seventh framework project financed by the European Union and conducted between 2010 and 2014.[2] Additional information has been included.

The regulatory scope and policy context of the project was assessed and developed with the help of the Policy Advisory Board. This was an Advisory Group created within Work Package 10 and comprised legislators and experts from the EU Member States and non-EU countries. Its aim was to discuss and provide input in the work of the PlantLIBRA project from a policy perspective.

2.2 Legal Framework for Botanicals in the EU

2.2.1 European Harmonisation

FS, including those containing botanicals or botanical preparations are covered by Directive 2002/46/EC of the European Parliament and of the Council of 10 June 2002 on the approximation of the laws of the Member States (MS) relating to FS. This Directive partially harmonises the rules applicable to the placing of FS on the market in the EU MS.

The scope of this Directive covers all FS and includes certain requirements, in particular concerning labelling information and notification, applying to all FS, regardless of their composition.

However, the detailed rules contained in the Directive are only applicable to vitamins and minerals used in food supplements. The use of substances other than vitamins or minerals in FS therefore continues to be subject to the rules in force in national legislation. Products, lawfully marketed in accordance to such national rules are subject to mutual recognition (see further) under Articles 30 and 34 of the EC Treaty[3].

Recital 8 of the Directive states that specific rules concerning nutrients, other than vitamins and minerals, or other substances with a nutritional or physiological effect used as ingredients of FS should be laid down at a later stage. This is not yet the case.

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[2] www.plantlibra.eu.

[3] Consolidated versions of the Treaty on European Union and the Treaty on the Functioning of the European Union. Official Journal of the European Community C115/01, 9 May 2008.

In 2008, the EC issued a report on the use of substances other than vitamins and minerals in FS[4], in which it indicated that it is not feasible nor necessary to engage in further harmonisation on the use of substances other than vitamins and minerals in FS until adequate and appropriate scientific data become available.

It must be stressed that the substances in question, including botanicals and botanical preparations, are already covered by various Community horizontal legislative texts of general application (i.e. covering all foods or aspects also relevant for FS)

2.2.2 Horizontal EU Legislation Applicable to Botanical Food Supplements

FS containing substances other than vitamins or minerals are foodstuffs within the meaning of Article 2 of Regulation (EC) No 178/2002 (the General Food Law Regulation (GFLR)), which states that "foodstuff" (or "food") means "any substance or product, whether processed, partially processed or unprocessed, intended to be, or reasonably expected to be ingested by humans."

Article 2 also explicitly excludes from the definition of foodstuff a series of product categories, including medicinal products within the meaning of Directive 2001/83/EC on the Community code relating to medicinal products for human use (the Medicinal Product Directive (MPD)).[5]

This Regulation also lays down the responsibilities of food business operators in relation to food safety. These responsibilities include the obligation to place on the market only food that is safe, to ensure traceability of food and food ingredients, to be able to immediately initiate procedures to withdraw foods that are not or suspected not to be in compliance with the food safety requirements and to inform the competent authorities thereof.

It also specifies the missions and tasks of the European Food Safety Authority (EFSA), which is now involved in many activities that are directly relevant to FS, e.g. the establishment of tolerable upper levels of vitamins and minerals; guidance on the scientific evaluation of health claims and subsequent assessments; involvement in risk assessment under article 8 of the food fortification legislation; assessment of nutritional substances submitted in conformity with article 4.6 of the FSD; the self-tasking mandate on botanicals and botanical ingredients, etc.

It is generally considered that the establishment of the GFLR creates a legal counterpart of medicinal law, effectively regulating the safety aspects of foodstuffs, including FS.

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[4] European Commission. Report from the Commission to the Council and the European Parliament on the use of substances other than vitamins and minerals in food supplements. COM(2008) 824 final; Brussels, 5.12.2008.

[5] Directive 2001/83/EC of the European Parliament and of the Council of 6 November 2001 on the Community code relating to medicinal products for human use. Official Journal of the European Union: L311/67 28 November 2001.

In addition to this general framework legislation, BFS are also subject to the main legislation applicable to foodstuffs. This includes:

2.2.2.1 Novel Food Regulation (EC) 258/97[6] and Regulation (EU) 2015/2283[7]

The Novel Foods Regulation (NFR) specifies the requirements for putting on the market novel food ingredients, i.e. ingredients corresponding to the definition of novel foods that were not marketed in the EU to a significant degree prior to May 1997.

It provides for the requirement of a pre-marketing authorisation procedure based on the submission of a safety dossier followed by an assessment by a national authority. It may lead to an assessment by EFSA if the national authorities do not agree on the outcome of this assessment.

There is also a notification procedure for novel foods that are substantially equivalent to other foods. The outcome of both the authorisation and notification procedures can be found on-line.[8]

From 1 January 2018, the NFR 2015//2283 enters into force. It foresees a centralised assessment of applications by EFSA and the possibility for a substantial equivalence notification procedure is removed.

2.2.2.2 Health Claims Regulation (EC) 1924/2006[9]

The Nutrition and Health Claims Regulation (NHCR) provides for a pre-marketing approval procedure for nutrition and health claims for all foods, including food supplements. It is fully applicable to FS, lays down the definition of health and reduction of disease risk claims and the modalities for their approval. This legislation covers communication to the consumer on the product's health effects.

It led to the establishment of a positive list of health claims. This list contains the nutrient or other substance, the health claim and any conditions of use. It is published by the EC as a register.[10]

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[6] Regulation (EC) No 258/97 of the European Parliament and of the Council of 27 January 1997 concerning novel foods and novel food ingredients. Official Journal of the European Union: L043/1, 14 February 1997.

[7] Regulation (EU) 2015/2283 of the European Parliament and of the Council of 25 November 2015 on novel foods, amending regulation (EU) No 1169/2011 of the European Parliament and of the Council and Repealing Regulation (EC) No 258/97 of the European Parliament and of the Council and Commission Regulation (EC) No 1852/2001. Official Journal of the European Union: L327/1, 11 December 2015.

[8] http://ec.europa.eu/food/safety/novel_food/index_en.htm.

[9] Corrigendum to Regulation (EC) No 1924/2006 of the European Parliament and of the Council of 20 December 2006 on nutrition and health claims made on foods. Official Journal of the European Union: L12/3, 18 January 2007.

[10] http://ec.europa.eu/nuhclaims/.

To date, no claim for botanicals or botanical preparations has been approved because the application of the NHCR to botanicals has lead to a moratorium until a number of issues have been resolved (see Sect. 2.2.6).

The legislation also foresees the possibility for the approval of new health claims following an application for authorisation and EFSA assessment of the scientific justification. EFSA has published various guidance papers in this context[11].

2.2.2.3 Food Fortification Regulation (EC) 1925/2006[12]

The Food Fortification Regulation (FFR) covers detailed rules on the addition of vitamins and minerals to foods. However, article 8 provides for a process to address safety concerns of all food components, including botanicals and botanical preparations. It is therefore also applicable to other substances that are used in FS. It provides for a system whereby substances can be subjected to a EFSA risk assessment when they are added to foods or used in the manufacture of foods under conditions that would result in the ingestion of amounts of these substances greatly exceeding those reasonably expected to be ingested under normal conditions of consumption of a balanced and varied diet and/or would otherwise represent a potential risk to consumers.

The EC itself, or following a request from MS may initiate the procedure in order to include a certain substance in a list to prohibit or restrict its use. The EC has developed and published implementing rules for this procedure in 2012.[13] Since then, two substances have been introduced into the process: Yohimbe (*Pausinystalia yohimbe* (K. Schum.) Pierre ex Beille) and *Ephedra* ssp. On both EFSA has published its opinion[14,15.] On that basis the EC has decided to add *Ephedra* ssp to the list of prohibited substances and *Yohimbe* to the list of substances for which further data are requested. Assessments for hydroxyanthracene derivates containing botanicals, green tea catechins and monacolin K from red yeast rice are currently in the process.

The above procedure is a case-by-case assessment of food components and is not intended as a tool to develop negative or positive lists.

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[11] https://www.efsa.europa.eu/en/topics/topic/nutrition.

[12] Regulation (EC) No 1925/2006 of the European Parliament and of the Council of 20 December 2006 on the addition of vitamins and minerals and of certain other substances to foods. Official Journal of the European Union: L404/26, 30 December 2006.

[13] Commission Implementing Regulation (EU) No 307/2012 of 11 April 2012 establishing implementing rules for the application of Article 8 of Regulation (EC) No 1925/2006 of the European Parliament and of the Council on the addition of vitamins and minerals and of certain other substances to foods. Official Journal of the European Union: L102/2, 12 April 2012.

[14] EFSA 2013. Scientific Opinion on the evaluation of the safety in use of Yohimbe (Pausinystalia yohimbe (K. Schum.) Pierre ex Beille). EFSA Journal 2013;11(7):3302.

[15] EFSA 2013. Scientific Opinion on safety evaluation of Ephedra species in food. EFSA Journal 2013;11(11):3467.

2.2.2.4 Food Information to Consumers Regulation (EU) 1169/2013[16]

The Food Information to Consumers Regulation (FICR) lays down labelling requirements for all foodstuffs. It specifies the mandatory particulars and the modalities for correct labelling.

Although some specific labelling requirements for FS have been specified in FSD, the general labelling requirements that are applicable to all foodstuffs are also applicable to FS. These relate to the name, list of ingredients, best before date, (quantitative) ingredient declaration, presence of allergens, etc.

FS are excluded for the nutrition labelling rules as specific requirements have been laid down in the FSD.

2.2.2.5 Food Hygiene Regulation (EC) 852/2004[17]

The European Food Hygiene Regulation (FHR) lays down requirements for the safe manufacturing of foods, including food supplements based on the principles of Hazard Analysis and Critical Control Points (HACCP).

The main principles are:

- The primary responsibility for food safety lies with the food business operator;
- Food safety should be ensured throughout the food chain, starting with primary production;
- General implementation of procedures by companies based on the HACCP system whereby the manufacturer is obliged to assess his whole production process, identify those points in the process where safety risks can occur or that are essential to be controlled, apply measures to make sure these points are sufficiently controlled and monitor and document this during each production run;
- Registration or approval for certain food establishments;
- Development of guides for good practice for hygiene or for the application of HACCP principles as a valuable instrument to help food business operators at all levels of the food chain to comply with the safety rules. Several such guides have been developed specifically for food supplements, including botanical food supplements. One such guide has been developed by Food Supplements Europe[18];
- Flexibility is provided for food produced in remote areas (high mountains, remote island) and for traditional production and methods.

Microbiological criteria are specified in Regulation (EC) No 2073/2005[19].

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[16] Directive 2000/13/EC of the European Parliament and of the Council of 20 March 2000 on the approximation of the laws of the Member States relating to the labelling, presentation and advertising of foodstuffs. Official Journal of the European Union: L109/29, 6 May 2000.

[17] Corrigendum to Regulation (EC) No 852/2004 of the European Parliament and of the Council of 29 April 2004 on the hygiene of foodstuffs. Official Journal of the European Union: L226/3, 25 June 2004.

[18] www.foodsupplementseurope.org.

[19] Commission Regulation (EC) No 2073/2005 of 15 November 2005 on microbiological criteria for foodstuffs. Official Journal of the European Community L338/1, 22 December 2005.

2.2.2.6 Pesticide Residues Regulation (EC) 396/2005[20]

Pesticides are used to protect crops before and after harvest from infestation by pests and plant diseases. This Regulation harmonises maximum residue levels (MRLs) in the EU to protect consumers from exposure to unacceptable levels of pesticides residues in food and feed.

2.2.2.7 Contaminants Regulation (EC) 1881/2006[21]

This Regulation establishes maximum levels for certain contaminants in foods, including food supplements. This includes maximum levels in certain foods for the following contaminants: Nitrates/Mycotoxins (aflatoxins, ochratoxin A, patulin, deoxynivalenol, zearalenone, fumonisins, citrinine ergot sclerotia and ergot alkaloids, tropane alkaloids)/Metals (lead, cadmium, mercury, inorganic tin, arsenic)/3-MCPD/Dioxins/Dioxin-like PCBs and Non dioxin-like PCBs/Polycyclic Aromatic Hydrocarbons (PAH) (benzo(a)pyrene)/Melamine/Erucic acid.

2.2.2.8 Food Additives Regulation (EC) 1333/2008[22]

The Additives Regulation (AR) provides for a pre-marketing approval procedures for additives to be used in foods, including FS. It also specifies the additives permitted and their conditions of use. This includes substances used for technical purposes, such as colours, preservatives, antioxidants, emulsifier, thickener, gelling agents, stabilisers, flavour enhancers, acids, acidity regulators, anti-caking agents, modified starches, sweeteners, raising agents, anti-foaming agents, glazing agent, emulsifying salts, flour treatment agents, firming agents, humectants, bulking agents and propellant gasses.

For most of the additives permitted, purity criteria have also been established in Regulation (EU) No 231/2012.[23]

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[20] Regulation (EC) No 396/2005 of the European Parliament and of the Council of 23 February 2005 on maximum residue levels of pesticides in or on food and feed of plant and animal origin and amending Council Directive 91/414/EEC. Official Journal of the European Union: L70/1 16 March 2005.

[21] Commission Regulation (EC) No 1881/2006 of 19 December 2006 setting maximum levels for certain contaminants in foodstuffs. Official Journal of the European Union: L364/5, 20 December 2006.

[22] Regulation (EC) No 1333/2008 of the European Parliament and of the Council of 16 December 2008 on food additives. Official Journal of the European Union: L354/16, 31 December 2008.

[23] Commission Regulation (EU) No 231/2012 of 9 March 2012 laying down specifications for food additives listed in Annexes II and III to Regulation (EC) No 1333/2008 of the European Parliament and of the Council. Official Journal of the European Union: L83/1 22 March 2012.

2.2.2.9 Extraction Solvents Dir 2009/32/EC[24]

This Directive specifies permitted extraction solvents for the manufacture of food, including, where appropriate residue limits.

2.2.2.10 Food Irradiation Directive 1999/2/EC[25] and Directive 1999/3/EC[26]

This legislation specifies the foods that are permitted to be irradiated. It should be noted that most botanicals used in food supplements are not on this list. Only dried aromatic herbs, spices and vegetable seasonings are on the approved list.

2.2.3 National Legislation

Although a harmonized framework has been ensured by the FSD, major differences exist between EU MS in the way rules for the use of botanicals have been implemented.

A large majority of the MS have drawn up positive or negative lists of substances other than vitamins and minerals, which can be used in food supplements. In some cases, use of the substances in question is subject to compliance with technical conditions, such as maximum limits, type of extract or combination of ingredients. Furthermore, entry of new substances onto these lists is often subject to an assessment.

Table 2.1 illustrates the different approaches applied to a number of selected botanicals used in BFS (EU 2008 Report).[27]

Based on the 2008 EU report, research intelligence by EAS-Strategies (EAS), and input from the PlantLIBRA project, the main elements of existing regulations in the EU Member States are highlighted below.

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[24] Directive 2009/32/EC of the European Parliament and of the Council of 23 April 2009 on the approximation of the laws of the Member States on extraction solvents used in the production of foodstuffs and food ingredients (recast) Official Journal of the European Union: L141/3, 6 June 2009.

[25] Directive 1999/2/EC of the European Parliament and of the Council of 22 February 1999 on the approximation of the laws of the Member States concerning foods and food ingredients treated with ionising radiation. Journal of the European Union: L66/16, 13 March 1999.

[26] Directive 1999/3/EC of the European Parliament and of the Council of 22 February 1999 on the establishment of a Community list of foods and food ingredients treated with ionising radiation. Journal of the European Union: L66/16, 24 March 1999.

[27] Commisison of the European Communities. Report from the Commission to the Council and the European Parliament on the use of substances other than vitamins and minerals in food supplements. 05/12/2008. COM(2008) 824 final.

Table 2.1 Illustration of the variation of permission approaches in Member States

Botanicals & botanical extracts	AUSTRIA	BELGIUM	BULGARIA	CYPRUS	CZECH REPUBLIC	DENMARK	ESTONIA	FINLAND	FRANCE	GERMANY	GREECE	HUNGARY	IRELAND	ITALY	LATVIA	LITHUANIA	LUXEMBOURG	MALTA	NETHERLANDS	POLAND	PORTUGAL	ROMANIA	SLOVAKIA	SLOVENIA	SPAIN	SWEDEN	UNITED KINGDOM
Aloe (*Aloe vera* (L.))	E	✓	E	E	C	L	✓	C	A	C	✖	C	✓	✓	✓	E	✓	✓	✓	✓	✓	✓	C	L/C	✓	✓	C
Ginkgo (*Ginkgo biloba*)	E	L	E	E	L	✓	✖	C	A	✖	✖	L	✖	C	L	E	✓	✓	✓	✓	✓	✓	L	✖	✖	✖	C
Ginseng (*Panax ginseng*)	E	✓	E	E	L	L	✓	C	A	✖	L	L	C	✓	L	E	✓	✓	✓	✓	✓	✓	✓	L	✓	✓	C
Garlic (*Allium sativum* (L.))	✓	✓	E	E	C	✓	✓	✓	A	C	✓	L	C	✓	✓	✓	✓	✓	✓	✓	✓	✓	L	✖	✖	C
Green tea extract (*Camellia sinensis*)	E	L/C	E	E	C	E	✓	✓	A	C/A	✓	L	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✖	✓	C
Garcinia extract (*Garcinia cambogia*)	E	✓	E	E	L	✖	C	✓	A	✖	✖	L	✖	✓	✓	E	✓	✓	✓	✓	✓	✓	✓	C	✖	E	✖
Guarana extract (*Paullinia cupana*)	C	✓	E	E	C	L	✓	✓	A	C/A	✖	L	C	✓	L	E	✓	✓	✓	✓	✓	✓	✓	✓	✖	✓	✓

Symbols	
✓	Permitted for use in food supplements either under national law or internal guidelines.
L	Permitted for use in food supplements - maximum level established.
C	Permitted for use in food supplements under specific conditions (eg type of extract, ingredients combination in the final product, etc)
E	Permission may be given on a case by case basis following evaluation, considering issues such as ingredient function.
A	Not currently pemitted. May be permitted following a pre-marketing authorisation.
✖	Not permitted for use in food supplements, or regarded as medicinal.

2.2.3.1 Austria

In July 2005, the Federal Ministry of Health and Women issued under the framework of the Austrian Codex Alimentarius the "Recommendation for food supplements concerning content of vitamins and minerals, overages and use of plant parts" which, among others, includes:

- A list of herbs prohibited for use in food supplements,
- A short list of herbs and parts thereof for which there are generally no safety concerns and which can be used in food supplements.

Herbs not covered in the Recommendations and other bioactive substances are evaluated on a case-by-case basis. Committees for food supplements and tea have been created from academia and medicinal controls.

A positive and negative list of botanicals for the use in the production of herbal infusions is included in the Chapter B31 of Austrian Food Codex.

2.2.3.2 Belgium

The Royal Decree of 29 August 1997 on the production and marketing of foods composed of plants or containing plant preparations includes a list of prohibited plants, a list of permitted mushrooms and a list of plants permitted in food supplements specifying, in some cases, their conditions of use.

A national plant committee is charged with assessing safety and inclusion of conditions of use in the list of allowed plants. In 2005 the use of maximum levels for active ingredients of certain plants used in food supplements was integrated in this list.

The Royal Decree was further updated in March 2012 and April 2014, based on the scientific opinion of the Belgian Advisory Commission on Plant Preparations. The most important changes in this list are the specification of the plant parts and the additional warnings for labelling. Concerning the plant parts: only the traditionally and safely used plant parts are retained in the list.

Together with France and Italy, Belgium has been working on a consolidated list for the three countries. This so-called BELFRIT list is now finalised and a proposal to include the BELFRIT list in the Royal Decree was notified to the EC. The final new decree was published in February 2017.

2.2.3.3 Bulgaria

Ordinance No 47 (December 2004), as amended on requirements related to food supplements transposes the EU Food Supplement Directive into national law. Annex 4 includes a list of about 120 botanicals that are prohibited for use in food supplements.

According to the Bulgarian food supplement Ordinance standardized plants, plant parts and extracts with a beneficial effect on the health and safe at the daily dose recommended by the manufacturer are permitted to be added to the composition of the food supplements.

2.2.3.4 Croatia

Croatia joined the EU on 1 July 2013. Food supplements need to be approved following a simplified or full notification procedure (depending on their classification), for efficient monitoring.

A list of permitted plant species and mushrooms for use in food supplements, including where applicable, additional restrictions or conditions of use and mandatory warning statements, is provided in Annex II to Croatian Ordinance (No 160/13). Annex 3 of this law lays down the negative list of plants, which are not permitted for use in food supplements. In addition, an application procedure for plants not listed is foreseen, entailing a safety evaluation.

2.2.3.5 Cyprus

The 2004 Regulation on Food Supplements transposes the EU Food Supplement Directive. There are no positive or negative lists of botanicals and other bioactive substances.

The use of substances other than vitamins and minerals in food supplements is evaluated by a scientific committee following one of the two established procedures:

– Mutual recognition if the food supplement product is lawfully sold in another EU Member State
– An authorisation procedure by submission of a detailed dossier to gain a license prior to marketing.

2.2.3.6 Czech Republic

The Czech General Food Law 316/2004 (codified as Food Law 456/2004) includes the definition of food supplements. The Czech Decree No. 225/2008 Coll. (as amended by Ordinance 352/2009) stipulates requirements for botanical food supplements and includes two lists:

1. Annex 3: Conditions for the use of certain other substances in food supplements: with maximum daily levels for certain botanicals;
2. Annex 4: List of plants prohibited in the manufacture of foodstuffs: Latin names and parts of plants.

A new decree was notified to the EC in June 2016. This proposal includes

– A list of botanical and other substances with maximum permissible amounts
– A list of botanicals and substances not permitted for use.

2.2.3.7 Denmark

The 2003 Danish law on food supplements (BEK no 683) permits the use of vitamins, minerals, botanicals and other bioactive substances in food supplements.

Food supplements may contain plants, mushrooms or parts of these. Since 1989, a ‘Drogeliste’, a Danish list of plants, mushrooms etc., that have been toxicologically evaluated, has been published. The latest version is from May 2000 and later evaluations are found as an addendum.

The Danish authorities have published guidance on the safety of food supplements and there are different rules for ingredients that are dried or slightly concentrated.

In addition, the Danish authorities have published a law on the addition of substances other than vitamins and minerals to foods, including food supplements, with a nutritional and/or physiological effect in 2011. This law has been amended twice in 2013 and the latest amendment was published in August 2013.

In case of non-water extracts or extracts with purity of at least 50% or concentrated 40 times or more this order will apply and an authorisation may be required to assess the safety of the extract before product marketing.

2.2.3.8 Estonia

The Food Act of 1999 and Regulation nr 100 of 12 November 2014 on Composition and quality requirements for food supplements and requirements for the provision of food information, apply in Estonia.

In addition, the State Agency of Medicines published a list of botanicals that are generally regarded as medicinal and therefore cannot be used in food supplements.

2.2.3.9 Finland

The Finnish Regulation of the Ministry of Trade and Industry on Food Supplements 571/2003 permits the use of botanicals and other substances with a nutritional or physiological effect. The notification procedure is electronic.

There are no legal lists specifying the permission or prohibition of botanicals or other bioactive substances in food supplements. The permission of botanicals and other bioactive substances is evaluated on a case-by-case basis.

The Finnish Medicines Agency (Fimea) maintains a list of the substances and herbals that may make a product a medicinal product. However, herbals included in this list can also be used in food supplements depending on the level and the extracted active substances and provided that no medicinal claims are made for the final product.

2.2.3.10 France

The provisions of the EU Food Supplement Directive are transposed by the 2006 Food Supplements Decree No 2006-352 which permits the use of botanicals and other bioactive substances.

France adopted an Order on the use of plants and plant preparations (other than mushrooms) permitted in food supplements and their conditions of use on 24 June 2014. Annex I is a list of approximately 600 plants whose use is authorised in food supplements. Annex II is on information to be communicated by food business operators in relation to the characterisation of plant preparations (mandatory information), while Annex III is in relation to the safety of plant preparations (only when their nature or conditions of use significantly differ from the traditional use). Food supplements containing plants not included in the Annex I or plants deviating from the set conditions of use require declaration in accordance with Article 16 of the French food supplement Decree (based on the so-called “mutual recognition”), or in accordance with Article 17 (pre-marketing authorisation procedure, involving an ANSES scientific assessment).

France also adopted a Decree on 22 August 2008 on medicinal plants or parts of plants entered in the Pharmacopoeia, which may be sold to the public by persons other than pharmacists. It includes a list of around 184 ‘released’ medicinal plants. They can be used under certain restrictive conditions (e.g. raw state, powder, aqueous extract) in food supplements.

France is one of the three countries that participated in the so-called BELFRIT project. The BELFRIT list of 1029 plants is now finalised. The French DGCCRF published it at the end of April 2014. This list is meant to help food supplement manufacturers but has no legal value yet.

2.2.3.11 Germany

The 2004 Ordinance on Food Supplements ("Verordnung über Nahrungsergänzungsmitter") with which Directive 2002/46/EC on Food Supplements was transposed into German law permits the use of certain vitamins and minerals in food supplements. The Ordinance however, does not cover any other substances than vitamins or minerals with a nutritional or physiological effect such as amino acids, essential fatty acids or botanicals ("other substances"). Food supplements containing such "other substances" are verified on a case-by-case basis to decide whether they comply with the general legal provisions. In Germany, "other substances" are treated in the same way as food additives. This means they are subject to authorisation for the use in food supplements and other foods.

Experts from Federal Authorities and from the Federal States (Länder) have undertaken a joint project on categorisation of "other substances" to simplify and unify their evaluation. This list of more than 600 botanicals ("Stoffliste") for which by reason of health objections restrictions might have to be considered with respect to their use in food is available on the homepage of the Federal Office of Consumer Protection and Food Safety since 2015.

The "Stoffliste" is only indicative and has no immediate legal effect.

2.2.3.12 Greece

The 2004 Food Supplement Ministerial Decision (AR. U1/ GP. 127962/03) implements the EU Food Supplement Directive. There is no legal positive or negative list of botanicals.

The permission to market botanicals or other bioactive substances in food supplements is evaluated by the Greek Organisation of Medicines (EOF) on a case-by-case basis during product notification.

2.2.3.13 Hungary

The Hungarian Decree of the Ministry of Health 37/2004 (IV. 26.) on food supplements permits the use of substances with physiological or nutritional effects in food supplement products. The use of herbal ingredients and preparations in food supplements is currently not specifically regulated by a legal act and herbals are subject to the assessment of the National Institute for Food and Nutrition Science (OÉTI).

The Hungarian Institutions involved in assessing herbal ingredients adopted their first negative list in 2007. This internal negative list of herbs contained 243 entries and was published on the OÉTI website. This negative list is under continuous revision. It is indicative and has no legal value. The last update was performed in December 2013.

In order to establish a national legislation on the quality requirements of botanicals intended for use as food and/or food ingredients there is draft legislation in the pipeline proposed by the Ministry of Rural Development. This legislation has not been implemented yet.

2.2.3.14 Ireland

Directive 2002/46/ is transposed into national legislation by the European Communities (Food Supplements) Regulations 2007 (S.I.No. 506 of 2007). These regulations require any person placing a food supplement on the market in Ireland to notify the Food Safety Authority of Ireland (FSAI) and provide a copy of the label.

Irish national law on food supplements does not include any negative and/or positive lists of botanicals and other bioactive substances.

In 2013 FSAI has published "Guidance Note No 21—Food Supplements Regulations and Notifications (Revision 2)".

2.2.3.15 Italy

The Circular 18 July 2002, published by the Health Ministry in the Italian Official Gazette, General Series on 12 August 2002, extended the pre-marketing notification procedure mentioned in art. 7 of the Legislative Decree 111/1992, also to herbal food supplements. A botanical ministerial guideline on documentation to be maintained by companies for their botanical ingredients, in case the Ministry would request such information was also published and updated in January 2015.

The Italian Ministry of Health has issued a positive and negative list of plants and their derivatives that have been evaluated by the Commission on Dietetic Foods and Nutrition (CUDN). In 2012 the Italian Ministry of Health adopted the Ministry Decree of 9 July 2012-G.U. 21-7-2012 including in its Annex an extensive positive list of botanicals with an indication of their permitted plant parts that may be used in food supplements. During 2013–2014 the Annex of the decree was regularly updated via the publication of a ministerial guideline published on the Ministry of Health's website which also includes indications or references to physiological effects.

In March 2014, the Italian Ministry of Health has adopted a new plant Decree revising the Decree of 9 July 2012. The aim of the new Decree was to gradually include the BELFRIT project list (that was developed by three countries Belgium, France and Italy) into Italian legislation. The 2014 Decree therefore includes two

positive lists (the latest Italian plant list in Annex 1 and a new Italian BELFRIT list in Annex 1bis), which have been applicable in parallel and which the Ministry intends to finally merge into one positive list after further revisions. A new plant Decree to complete the introduction of the BELFRIT list is in progress.

2.2.3.16 Latvia

There is currently no specific national list of permitted or prohibited herbs, bioactive substances or maximum and minimum levels for the addition of vitamins and minerals.

The addition of some components may be evaluated case-by-case by the State Agency of Medicines. It is possible to prohibit the commercialisation of a new food supplement, as the firm has to notify the product to the public administration.

2.2.3.17 Lithuania

The 2003 Lithuanian Decree on Food Supplements HN 17/2003 permits the use of botanicals and other bioactive substances. It does not include any negative and/or positive lists of botanicals.

Lithuania applies a national notification system for food supplements, The State Food and Veterinary Service (SFVS) takes samples for laboratory analysis of each consignment of food supplements from non-EU countries.

A draft Order of the Minister for Health amending Order No V-432 of 13 May 2010 approving the Lithuanian Hygiene Norm HN 17:2010—Food Supplements was notified on EU level in April 2014. The Order envisages a negative list of botanicals that are prohibited for use in food supplements. The list contains 188 botanical ingredients. To date this Decree has not been published.

2.2.3.18 Luxemburg

The Food Supplement Regulation does not include any negative and/or positive lists of botanicals and other bioactive substances. The authorities evaluate the use of botanicals and other bioactive substances in food supplements on a case-by-case basis and generally apply the mutual recognition principle if proof is available that the same food supplement product is already lawfully sold in another EU Member State.

2.2.3.19 Malta

The Food Safety Act (ACT NO. XIV OF 2002) and Food Supplements Regulations 2003 (L.N. 239 of 2003) permit the use of botanicals in food supplements. It does not include any negative and/or positive list of botanicals and other bioactive substances.

The Maltese authorities evaluate the permission to market botanicals and other bioactive substances in food supplements on a case-by-case basis following a risk assessment by the Malta Standards Authority.

2.2.3.20 The Netherlands

The Decree of 15 March 2003 on Food Supplements implements the EU Food Supplement Directive, completed by several Commodities Act Decrees including that on 'Herbal preparations' of January 2001, which covers herbal preparations that are brought on the market as foods and non-food products. The Decree limits the amount of toxic pyrrolizidine alkaloids in herbal preparations to 1 μg/kg. In addition, part I of the annex to the Decree lists plants that are known to contain toxic pyrrolizidine alkaloids. However, the limit for toxic pyrrolizidine alkaloids extends to all plants with these constituents that are used in herbal preparations. Furthermore, the Decree forbids the presence of aristolochic acids and yohimbine alkaloids in herbal preparations. Part II of the annex to the Decree defines plants that are too toxic to be used in food or in other commodities, and this part of the annex is currently comprised of 46 plants and fungi.

The Dutch authorities have also published a guideline lists of traditional Chinese herbal preparations and Ayurvedic herbal preparations in which harmful substances may be present.

2.2.3.21 Poland

Food supplements are covered by the Polish Decree on the composition and labelling of dietary supplements of 9 October 2007 and by the Polish Act on Food Safety. The definition of food supplements mentioned in the Polish laws permits the use of botanicals and other bioactive substances in food supplements. It does not include any negative and/or positive lists of botanicals.

The status of certain botanicals as ingredients in food supplements needs assessment by the Polish Medicinal Authorities prior to notification.

Poland is currently revising its legislation after the EC launched infringement procedures in 2013 against Poland because of the non-application of mutual recognition.

2.2.3.22 Portugal

The Portuguese Decree No 136/2003 on food supplements (as last amended by the Decree 118/2015) permits the use of botanicals and other bioactive substances in food supplements. It does not include any negative and/or positive lists of botanicals and other bioactive substances. The permission to market botanicals and other bioactive substances in food supplements is evaluated on a case-by-case basis.

The DGAV (Direção-Geral de Alimentação e Veterinária) uses as guidance their internal database of food supplement notifications.

2.2.3.23 Romania

The 2007 Order No 1069 on food supplements (Norma din 19/06/2007 privind suplimentele alimentare) permits the use of botanicals and other bioactive substances in food supplements. The 2005 Common Order of the Ministry of Health and Ministry of Agriculture, Forests and Rural Development no. 401/244 regulates the use of botanicals in food supplements and includes a positive and negative list of herbs and plants, and a positive list of cultivated and wild mushrooms. Moreover, the Order 1228/2005 specifies rules on the approval of food supplements containing animal or herbal products (extracts), alone or in combination with vitamins and minerals.

Ordnance 1228/2005/244/63 of 2006 specifies rules for placing on the market botanicals, botanical/animal extracts or mixtures of them and/or with vitamins, minerals and other substances with nutritional and physiological effects intended for human consumption as food supplements.

In March 2015 a draft legislation with new lists of botanicals (based on the French, Belgian, Italian lists) was notified to the European Commission. If the draft will be approved the Order 244/401/2005 on herbals, processed and partially processed herbals used in food supplements would be repealed and a new lists of botanicals would be implemented.

2.2.3.24 Slovak Republic

The relevant EU legislation in the field of food supplements (Directive 2002/46/EC) has been fully implemented into the Slovak food legislation—i.e. in to the Decree of the Ministry of Agriculture and the Ministry of Health of the Slovak Republic No. 16826/2007-OL, in the Slovak Food Code on foodstuffs intended for particular nutritional uses and food supplements as amended further. Currently, as there is no national legislation related to food supplements in the Slovak Republic, there are no negative or positive lists of botanicals or other substances related to food supplements.

The Decree No. 2089/2005-100 establishing a Chapter of the Slovak Food Codex governing coffee, tea and similar food products provides a list of herbs and their parts permitted for use in tea.

2.2.3.25 Slovenia

Regulation 82/2003 on Food Supplements as recently amended by the Regulation 66/2013 permits the use of botanicals and other bioactive substances in food supplements. The use of herbs and their parts is regulated under Decree 103/2008 on the classification of medicinal herbs, and includes four categories of herbs:

- Herbs permitted for use in foods, including food supplements, provided that no medicinal claims are made,
- Herbs permitted for use in OTC medicines,
- Herbs permitted for use in prescription only medicines,

– Herbs prohibited from use in all types of food and medicinal products.

Herbs included in the list of plants permitted for use in food can in principle be used in food supplements as long as their safety can be proven and no medicinal claims are made for the final product. Products containing high levels of concentrated herbal extracts of herbs from the category 1 generally require a pre-marketing authorisation from the competent health authority and are evaluated on a case-by-case basis.

2.2.3.26 Spain

The Royal Decree 1487/2009 on food supplements permits the use of botanicals and other ingredients in food supplements in Spain. The authorities have however not issued any lists. A couple of negative/positive lists of plants are used as guidance documents by the Spanish authorities.

In December 2013, a draft Royal Decree amending the Spanish Royal Decree 1487/2009 on food supplements and inserting a list of other substances permitted in food supplements was notified via TRIS to the European Commission and other EU Member States. This decree has not been implemented yet.

2.2.3.27 Sweden

The Food Supplement Ordinance (LIVSFS 2003:9) permits the use of botanicals and other bioactive substances in food supplements. The authorities tolerate the use of other bioactive substances in food supplements as long as they are not classified as medicines or natural remedies by the Medicinal Products Agency.

The authorities used to use as a guideline the negative list of plants and plant parts unsuitable for use in food (VOLM). This list is non-exhaustive and subject to modification. Plants contained therein may be unsuitable under specific conditions and due to specific plant parts. In addition, plants not listed in the VOLM list ccannot be ensured not to be harmless. In borderline cases, the advice of the Medicinal Products Agency (MPA) may be required for the final product classification. The Swedish Medical Products Agency has a list published on its website (as a guidance) with substances and plants on which the agency has regularly received questions.

2.2.3.28 United Kingdom

The Food Supplement (England) Regulations 2003 (S.I. 2003 No.1387) permit the use of substances with nutritional or physiological effects in food supplements. It does not include positive and/or negative lists of botanicals or other bioactive substances.

To assist companies in determining the likely status of their product, a list of herbal ingredients has been compiled by regulatory bodies and industry in the UK. This non-exhaustive list, which has no legal status, includes plants specifying their recorded uses in the UK (i.e. food, medicines, cosmetics and aromatherapy). There is also a list of herbal ingredients prohibited or restricted in medicines, which are under the responsibility of the Medicines and Healthcare Products Regulatory Agency (MHRA). Both lists and the 'guide to what is a medicinal product' are useful tools, and used by the authorities in determining product classifications on a case-by-case basis.

2.2.3.29 Norway

Herbs are mostly classified as medicines in Norway, but there are some exceptions.

The Norwegian Regulation 1565/1999 on the classification of medicinal products (Forskrift om legemiddelklassifisering) includes a list of herbs that was issued by the Norwegian Medicines Agency and is generally also used as guidance by the Norwegian Food Safety Authority. The list is divided into the following three herbal categories:

- Herbs for free sale in food,
- Herbal medicines, and
- Herbal medicines on prescription.

Herbs not appearing on this list are assessed on a case-by-case basis by the Norwegian Medicines Agency.

2.2.3.30 Switzerland

Food supplements are regulated by the Swiss EDI Regulation on Special Foods (Art. 22). The Annexes of the Swiss EDI Regulation are listing vitamins, minerals, including their sources, and some other bioactive substances authorised for use in food supplements. Herbal ingredients and extracts thereof are not included in the Annexes of the Swiss EDI Regulation and would therefore require an individual authorisation ("Bewilligung") from the Swiss Federal Office for Public Health (BAG) before their marketing in food supplements.

Swiss Institute for Remedies, Swissmedic and Swiss Federal Office for Public Health (BAG) have issued in a co-operation a document on the classification of herbs and herbal substances: "Classification of herbal materials and preparations as drugs or as food". The document includes a list of herbs with an indication of their classification as medicine or food and their general appropriate purpose of use in specific food sectors. This document is used as a guideline by the Swiss authorities while evaluating herbal ingredients during the authorisation procedure.

2.2.4 Mutual Recognition

In the light of the diverging national rules on BFS, Mutual Recognition (MR) remains an important tool for ensuring the free movement of products, including food supplements, on the European market.

MR means that a MS is obliged to accept on its territory products that are lawfully marketed in another Member State, even when such products would not comply with their national domestic rules. This is a direct consequence of Article 30 of the Treaty.

MR does not prevent MS to still object to the marketing of such product, provided they would pose danger to health. In such cases, it is upon the MS to prove that such is the case.

The principles of MR have been included in Regulation 76/2008 that came into application on 13 May 2009[28]. This Regulation specifies clear procedures and rules to govern refusals of MR.

In addition, the Court of Justice of the European Union (CJEU), as part of its judicial supervision, has set precise limits within which the MS may validly exempt themselves from MR. The CJEU has consistently ruled it is for the MS, to decide on their intended level of protection of human health. However, in exercising this discretion they must comply with the principle of proportionality. This means that the measures and decision they take need to be confined to what is actually necessary to ensure the safeguarding of public health. In addition, such measures must be proportional to the objective thus pursued, which could not have been attained by measures which are less restrictive of intra-Community trade (see as an example paragraphs 86 to 88 of the judgment in the case C-319/05, Commission v Germany)[29].

In practice however, MS still often deny mutual recognition in the area of food supplements without observing these legal principles. This and a number of other elements may pave the way to further harmonisation in this area at EU level.

2.2.5 Towards Further Harmonisation?

Considering all the issues described and analysed in its 2008 report, the EC concluded that laying down specific rules applicable to substances other than vitamins and minerals for use in food supplements was not justified at that time. The EC doubted the feasibility of such a measure, which, in any case, in its view was not necessary in the short term.

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[28] Regulation (EC) No 764/2008 of the European Parliament and of the Council of 9 July 2008 laying down procedures relating to the application of certain national technical rules to products lawfully marketed in another Member State and repealing Decision No 3052/95/EC. Official Journal of the European Union L218/21. 13.08.2008.

[29] Case 319/05: Judgment of the Court (First Chamber) of 15 November 2007. Commission of the European Communities v. Federal Republic of Germany. Official Journal of the European Community C8/3, 12 January 2008.

The EC highlighted the complexity and different approaches by national authorities and the limited scientific information available on other substances. It also indicated that a number of new or recent legal instruments, adopted or in the process (including the NFR, NHCR, FFR) would already harmonise part of the aspects relating to these products.

Finally, the EC pointed out that, in general terms, despite certain limitations, mutual recognition is a useful instrument for facilitating the free movement of the products concerned.

The EC therefore concluded that the legal instruments described in its report already constitute a sufficient legislative framework for regulating this area and it did not consider it opportune to lay down specific rules for substances other than vitamins or minerals for use in foodstuffs.

However, since substances other than vitamins or minerals, including substances derived from plants, are now being added to ordinary foodstuffs and not only to food supplements, the Commission did not rule out the possibility, at a later state, of carrying out a supplementary analysis to the report, examining the conditions for the addition of these substances to foodstuffs in general.

The current status created by the application of the NHCR to botanicals is further described in Sect. 2.2.6.

2.2.6 Borderline with Medicinal Law: Traditional Herbal Medicinal Products

Botanicals are also used in medicinal products for their medicinal purposes. The EU legal system accepts this dual use, provided a product is in conformity with the legal framework chosen.

In principle, medicinal product legislation is based on pre-marketing approval of individual medicinal products. This is based on the demonstration of safety, quality and efficacy. For some medicinal products, efficacy can be demonstrated on the basis of bibliographic evidence showing well-established use. However, for herbal medicinal products, a specific legislation was adopted in 2004: Directive 2004/24/EC relating to herbal medicinal products (the Traditional Herbal Products Directive (THMPD))[30]. The reason is that no traditional medicinal product could have been authorized under the MPD existing at the time, mainly due to limitations of available data on efficacy.

For this reason for a traditional herbal medicinal products (THMPs) it has to be demonstrated that the product is "non toxic under the specific conditions of use and the pharmacological effects and efficacy are plausible on the basis of long term use

[30] Directive 2004/24/EC of the European Parliament and of the Council of 31 March 2004 amending, as regards traditional herbal medicinal products, Directive 2001/83/EC on the Community code relating to medicinal products for human use. Official Journal of the European Union: L136/85 30 April 2004.

and of available experience". By this the legislation specifically accepted that efficacy requirements for herbal traditional medicinal products are lower than for the other categories of medicinal products.

A licence as THMP according to Directive 2004/24/EC can be granted, in general, only for products present on the market for at least 30 years, of which 15 years in a EU Member State. To this end, any products containing the same active substances, regardless of excipients, with the same or similar indications, dosage and posology equivalent and identical administration routes are considered equivalent.

In a number of MS certain botanicals are not permitted for use in FS but are restricted to medicinal products under the THMPD rules. This is despite these botanicals being accepted for use in FS in other MS and therefore MR should apply. In most cases no reasons are given for such practice and in a number of cases MS have been challenged before the CJEU and have lost these cases. Nevertheless, product classification issues remain a reality despite such extensive case law.[31]

To distinguish the borderline between food and medicinal products, the Court of Justice of the EU has established extensive case law.

The main principles established by the CJEU in its various judgments can be summarised as follows:

- Member Sates have the competence to determine whether a certain product is a medicinal product or not, but have to base that decision on a case-by-case assessment of all of the product's characteristics, particularly its composition, its pharmacological properties as they may be ascertained in the current state of scientific knowledge, the way in which it is used, the extent to which it is sold, its familiarity to the consumer and the risks that its use might entail. This means that a decision cannot be taken solely on the basis of the composition, the form or the nature of the ingredients of a product but must be based on all of its characteristics.
- All products that are presented as having therapeutic or preventative effects in relation to diseases should be subject to medicinal law in order to be able to ascertain the efficacy of the product in relation to its claimed effects on the basis of the clinical studies performed. It is the aim of medicinal law that if efficacy cannot be established, a marketing licence can effectively be refused.
- However, medicinal law is not intended to cover products that have an effect on the body or health but are not presented for the treatment, prevention of cure of diseases. The concept of a physiological effect is not specific to medicinal products only but is also among the criteria used for the definition of food supplements. Products having an effect on the human body, but which do not significantly affect the metabolism and thus do not strictly modify the way in which it functions, cannot be considered as medicinal products by function. This is the case with many botanicals and botanical preparations.

[31] www.curia.eu. Relevant cases include: C112/89, C60/89), C219/91, C369/88, C290/90, C227/82, C211/03, C319/05, C140/07, 88/07, C27/08, C308/11.

– The fact that a risk to health may be present is not sufficient to classify a product as medicinal by function. The legal framework of food (GFLR) and national legislation in place contain sufficient provisions to ensure the safety of any food, including botanical food supplements.
– The fact that similar products are registered as medicinal products is also not a determining factor to consider all similar products as medicinal products.

A correct implementation of both food and medicinal law and observance of these principles established by the CJEU would already eliminate many problems that hinder the free movement of botanical food supplements in the EU.

2.3 Legal Framework For Botanicals in Selected Non-EU Countries

Given that the regulatory situation relating to botanicals is so diverse in the EU Member States, it should not be a surprise that in non-EU countries there are even less similarities. Botanicals have traditionally been used in food and medicinal products and regulations have evolved over time to cover such traditional use.

2.3.1 ASEAN (Association of South-East Asian Nations)

The 10 countries of the ASEAN have developed common regulatory requirements for food supplement products. One of the areas for harmonisation is the development of a list of prohibited active ingredients. In future, botanicals widely used in food supplements in certain ASEAN countries will likely be available in all of the ASEAN countries.

2.3.2 China

Food supplements are regarded as “health food” in China. Health food is regulated according to the Administrative Measures for Health Food Registration and Filing”, which was recently implement on 1st July 2016 and other relevant regulations, which will be issued by the China Food & Drug Administration (CFDA).

According to this new regulation, there will be two routes of product placement: Registration and Filing (notification).

Registration is applicable for

- Health Food with ingredients not on the Health Food Raw Materials Catalogue list (intended health claims are on the permitted list)
- Health Food imported into China for the first time (excluding Nutrient Supplements containing vitamins and minerals)

Whereas Filing (notification) is applicable for:

- Health Food with ingredients on the Health Food Raw Materials Catalogue list (intended health claims are on the permitted list)
- All Nutrient Supplements containing vitamins and minerals listed on the Health Food Raw Materials Catalogue list

Currently, the CFDA is still developing the list of ingredients Health Food Raw Materials Catalogue that qualify for the fast track Filing (notification) route. So far, they have only issued a draft list of permitted vitamins and minerals ingredients for public consultation.

Though it is still not clear when will the botanical ingredients be included in the catalogue, it is likely that the CFDA will consider the following types of botanical ingredients (mainly Chinese herbs), which were previously permitted for use in Health Food in the catalogue.

- Ingredients that can be used in health food—e.g. ginkgo leaf, ginseng, saffron;
- Ingredients that can be used in both conventional food and medicine—e.g. cinnamon, mint, ginger, dates, Chinese wolfberry.

Those botanical ingredients that are not on the list will be subject to the full registration process.

2.3.3 Customs Union of Belarus, Russia and Kazakhstan

2.3.3.1 Eurasian Economic Union (EAEU) of Belarus, Russia, Kazakhstan, Armenia and Kyrgyzstan

According to the Customs Union Technical Regulation TR CU 021/2011 "On safety of foods", food supplements are defined as natural (or identical to natural) biologically active substances, including pro-biotic microorganisms, designed to be taken with food, or made part of food products

Food supplement ingredients may originate from herbs, animals and minerals and can be produced by chemical or biochemical processes.

Food supplements must not contain: strong, narcotic or poisoning substances and herbal substances which are not permitted for use in medicines and/or foods and doping substances from the WADA list. In addition, there are regulatory restrictions on food supplements intended for children. There is a list of

botanicals which are allowed in food supplements and herbal teas for children of 3–14 years of age.

The TR CU 021/2011 "On safety of foods" specifies the list of 339 botanicals (Annex 7) which are not allowed in food supplements. The document also bans use of specific animal products, synthetic analogues of natural substances of medicinal plants, human tissues, some microorganisms and fungi.

2.3.4 Latin America

2.3.4.1 Argentina

In Argentina, food supplements are designated by law as 'suplementos dietarios' (dietary supplements) and are regulated under the Article 1381 of the Argentine Food Code (CAA) (in the overall Chapter XVII on 'Dietetic Foods').

As a general principle, food supplements are subject to a pre-market authorisation (valid for 5 years) .The Argentine food supplements legal definition explicitly provides for the possibility of using herbal ingredients in food supplements. Such products should however additionally contain vitamins, minerals, amino acids, fibre, proteins etc. in order to comply with the legal definition of food supplements laid down in Art. 1381 CAA.

'Disposición' ANMAT N° 1637/2001 contains two annexes relating to herbal ingredients used in food supplements:

- Positive list of 35 herbs and other substances of plant origin that can be used in food supplements.
- List of 118 herbal ingredients prohibited for use in food supplements.

In addition to this regulation on herbals, the plants regulated under the Food Argentine Code are also permitted to be used in food supplements since their food use is acknowledged. Guarana (*Paullinia cupana*) is for instance one of these plants regulated under the CAA and allowed to be used in food supplements in Argentina.

In practice, the Argentine authorities ANMAT (the National Administration of Medicines, Foods and Medical Technology) seem to follow a rather restrictive approach when assessing imported food supplement products containing botanicals at the time of food product registration (stability issues). The tradition of use of herbs in medicinal products in Argentina—and overall in Latin America—is not to be neglected.

Argentina is currently revising its regulation on dietary supplements, including the list of permitted botanical ingredients. In 2015 the revised regulation was under public consultation, where the list of permitted herbs was proposed to be included in the CAA and the information to be submitted at the time of requesting the inclusion of a new herb was also detailed. The sanitary authorities are still drafting the new regulation.

2.3.4.2 Brazil

In Brazil, various regulations cover foods presented in the form of tablet, capsule, powder and powder to be diluted. The regulation "Portaria n°32/98" applies to vitamin and/or mineral supplements only. The concept of food supplement with combination of vitamin and/or minerals with other substances such as herbs does not exist.

Plants/herbs can be regulated under the drug or food law, depending on their traditional use. On one hand, plants under the medicine umbrella can fall in one of the following two categories:

- "Fitoterápicos" (phytotherapeutics);
- "Drogas vegetais" (plant drugs), also commonly known as "medicinal plants of popular tradition".

The difference between these two is that plant drugs can be distributed in their integral form, in pieces, crushed or in powder, and that phytotherapeutics are marketed in pharmaceutical forms.

On the other hand, herbs/plants falling under the food umbrella are covered by the ANVISA Resolution n°16/99 on "New Foods and Ingredients". In Brazil, "New Foods and Ingredients" are defined as: "foods or substances with no history of use in Brazil, or foods with substances already in use, but that will be added to foods or used at levels much higher than those currently observed in foods used in a regular diet".

Various plants, mainly preparations of vegetables and fruits, and other bioactive substances in a dose form have been approved as "New Foods".

Nutrients or non-nutrients associated with any vegetal species that are included in the list of "phytotherapeutics" or "medicinal plants of popular tradition" cannot be regarded as food. Therefore, these will not be able to be marketed as a "food supplement" product.

Brazil is currently revising the "Portaria n°32/98" in order to develop a single piece of regulation for food supplements, which would address all types of permitted ingredients, including botanicals.

2.3.5 United States

The Dietary Supplement Health and Education Act (DSHEA) was signed into law in 1994 creating a new regulatory framework for dietary supplements as a separate category of foods and establishing requirements for safety and labelling.

Under this framework a company is responsible for determining that the dietary supplements it manufactures or distributes are safe and that any representations or claims made about them are substantiated by adequate evidence to show that they are not false or misleading. Dietary supplements do not need approval from FDA before being marketed. Companies that manufacture or distribute dietary supple-

ments containing "new dietary ingredients" are required to submit pre-market safety notifications. Dietary supplements containing only ingredients that are "not new" used previously as conventional foods before the law of 1994 are exempt from the notification requirement. FDA can take regulatory action to remove unsafe products from the market, including products containing new dietary ingredients for which there is inadequate evidence of safety in a pre-market safety notification.

A company does not have to provide FDA with the evidence it relies on to substantiate "structure/function claims" as a statement of nutritional support or effectiveness before or after it markets its products. FDA has published guidelines on "substantiation" of "structure/function claims" for use by manufacturers that want to substantiate a structure /function claim by the application of a substantiation standard of competent and reliable scientific evidence to claim about the benefits and safety of dietary supplements. Within 30 days of making a structure/function claim, the manufacturer/distributer must notify FDA of the wording of the claim. "Health claims" (e.g. reduction of risk of disease) can only be made under the authorisation of a specific regulation, which may be initiated by a petition to FDA. If a manufacturer or distributor wishes to claim that a product be used to diagnose, treat, cure or prevent a disease then the product by law is a drug and must meet the requirements for drugs. The U.S. has no separate category for traditional medicines.

In July 2011 FDA published draft guidelines on how to comply with the regulatory requirement to provide a pre-market safety notification for dietary supplements containing new dietary ingredients. This draft contains criteria on how to determine the identity of plant-based ingredients and how to use history of use or other evidence to demonstrate the safety of plant based ingredients.

2.4 The Regulatory Framework for Safety Assessment

2.4.1 Safety Management in the EU

Botanicals are not subject to a systematic pre-market safety assessment in the EU. The horizontal food legislation in place, both harmonised and national and the long history of use of many botanicals, covered by positive and negative lists, together with the Rapid Alert System for Food and Feed (RASFF) and a national notification system, constitute a substantial legal framework consistent with the requirements of food safety.

Safety is covered by many legal texts that are fully applicable to BFS. This includes:

– The GFR containing obligations to ensure that food put on the market is safe, notification whereby the competent authorities must be informed in cases where a food may not be in conformity with the food safety requirements and companies must have procedures in place to be able to recall or withdraw such products from

the market. Additionally, full traceability of the product and all of its ingredients—the tracking of the food/food ingredient from 'farm to fork'—is mandatory.
- The NFR, ensuring that foods and food ingredients, including new botanicals, which have not been used for human consumption to a significant degree within the EU prior to May 1997, are subject to a pre-market authorisation procedure, involving an assessment of the safety of these foods following an application for authorisation.
- The FFR, establishing in Article 8, a procedure to be used in cases where a substance other than vitamins or minerals, or an ingredient containing a substance other than vitamins or minerals, is added to foods or used in the manufacture of foods under conditions that would result in the ingestion of amounts of this substance greatly exceeding those reasonably expected to be ingested under normal conditions of consumption of a balanced and varied diet and/or would otherwise represent a potential risk to consumers. In such cases, on its own initiative or on the basis of information provided by the MS, the EC may take a decision, following an assessment of available information by EFSA, to include the substance in Annex III of the FFR. This will make it possible to draw up a list of substances whose use in foods is prohibited, restricted or under EU scrutiny. Yohimbe (Pausinystalia yohimbe (K. Schum.) Pierre ex Beille) and Ephedra ssp. Have already included in this annex.

A number of tools also are in place at EU level to help enforcement ensure the safety of the food chain.

- The GFR and the Official Controls Regulation 882/2004 (OCR) specify the obligations of enforcement authorities in terms of food safety[32].
- Furthermore, safeguard clauses in many of the applicable EU legislation allow Member States to take action in case of unexpected emerging safety risks. This means that when, as a result of new information or reassessment of existing information, there are detailed grounds for establishing that a product endangers human health, a MS may temporarily suspend or restrict its availability/use, even if it fully complies with the relevant EU legislation. It must inform then the other MS and the EC, who must then examine the grounds for the decision and deliver its opinion and take any appropriate measures.
- The RASFF, created by the GFR provides for an effective tool to monitor and communicate health risks among the Member States[33]. Rapid Alert notifications are sent in a number of cases, including when a food or feed presenting a serious risk is on the market and when immediate action is required. Alerts are triggered

[32] Regulation (EC) No 882/2004 of the European Parliament and of the Council of 29 April 2004 on official controls performed to ensure the verification of compliance with feed and food law, animal health and animal welfare rules. Official Journal of the European Community L165/1, 30 April 2004.

[33] Commission Regulation (EU) No 16/2011 of 10 January 2011 laying down implementing measures for the Rapid alert system for food and feed. Official Journal of the European Community L6/7, 11 January 2011.

by the Member State that detects the problem and initiates the relevant measures, such as withdrawal/recall. The notification aims to give all the members of the network the information to verify whether the product concerned is on their market, so that they also can take the necessary measures. The Commission publishes on its website a weekly overview of Rapid Alert notifications, information notifications, and border rejections.[34]

2.4.2 Safety Management at National Level

There is no systematic safety approval of botanicals for use in foods in the EU and there are no specific requirements for botanicals in the FSD.

Still the FSD has given MS the possibility to impose a notification procedure by which companies are required to inform the authorities of the marketing of a product by means of the product label.

The way in which this notification is applied differs between the MS. In some cases it is a well developed and managed system by which the authorities request more detailed information, assess the information and take actions as appropriate (e.g. in Belgium, France, Italy). In other MS the notification acts as information for enforcement authorities to monitor the market. Five of the MS have not even considered it necessary to impose this notification (Austria, Netherlands, Slovenia, Sweden, UK).

Although it is not permitted for MS to impose a pre-market authorisation process for such products, in the MS where notification requirements are extensive, it nevertheless acts as a sort of pre-market verification as most companies await the assessment of the authorities.

Furthermore, the use of botanicals falls under national law and a number of risk management measures have been applied to botanical food supplements by specific MS. These are mostly under the form negative lists (containing botanicals the use of which is prohibited) and/or positive lists, including botanicals that are allowed, with or without conditions of use, maximum levels and/or advisory labelling statements.

Guidance and conditions for appropriate processing and quality assurance, based on the FHR, often developed by the sector and approved by authorities may complement these national lists and notification requirements. This is important as botanicals can carry inherent safety risks that can be managed by appropriate processing and quality assurance, for example:

– The harmful components may be associated only with one of the plant's components (e.g. the leaves, fruits, seeds, roots). Removing it makes the plant fit for consumption, as is the case with potatoes, where the leaves are toxic but the tubers valuable foods.

[34] http://ec.europa.eu/food/safety/rasff/index_en.htm.

- A plant may be used as raw material for the production of additives, flavours, and functional food ingredients using processing techniques such as isolation, extraction and purification and appropriate controls to remove undesirable components. For example it is generally accepted that the oil from the borago species is acceptable for food use when it can be demonstrated analytically that the oil does not contain pyrrolizidine alkaloids.
- A plant may be subjected to a treatment that inactivates or destroys the undesirable components. For instance, it is well known that it is necessary to cook beans (Phaseolus vulgaris) at adequate temperature to destroy the phytohaemaglutin or lectins they contain.
- A plant may show harmful effects at high doses but not at a lower dose. Assessment of the dose and ways in which to ensure that such doses are not exceeded are part of the safety assessment. Some Member States have established maximum levels for plant components.
- A plant may show undesirable effects for specific population groups, even when used in an appropriate way, or it may interact with other foods or medicinal products. In such cases the labels of the botanical products carry appropriate warning information.
- Undesirable properties may be restricted to one single species of an entire plant family. In such a case appropriate methodology or measures are required to make sure that the toxic species is identified and separated from other members of the same family and contamination thus avoided.

Finally, another tool that is increasingly established to help ensure safety of FS is a system for nutrivigilance. Such a system collects information about adverse effects experienced by users of FS and other products. In that way causal relationships can be identified and where appropriate enforcement steps taken.

The combination of these measures in combination with EU and national law are considered a strong framework ensuring the safe use of BFS.

2.5 The Regulatory Framework for Benefit Assessment

2.5.1 Nutrition and Health Claims in the EU

Since 2006 all nutrition and health claims made on foods, including food supplements are subject to a pre-marketing authorisation after an assessment of the scientific justification by EFSA.

The criteria for this assessment have not been specified in the law. The only requirement is mentioned in recitals 17 and 23 of the Regulation, which state respectively that “Scientific substantiation should be the main aspect to be taken into account for the use of nutrition and health claims and the food business operators using claims should justify them. A claim should be scientifically substantiated by taking into account the totality of the available scientific data, and by weighing

the evidence" and that "Health claims should only be authorised for use in the Community after a scientific assessment of the highest possible standard. In order to ensure harmonised scientific assessment of these claims, the European Food Safety Authority should carry out such assessments."

The methodology EFSA would apply was not disclosed until after the adoption of the law, when EFSA published guidance on how to compile a submission. This guidance and the learnings from the opinions published showed that, apart from essential nutrients, EFSA only accepted human intervention studies as sufficient justification for a claim.

In assessing the scientific evidence, EFSA verifies the following elements:

- That the food/constituent is well defined and sufficiently characterised.
- That the claimed effect is well defined, is a beneficial physiological effect for the target population, and can be measured in vivo in humans.
- That a cause and effect relationship is established between the consumption of the food/constituent and the claimed effect in humans (for the target group under the proposed conditions of use), by considering the strength, consistency, specificity, dose–response, and biological plausibility of the relationship.
- That the quantity of the food/constituent and pattern of consumption required to obtain the claimed effect could reasonably be achieved as part of a balanced diet.
- That the wording of the claim reflects the scientific justification provided.
- The conditions or restrictions of use of the food and whether additional statements or warnings that should accompany the health claim on the label and in advertising are required.

It is clear that EFSA focuses extensively on the validity of end-points used (biomarker, physiological or clinical effect) and the size of effect.

This has proven problematic for botanicals as such studies have not been required before, not under national, nor under medicinal regulations. When EFSA therefore started its assessment of the submitted health claims for botanicals, it delivered only negative opinions.

2.5.2 Standard for Scientific Assessment Vs. Tradition of Use

Botanicals are also used in medicinal products by virtue of the THMPD. In this legislation the problem of the scientific justification of the benefits of botanicals was directly addressed. The Regulator excluded such products from the requirement of demonstrating efficacy if traditional use for 30 years (of which 15 years in the EU) could be demonstrated.

Or as it is stated in whereas 5: "The long tradition of the medicinal product makes it possible to reduce the need for clinical trials, in so far as the efficacy of the medicinal product is plausible on the basis of long-standing use and experience. Pre-clinical tests do not seem necessary, where the medicinal product on the basis of the information on its traditional use proves not to be harmful in specified conditions of use."

Table 2.2 Accepted indications for medicinal products in HMPC monographs and for foods in EFSA opinions

THPM Monograph indications	EFSA beneficial physiological effects
Symptoms of temporary fatigue and sensation of weakness	Reduction of tiredness and fatigue is
Symptomatic relief of digestive disorders such as dyspepsia […], bloating and flatulence	Reduction of gastro-intestinal discomfort is
For relief of mild symptoms of mental stress	Resistance to mental stress might be
Used to aid sleep	Reduction of sleep onset latency and improvement of sleep quality might be
For relief of […] heaviness of legs related to minor venous circulatory disturbances	Maintenance of elasticity and strength of the venous walls is
For the prophylaxis of migraine headaches after serious conditions have been excluded	Relief from stress-induced headache is
For the relief of minor symptoms in the days before menstruation (premenstrual syndrome)	Reduction of menstrual discomfort is
For the relief of menopausal complaints such as hot flushes and profuse sweating	Reduction of menopausal discomfort is
For the treatment of habitual constipation or in conditions in which easy defecation with soft stool is desirable	Changes in bowel function such as reduced transit time, more frequent bowel movements, increased faecal bulk, or softer stools may be

Thus THMPs can be registered on that basis and are permitted to claim their intended use without the reliance on intervention trials showing an effect on appropriate biomarkers or physiological or clinical functions.

This tradition of use is specific for botanicals, given their long-standing use. The Herbal Medicinal Product Committee (HMPC) within the European Medicinal Agency (EMA) has since then developed over 100 monographs in which they describe the traditional benefits and the conditions of use of the products based on tradition of use. The outcome of this work shows that the traditional effects of many plants described can be classified as beneficial physiological effects, rather than effects that relate to the treatment or prevention of disease. Table 2.2 gives examples of such claims and shows the similarity of the effect described in the monographs as compared to effects for which EFSA has confirmed these are beneficial health effects.

Because of this inconsistency, the European Commission decided in September 2010 to stop the assessments of health claims for botanicals. Indeed, given that all assessments would have resulted in negative opinions because tradition of use was not accepted, this would have led to the prohibition of any health benefit communication for botanicals used in food supplements, while at the same time, the same effects could continue to be used on THMPs solely on the basis of tradition of use.

To date, these claims for botanicals in food and food supplements have remained on hold and the Commission has started in 2013 discussions with the Member States on what route to take to solve this problem:

– Either continue with the assessments as foreseen, leading to the probable rejection of all claims.
– Either to exempt botanicals and accept tradition of use by changing the Claims Regulation or developing a new legislative framework for these products. In this latter case, it would also be possible to address specific aspects of safety and quality.

In 2015 this discussion has been formalised in a study covered by the Commission's better Regulation initiative. This so-called REFIT (Regulatory Fitness and Performance programme) assessment will gather views on the above and assess the consequences of each of the proposed scenarios.

This assessment is carried out by an independent contractor and is scheduled to start in September 2016, with the report expected end 2017.

On the basis of this report, the Commission is expected to come forward with a proposal. In the mean time, consumers still can be informed about the traditional benefits of botanicals on food supplements labels.

Chapter 3
Assessment of Food Supplements Containing Botanicals in Epidemiological Research

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Abstract The purpose of this chapter is to present an overview of how food supplements containing botanicals (or PFS) are being assessed in epidemiological research. The chapter is organized in six sections: Sect. 3.1 is the introduction, which provides a background to the topics explained in the subsequent sections, and also describes the objectives of the chapter; Sect. 3.2 describes the market structure of the food supplements containing botanicals in EC Member States, for which a revision of recent reports on market data, trends and main distribution channels, is presented; Sect. 3.3 describes the methods and administration techniques used to assess individual food consumption, which are adapted to design the methods and techniques for the assessment of individual intake of food supplements containing botanicals; moreover, the methodology designed for data collection on PFS consumption in a six-European-country survey within the PlantLIBRA project is presented as an example of tools used in the assessment of these products; Sect. 3.4 describes the methodology used in the PlantLIBRA Consumer Survey in detail; Sect. 3.5 presents selected results of the first analyses of the PlantLIBRA Consumer Survey, also highlighting the issues associated with measuring usage of PFS in European populations and making recommendations for future research; finally, Sect. 3.6 presents a systematic review conducted with the purpose of analysing the uses that PFS have in gastrointestinal health and disease through selected plants.

Keywords Food supplements • Botanicals • Intake assessment • Epidemiological research • European survey

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3.1 Introduction

Botanicals and their derivatives/preparations are used throughout Europe for health purposes, with increased usage in the general population as well as among specific subgroups encompassing children and pregnant women or those suffering from diseases such as cancer among others (Menniti-Ippolito et al. 2002; Ritchie 2007; Adams et al. 2009; Bishop and Lewith 2010). Botanicals are used in many different types of products, including foods, (teas and juices), food supplements such as plant food supplements (PFS), herbal medicinal products (HMP), homeopathic products, cosmetics, biocides etc. (Larrañaga-Guetaria 2012). These different product categories are regulated by specific legislation, depending on the intended use of the product.

The European Union (EU) Directive on Food Supplements (2002/46/EC) defines dietary supplements (which include PFS) as (European Parliament 2002a):

> "…foodstuffs the purpose of which is to supplement the normal diet and which are concentrated sources of nutrients or other substances with a nutritional or physiological effect, alone or in combination, marketed in dose form, namely forms such as capsules, pastilles, tablets, pills and other similar forms, sachets of powder, ampoules of liquids, drop dispensing bottles and other similar forms of liquids and powders designed to be taken in measured small quantities".

The marketing of a product as a PFS however, depends on national legislation, which differs widely across Member States. Countries vary in the extent to which products are regulated, as well as in the process of regulatory control. Some countries have regulated the use of botanicals in detail (including negative and positive lists), some apply specific conditions of use, (including maximum usage levels or warnings for the consumer), and in others less specific requirements exist. An added complexity lies in the application of the basic European "principle of mutual recognition", whereby any product that is lawfully marketed in one Member State can be sold in all 27 Member States (Larrañaga-Guetaria 2012).

Moreover, the same botanical may be used as a food supplement and as a medicinal product, depending on the intended use of the product and both food supplements and medicinal products often share the same form of presentation (powders, pills or tablets). Hence the legal status of products differs from one country to another, resulting in a complex market environment. This so-called borderline issue between PFS and HMP is a major obstacle to the marketing of PFS in the European Union (Larrañaga-Guetaria 2012).

Plant food supplement usage data at EU level are scarce with reports providing PFS market data as opposed to data reported directly by the consumer (EAS 2007). Surveys on the intake of botanicals have been conducted primarily in the context of the intake of dietary supplements in general (Skeie et al. 2009) or as part of surveys of complementary and alternative medicine (CAM) therapies (Vargas-Murga et al. 2011), and issues such as the legal distinction between HMP and PFS have not been taken into account. A recent systematic review evaluating the demographic characteristics and health status factors associated with CAM use reported that the majority of population based consumption studies had been

conducted in the USA (64% of the 110 identified studies), and of these, 13% were in Europe, with the majority carried out in Scandinavia (7%) and the United Kingdom (5%) (Bishop and Lewith 2010). Studies have been limited by the heterogeneity of definitions used, study designs and objectives making it difficult to compare results and to extrapolate conclusions. The ambiguity of categories such as "natural medicine", "herbal remedies" or "herbal medicine" and what constitutes "dietary supplements" makes it nearly impossible to attain reliable estimates of the prevalence of PFS usage in Europe, with only limited data available at national levels (Vargas-Murga et al. 2011; Harrison et al. 2004; Centro de Investigación sobre Fitoterapia 2007) but not at the European level.

A study by the European Advisory Services (EAS) on "The use of substances with nutritional or physiological effect other than vitamins and minerals in food supplements" (EAS 2007), provided information on European market and regulation data, and highlighted the need for obtaining PFS usage data in order to plan, monitor and evaluate national and European policies, as in other regions of the world. One such example is the United States of America, where the Alternative Health/CAM supplement of the National Health Interview Survey (NHIS) has been collecting data on botanical dietary supplements for some years now (National Center for Health Statistics 2003; Bardia et al. 2007; Dwyer et al. 2013).

The European Food Safety Authority (EFSA) has recognised the lack of data in the sector and has published a number of reports addressing related issues, namely the recommendations for reporting the use of supplements and medicines by adults in any pan-European dietary survey or project (EFSA 2009), and the "Compendium of botanicals reported to contain naturally occurring substances of possible concern for human health", aimed to help with the safety assessment of botanicals and botanical preparations intended for use as food supplements (EFSA 2012).

The purpose of this chapter is to present an overview of how food supplements containing botanicals (or PFS) are being assessed in epidemiological research. It is organized in five sections: Sect. 3.2 describes the market structure of the food supplements containing botanicals in EC Member States, for which a revision of recent reports on market data, trends and main distribution channels, is presented; Sect. 3.3 describes the methods and administration techniques used to assess individual food consumption, which are adapted to design the methods and techniques for the assessment of individual intake of food supplements containing botanicals; moreover, the methodology designed for data collection on PFS consumption in a six-European-country survey within the PlantLIBRA project is presented as an example of tools used in the assessment of these products; Sect. 3.4 describes the methodology used in the PlantLIBRA Consumer Survey in detail; Sect. 3.5 presents selected results of the first analyses of the PlantLIBRA Consumer Survey, also highlighting the issues associated with measuring usage of PFS in European populations and making recommendations for future research; finally, Sect. 3.6 presents a systematic review conducted with the purpose of analysing the uses that PFS have in gastrointestinal health and disease through selected plants.

3.2 Market Structure of the Food Supplements Containing Botanicals in EC Member States

Food supplements (FS) are regulated by Directive 2002/46/EC, known as the Food Supplements Directive, and may be marketed within the Community only if they comply with the rules laid down in this directive (European Parliament 2002b). The objective of the document was to harmonize EC rules across Member States, but it did not provide for substances other than vitamins and minerals, such as amino and fatty acids, fibers, plants and plant extracts, to be used in FS and they continue being regulated by various national decrees. The manufacturer or the person placing the product on the market in the Member States territory is obliged to notify the competent authorities of these activities by forwarding a model of the label used. This process is free of charge in some European countries.

This section describes the PFS market structures in European Community (EC) Member States, for which a revision of recent reports on market data, trends and main distribution channels, is presented. The content presented in this section has been adapted from Vargas-Murga et al. (2011).

3.2.1 Market Data

Member States have a dynamic market for PFS, and in general for herbal products. Recent reports concerning the market data are published by Business Insights (BI), which cover the market for vitamins and minerals, herbs and botanicals, and sports and speciality supplements in Europe and the United States (US), and by Global Industry Analysts (GIA), which analyses the worldwide markets for herbal supplements and remedies (Tallon 2011; GIA 2011). According to BI, in the EC, the Nutrition and Health Claims Regulation (EC) No. 1924/2006 is highly controversial. For instance, up until 2011, the European Food Safety Authority (EFSA) had published 1851 opinions on 4951 submitted claims covering reduction of disease to basic structure function claims. 91% of the claims with published opinions submitted under the 13(3) route have received a negative evaluation by EFSA.

The global herbal supplements and remedies market exhibited robust growth over the first decade of the millennium, with little or apparently no significant decline on account of the worldwide recession and was forecasted to reach US$93.15 billion by the year 2015, according to GIA. The world market in fact, exhibited steady growth for the crisis-ridden period of 2008–2009 and beyond. Recession in the European economy and the increased capital requirements for registration under EC regulation of companies expanded the resources of small companies and provided opportunities for acquisitions in herbal supplement markets. In the US and Europe, herbal medicines represent a major share of the pharmaceutical market and are included in regular medicinal practice. However, the market is highly regulated and of difficult access, as companies need to pass rigorous tests before mass production.

In 2011, Europe represented the largest regional market, accounting for the single largest share of the world market. Asia-Pacific and Japan made up the other important markets for herbal supplements on a global basis. In terms of growth rate, the Asia-Pacific market, led largely by China and India, was set to pave the way with the highest Compound Annual Growth Rate (CAGR) of 10.7% through 2015. The market for herbal supplements varies by region based on factors such as consumer awareness, product availability and forms of delivery, product acceptance, and regional regulations (Global Industry Analysts 2011).

A study elaborated by the European Advisory Services (EAS) provides detailed data about the four largest EC Member States, in terms of sales, led by Italy, which is closely followed by Germany, UK and France (EAS 2007). According to this study, growth projections to 2010 provided an indication of the extent to which previous rapid growth could not be taken as an indicator of future rapid growth. However, market growth was not expected to reach the levels achieved in the previous decade. The reasons for the market growth decrease might have been due to changes of some important economic factors, for example market saturation. Other factors having a strong impact on the growth of the market of FS containing other substances might be the notification/authorisation of national requirements, restrictions on distribution channels and the extent to which the national authorities apply mutual recognition (EAS 2007).

Referring to herbal ingredients, the EAS reported that ginkgo followed by echinacea, garlic and ginseng were the four most commercially important botanicals in the combined markets of 17 EC Member States, although echinacea and gingko were part of the composition of products registered as medicines. The wide variations in the size of national markets are, in some cases, due to the regulatory origins.

3.2.2 Market Trends

According to the report by GIA (GIA 2011), a major trend observed in the market is a shift from a single ingredient market to multiple ingredient-based medications for a particular condition. There is also an increased demand for herbal and botanical products in multi formula and combination packed format, as well as for chewable capsules and tablets. Multi-herbs dominate as the largest segment, capturing a significant share of the overall herbal supplements and remedies market worldwide. The segment is also forecasted to surpass other markets, having the fastest compounded growth rate of 9.0% over the analysis period (2000–2006). Soy and specialty herbs are also expected to display strong growth potential in the future (GIA 2011).

Another important trend is seen in the type of consumer. According to the GIA, women, particularly in the middle-aged bracket, form the major consumer group

owing to their growing health-consciousness, increased concern for diet, and enhanced attention towards preventive healthcare. In addition, there is a greater urgency to maintain healthy lifestyles, focusing on alternatives for conventional medicine and general health. Some of the health benefit for which consumers consider herbal and botanical supplements as natural alternatives include: hormone replacement therapy, prostate health, brain health and cognitive function, and joint and connective tissue health.

GIA reports that the importance of a healthy diet and lifestyle reigns in the minds of the consumer, which is not affected even by the financial crisis witnessed in almost every other product segment worldwide. In fact, the recession may have actually prompted increased preference for dietary supplements. Escalating prices, tighter budgets and high health care and lifestyle costs have actually driven consumers towards the more economical and perceived healthier and safer options of alternative medicine and dietary supplements for relief of physical and mental disorders.

3.2.3 Distribution Channels

Direct sales and consumer sales channels or retailers are the two marketing techniques for PFS used by manufacturers, distributors and importers.

Direct sales include mail order, e-commerce, multilevel marketing and medical & alternative health practitioners, whereas consumer sales address drugstores, health/natural food stores, herbal shops, parapharmacies, pharmacies, supermarkets/mass market, and among others, specialized shops (e.g. gym, hairdresser, healthcare institutions, sporting goods store).

According to GIA (GIA 2011), there is an increase in the number of retail outlets along with e-commerce, coupled with efficient support and cooperation of medical and health professionals.

Mail order and Internet sales are expected to continue growing as a result of the increasing number of Internet websites selling PFS.

The common and widely distributed retail channels in the Member States are drugstores, health food stores, herbal shops, pharmacies and supermarkets. Most consumers prefer to buy PFS in herbal shops and pharmacies where they can receive advice on product benefits and dosage.

Multilevel marketing, also known as direct selling, party plans, relationship selling, person-to-person selling, and network marketing constitute another important channel. However, few are used by manufacturers/distributors.

Because consumer demand has increased greatly, larger pharmaceutical companies are entering the market, often by buying supplement firms. As a result, the structure of the market is changing and will continue to change as the PFS market matures.

3.3 Methods and Techniques for the Assessment of Individual Intake of Food Supplements Containing Botanicals

The use of PFS is on the rise around the world; however, there are many problems associated with botanical research. These include among other problems, defining the concepts and selecting the appropriate study methods. The methodology used for the assessment of PFS consumption is an area that has been little explored at the public health level, and in essence consists of using existing dietary survey methods and procedures that have mainly been developed with the aim of evaluating the nutritional status of a population i.e. the intake of energy, macronutrients and/or micronutrients. For instance, in the USA, there are some routinely conducted surveys that obtain some (limited) information on dietary supplements including botanicals (US DHHS 1997; Barnes et al. 2008; Slone Survey 1998–2007).

The present section includes an overview of the methods and administration techniques used to assess individual food consumption as a starting point, including their uses and limitations. Moreover, the methodology designed for data collection on PFS consumption in a six-European-country survey within the PlantLIBRA project (PLANT food supplements: Levels of Intake, Benefit and Risk Assessment), a project co-financed in the context of the 7th EU Framework Program (FP7 Ref. 245199) (http://www.plantlibra.eu/web/) is presented as an example of tools used in the assessment of these products. The content presented in this section has been adapted from Vargas-Murga et al. (2011).

3.3.1 Methods for the Assessment of Individual Food Consumption: Uses and Limitations

Dietary intake is a highly variable event, which experiences significant changes depending on, for example, the day of the week and the season, based on an underlying pattern of consumption. Thus, within a week an individual can consume hundreds of different foods. Additionally, the interviewed person may not know exactly what he/she is eating and/or how much if he/she did not prepare the food. Both intake variation and the error inherent in its assessment method may affect the quality of results (Serra-Majem et al. 2006).

The methods for collecting dietary information at the individual level are properly called food surveys (Serra-Majem et al. 2006) and can be classified into two main groups (Gibson 2005):

Group 1. Quantitative daily consumption methods—comprises recalls or records designed to measure the quantity of the individual foods consumed over a 1 day

period, including single and repeated 24 h recalls and estimated and weighed food records. By increasing the number of measurement days, quantitative estimates of the usual intakes of individuals can be obtained, using the same instruments. The number, selection, and spacing of the days depend on the food intake, the nutrients of interest, the day-to-day variation in nutrient intake, and the level of precision required. Determination of usual intake is particularly critical when relationships between diet and biological parameters or chronic disease are assessed. Estimates of usual intakes are also needed to evaluate the prevalence of inadequate intakes.

Group 2. Dietary history and the food frequency questionnaire—both obtain retrospective information on the patterns of food use during a longer, less precisely defined reference time period. They can be used to assess the usual intake of foods or specific classes of foods, and with modification, can also provide data on usual nutrient intakes.

Table 3.1 includes the uses and limitations of the food consumption assessment methods described above (Serra-Majem et al. 2006).

3.3.2 Administration Techniques of Food Consumption Assessment Methods

Food consumption assessment methods can be administered using two main types of techniques:

- By interview. The interview can be personal or by telephone. Uses of these interview techniques include that they ensure the completion of all questions, allow you to use complex and multiple questions and to clarify questions that are not understood (although this can introduce bias), facilitate cooperation and can be applied in illiterate populations. Limitations include that they are costly and time-consuming and can introduce interviewer bias.
- Self-administration. The respondent completes the questionnaire by her/himself, which could have been handed out or posted to the respondent. Self-administration techniques have fewer uses/strengths e.g. absence of interviewer bias and low cost—and more limitations—they tend to be partially completed, can not be used for multiple and complex questions, is difficult to ensure understanding of the question, have a low response rate, and present restriction of subjects (literacy is required).

The limitations and advantages of each method and administration technique have to be placed in the context of their cost and quality of the information obtained (Serra-Majem et al. 2006).

Table 3.1 Uses and limitations of the different food consumption assessment methods in individuals

Uses	Limitations
24-h recall	
– The administration period is short – The procedure does not alter the individual's usual intake – It is useful for any type of food pattern – A single contact is sufficient – Can be used in illiterate subjects – Its cost is moderate – High response rates	– A single 24-h recall cannot estimate the individual's usual intake – It is difficult to accurately estimate the portion size – Depends on the respondent's memory – Trained interviewers are needed for its administration – Limited application in children and the elderly
Repeated 24-h recall	
– Can estimate the individual's usual intake	– It is difficult to accurately estimate the portion size – Depends on the respondent's memory – Trained interviewers are needed for its administration – Limited application in children and the elderly
Dietary records (estimated and weighed)	
– Accuracy in the estimation or calculation of the portions consumed – The procedure does not depend on the individual's memory	– The individual must be able to read, write and count – Requires much time and cooperation by the respondent, especially the weighted dietary record – Codification and analysis cost are high
Dietary history	
– Can give a more complete and detailed description of the usual and past dietary intake than the other methods – Can be used in illiterate people	– Requires a highly trained interviewer, usually a dietician – It takes time and lots of cooperation from the interviewee – The administration cost is high – There is no standard way to do the dietary history
Food frequency questionnaire	
– Can estimate the usual intake of an individual – Fast and easy to administer – The habitual consumption pattern is not altered – Does not require trained interviewers – Very low administration costs, especially if conducted by mail – Ability to classify individuals into categories of consumption, useful in epidemiological studies	– The development of the instrument (questionnaire) requires considerable effort and much time – Literacy of the subjects is required – Doubtful validity of the estimated intake of individuals or groups with very different dietary patterns to those of foods from the list – Validity must be established for each new questionnaire and population – Requires memory of eating habits in the past – Low accuracy in the estimation and quantification of food portions – Remembering of the diet in the past may be biased by the current diet – The time and inconveniences to the respondent increase according to the number of food items and the complexity of the food list and quantification procedures – It is not useful in the elderly and children – Little validity for most vitamins and minerals – Does not allow assessment of intra-individual variation in intake, since only a single measure is available

Source: Adapted from Serra-Majem et al. (2006) and Gibson (2005)

3.3.3 *Plant Food Supplements Consumption Assessment Methods*

There are many difficulties associated with PFS research, in particular with establishing standardised concepts and definitions and with the choice of methodologies to assess botanical/plant food supplement consumption at the individual level. The dietary consumption assessment methods described above cannot detect consumption of PFS as they are, but can be adapted and used as a starting point in the design of assessment methods more suitable for PFS consumption.

The European Commission-funded PlantLIBRA project (http://www.plantlibra.eu/web/) addressed the abovementioned limitations in estimating PFS consumption by incorporating an activity to conduct a multi-country survey applying harmonised methodologies in the estimation of PFS usage. The aim was to obtain the most valid PSF intake data so as to estimate risk benefit of PFS consumption.

In summary, a cross-sectional retrospective method (consisting of two questionnaires: a screening and a main questionnaire) was used to evaluate the habitual consumption of PFS in the last 12 months on an individual level. This method allowed for the collection of data relating to the level of intake, some specific characteristics of each PFS consumed such as brand and manufacturer of PFS, the type of botanical preparations consumed, as well as the frequency, seasonality and duration of consumption of individual PFS over the last 12 months and over the lifespan. Sources of information about PFS, source of recommendations and the most frequent reasons for use of these products were also assessed. The PFS screening questionnaire was administered in person (face-to-face) or by telephone. Further details about the methodology used (definitions and instruments) are included in Sect. 3.4.

3.4 The PlantLIBRA PFS Consumer Survey 2011–2012: Definitions and Instruments

As mentioned previously, the European Commission-funded PlantLIBRA project has addressed the abovementioned limitations in estimating PFS consumption in their incorporation of an activity to conduct a multi-country survey applying harmonised methodologies in the estimation of PFS usage: the PlantLIBRA PFS Consumer Survey 2011–2012.

The purpose of this section is to present the methodology designed and implemented for the collection of PFS consumption data through the PlantLIBRA PFS Consumer Survey, including the main definitions and the most important instruments used. The content presented in this section has been adapted from Garcia-Alvarez et al. 2014.

3.4.1 Survey Methodology

3.4.1.1 Definition of Plant Food Supplements in the PlantLIBRA PFS Consumer Survey

Although there is a legal definition of Food Supplements (EU Directive (2002/46/EC) (European Parliament 2002a) under which PFS reside, for the purposes of this research it was necessary to develop a specific definition of PFS whose main characteristic is that they contain botanical preparations as ingredients for food supplementation.

Botanical preparations are obtained by subjecting botanicals (plants, algae, fungi or lichens) to treatments such as comminution, extraction, distillation, squeezing, fractionation, purification, concentration or fermentation. These include extracts, essential oils, expressed juices, powders, etc.

Botanical preparations can be considered as *nutrients* or *other substances*. Thus, the definition of PFS for the survey was as follows: PFS are "*foodstuffs the purpose of which is to supplement the normal diet and which are concentrated sources of botanical preparations that have nutritional or physiological effect, alone or in combination with vitamins, minerals and other substances which are not plant-based. PFS are marketed in dose form, such as capsules, pastilles, tablets, pills and other similar forms, sachets of powder, ampoules of liquids, drop dispensing bottles, and other similar forms of liquids and powders designed to be taken in measured small unit quantities*".

Products that did not meet this definition, such as herbal remedies and other medicinal products based on botanicals, and those that did not meet the PFS definition in terms of dosage, such as herbal teas or juices, were excluded.

3.4.1.2 Ethics Statement

Before initiating the fieldwork, approval for the conduct of the survey was obtained from four ethics committees: the Bioethics Commission of the University of Barcelona, Spain; the Ethics Committee of the University of Milano, Italy; the Ethical Committee of the Faculty of Medicine—Transilvania University of Brasov, Romania; and the Coordinating Ethics Committee, Hospital District of Helsinki and Uusimaa, Finland. Approval of the survey by these four ethics committees required submitting all survey material to their members for evaluation. No ethical approval for the survey was needed in Germany and the United Kingdom.

To ensure harmonisation and standardisation of the fieldwork and data collection across countries, a market research organization, *European Fieldwork Group* (EFG) was subcontracted to implement the survey. The survey was conducted by EFG in strict accordance with the ICC/ESOMAR Code on Market and Social Research. In all countries, informed consent was obtained verbally from all respondents after reading the survey information sheet. All data were recorded manually i.e. pen-and-paper. Recruitment of survey participants occurred in the selected cities in each country. Approximately the first 1000 individuals per country were systematically selected for

screening i.e. intercepting 1 in every 5 individuals passing by to ask him/her the initial screening questions; subsequent screening selection was performed on a convenience basis i.e. intercepting individuals in places where consumers were likely to be found, such as herbal shops, pharmacies etc. Eligible respondents who agreed to participate were given an appointment at their home/workplace to complete the main survey. The appointments of those willing to participate were later reconfirmed by phone.

The data were made anonymous when recorded electronically i.e. the respondents' contact details were not entered into the survey database. Instead, the market research organization assigned ID numbers to each respondent and provided PlantLIBRA partners only the database with the assigned ID numbers.

3.4.1.3 Sample Population and PFS Consumer Definition

A cross-sectional, 12-month retrospective survey was conducted in 24 cities in six European countries—Finland, Germany, Italy, Romania, Spain and the United Kingdom. An estimated sample size of 2000 screened individuals per country was calculated in order to obtain a final sample of approximately 400 consumers per country (total N = 2400 approximately). Per country, gender and age group quotas were set as follows: 300 adults (18–59 years) and 100 older adults (60-and-over years), with 30–50% male and 50–70% female. All individuals were screened by means of a brief questionnaire, which recorded PFS usage in the preceding 12 months. Individuals were considered eligible for inclusion if they were over 18 years old and met either of the following specified criteria, intended to capture the different usage patterns of PFS consumers:

1. They had taken at least one PFS in the last 12 months, in an appropriate dose form at a minimum frequency of either:
   (a) One daily dose for at least two consecutive or non-consecutive weeks, or
   (b) One or more doses per week for at least three consecutive weeks or
   (c) One or more doses per week for at least four consecutive or non-consecutive weeks
2. They had taken two or more different PFS, in an appropriate dose form, at a minimum frequency of one or more doses per week, with the sum of the usage period of the two or more products being equal to at least 4 weeks.

3.4.1.4 Instruments and Variables

A short screening questionnaire was used to identify consumers who met the survey inclusion criteria; it consisted of six questions which allowed interviewers to identify eligible consumers, based on the product(s) used, the frequency and duration of use and the dose form. Eligible consumers subsequently completed a more detailed questionnaire on their PFS usage in the preceding 12 months, providing details of product/plant names, dosage forms, frequency of use, reasons for use, adverse effects, places and patterns of purchase and information sources on products. These questions were asked for each of up to a maximum of five different PFS used. In

addition, respondents were asked to provide socio-demographic data including age, gender, level of education and employment status, as well as self-reported height and weight and further health-related lifestyle information.

3.4.1.5 Survey Administration and Data Collection

Fieldwork and data collection for the cross-sectional survey were conducted by the international market research company EFG, from May 2011 to September 2012. The duration of the fieldwork ensured that any seasonal variability in usage of products was captured. The survey protocols and instruments—training material, information sheet, informed consent, screening and usage questionnaires—were initially developed in English by consensus amongst the research team, and subsequently translated into the respective languages in each of the survey countries. Pilot interviews were conducted in each participating country to assess the comprehension of the questions and to determine the time required to complete the survey.

In each participating country, trained interviewers systematically screened approximately 1000 individuals during the first 3 months of the survey, which allowed the estimation of the prevalence rate. Subsequently, screening and recruitment were conducted on a convenience basis. The recruited eligible consumers were interviewed face-to-face and the more detailed PFS usage questionnaire completed.

3.4.1.6 Data Preparation and Statistical Analysis

All data from the completed surveys were entered into the statistical package SPSS for Windows v. 18 (IBM Corporation, Somers, NY, USA), which was also used for data analysis.

Following review of the completed interviews by the research team in each country, a database with botanical composition data for all PFS products reported was compiled for each country and then merged into a single database. Potential product duplicates between countries were not removed. Each product was coded for its botanical ingredients in scientific, English and local names and botanicals were coded after removing duplicates between countries. Additionally, each product was categorised as a single- or multi-botanical product. To indicate the certainty of the matching of products, a series of numerical codes were used, based on those used in the National Health and Nutrition Examination Survey 2005–2006 (NCHS 2009). Values ranged from 1 to 5, where “1” indicated an exact match, “2” a probable match, “3” a reasonable match, “4” a default match and “5” no match. Only products with certainty values 1–4 have been included in the analyses.

Respondent data were recorded in a separate database. A number of variables were created and/or recoded to facilitate reporting and analysis, including: (1) “education level”, defined as low, medium, and high; (2) “BMI”, which was calculated from self-reported weight and height, and for which WHO cri-

teria (WHO 2013) were used to categorise individuals as underweight (BMI < 18.5 kg/m^2), normal weight (BMI 18.5 ≤ 25 kg/m^2), overweight (BMI 25 ≤ 30 kg/m^2) and obese (BMI ≥ 30 kg/m^2); (3) "physical activity", calculated using the short version of the IPAQ (Craig et al. 2003) and defined as low, moderate or high.

Absolute frequencies and percentages for each of the variable categories were used to describe the qualitative nominal/ordinal and discrete quantitative survey data. In turn, all data have been stratified by gender, age range and country—also using absolute frequencies and percentages and 95% confidence intervals. When describing the association between two qualitative variables (nominal or ordinal), contingency tables were used. The continuous quantitative variables (e.g. BMI, alcohol) were recoded into categorical variables.

It is important to note that when reporting the main results of the survey, the unit of analysis varies depending on the variables used, i.e. for certain variables the unit is an individual respondent, however, given the potential intake of multiple supplements by one respondent, the unit of analysis may change to the supplement level. Furthermore, all results presented in the tables represent the analysis of raw data as opposed to data weighted by the population size. Data were not weighted because of the study methodology selected, whereby all country samples were very similar in size and included only PFS consumers.

3.4.1.7 Validation Study

In order to validate the PFS usage questionnaire, a validation study was conducted in which the data collected using the survey instruments were compared with a 30 to 180-day diary (used as the gold standard). The study was conducted in two of the PlantLIBRA consumer survey cities: Las Palmas de Gran Canaria (Spain) and Milan (Italy), where 48 and 49 consumers respectively were recruited using convenience sampling. The PFS usage questionnaire was completed by the respondents at the beginning and at the end of the 6-month period of the validation; during this time the consumers also completed the usage diary. Data from the last questionnaire and the diary were compared for concordance, and results indicated a good agreement for product consumed, dose form and doses per day.

3.5 Usage of Food Supplements Containing Botanicals Across Europe: Results from the PlantLIBRA PFS Consumer Survey 2011–2012

The purpose of this section is to present some selected results from the retrospective PlantLIBRA PFS Consumer Survey 2011–2012—conducted in consumers from six European countries, which include the characteristics of the PFS consumer sample, the type of PFS usage reported and the most frequently used botanical ingredients

in these products. A discussion is also included in which the issues associated with measuring usage of PFS in European populations are highlighted and recommendations for future research are made. The content presented in this section has been adapted from Garcia-Alvarez et al. 2014.

3.5.1 Characteristics of the PFS Consumer Sample

A final sample of 2,359 consumers (those eligible and willing to participate) was recruited from 11,783 screened individuals (Table 3.2). Due to different legal frameworks (different distribution of botanicals in food supplements and medicinal products), more individuals had to be screened in Finland in order to recruit the required 400 consumers. Table 3.2 also shows the sample used for the estimation of the usage prevalence rate. The estimated weighted overall PFS usage prevalence rate was 18.8% and per-country rates were as follows: Finland 9.6%, Germany 16.9%, Italy 22.7%, Romania 17.6%, Spain 18.0% and the United Kingdom 19.1%.

Survey respondents were recruited to fixed quotas for age and gender, which were achieved, with some differences within countries (Table 3.3). In Finland the proportion of adults aged 50-59 years was significantly higher (26.2%), whilst the opposite was true in Italy, where consumers in that age group constituted only 13.0% of adults. Romania had a significantly higher number of consumers in the youngest age group (30.5%), in contrast to Spain and the United Kingdom, where this age group represented only 9.5% and 9.0% of adult consumers, respectively. A significantly higher proportion of female consumers were recruited in Spain (56.7%) and in the United Kingdom marginally more males were recruited (50.3%). Across all countries, more than half of the participants (57.5%) were employed (Table 3.3), with the percentages slightly lower in Finland (50.9%) and in the United Kingdom (52.4%). The majority of participating consumers were educated to medium level (Table 3.3).

Respondents were asked a number of questions regarding health-related lifestyle factors (Table 3.3). Less than half of the consumers had never smoked (46.6%), less than one quarter were ex-smokers (23.1%) and less than one third were current smokers (30.3%).

More than half of the total respondents (59.3%) had not consumed alcohol or had consumed it less than once daily; more than a tenth (12.6%) reported daily alcohol consumption.

The proportion of overweight and obese people in the survey was 49.8% (Table 3.3). Some significant differences in levels of physical activity were noted between countries. High levels of activity were reported by 85.5% of Romanian respondents compared to a value of 42.9% across all countries.

Most of the respondents (65.1%) reported not being regular consumers of food supplements other than PFS in the preceding 12 months, except for

Table 3.2 Distribution of screened individuals, PFS consumers interviewed and prevalence sample by country and gender

			Finland	Germany	Italy	Romania	Spain	United Kingdom	Total
Total contacts (n)	Total individuals screened for the survey	Males	1405	1031	907	795	811	830	5779
		Females	1379	1028	1044	827	932	794	6004
	Total PFS consumers interviewed accepted	Males	193	197	187	199	174	191	1141
		Females	208	201	191	201	228	189	1218
Prevalence sample: systematically selected sample 1st 3 months of the Fieldwork (n)	Individuals screened	Males	486	564	439	502	551	454	2996
		Females	519	571	547	501	648	563	3349
	PFS consumers among Individuals screened	Males	33	90	99	95	55	65	437
		Females	71	111	156	124	133	144	739
PFS consumption prevalence (weighted) (%)			9.6	16.9	22.7	17.6	18.0	19.1	18.8

Table 3.3 PlantLIBRA's PFS consumer survey—socio-demographic sample characteristics, overall and by country

Characteristics	Categories	All countries		Finland		Germany		Italy		Romania		Spain		United Kingdom	
		n	% (95 % CI)	n	% (95 % CI)	n	% (95 % CI)	n	% (95 % CI)	n	% (95 % CI)	n	% (95 % CI)	n	% (95 % CI)
Gender	Male	1141	48.4 (46.4–50.4)	193	48.1 (43.2–53.0)	197	49.5 (44.6–54.4)	187	49.5 (44.4–54.5)	199	49.8 (44.8–54.7)	174	43.3 (38.4–48.1)	191	50.3 (45.2–55.3)
	Female	1218	51.6 (49.6–53.7)	208	51.9 (47.0–56.8)	201	50.5 (45.6–55.4)	191	50.5 (45.5–55.6)	201	50.3 (45.3–55.2)	228	56.7 (51.9–61.6)	189	49.7 (44.7–54.8)
Age	18–29 years	418	17.7 (16.2–19.3)	63	15.7 (12.1–19.3)	77	19.4 (15.5–23.2)	84	22.2 (18.0–26.4)	122	30.5 (26.0–35.0)	38	9.5 (6.6–12.3)	34	9.0 (6.1–11.8)
	30–39 years	445	18.9 (17.3–20.4)	65	16.2 (12.6–19.8)	57	14.3 (10.9–17.8)	88	23.3 (19.0–27.6)	65	16.3 (12.6–20.0)	101	25.1 (20.9–29.4)	69	18.2 (14.3–22.0)
	40–49 years	460	19.5 (17.9–21.1)	64	16.0 (12.4–19.6)	82	20.6 (16.6–24.6)	63	16.7 (12.9–20.4)	46	11.5 (8.4–14.6)	88	21.9 (17.8–25.9)	117	30.8 (26.1–35.4)
	50–59 years	441	18.7 (17.1–20.3)	105	26.2 (21.9–30.5)	80	20.1 (16.2–24.0)	49	13.0 (9.6–16.4)	67	16.8 (13.1–20.4)	76	18.9 (15.1–22.7)	64	16.8 (13.1–20.6)
	≥ 60 years	595	25.2 (23.5–27.0)	104	25.9 (21.6–30.2)	102	25.6 (21.3–29.9)	94	24.9 (20.5–29.2)	100	25.0 (20.8–29.3)	99	24.6 (20.4–28.8)	96	25.3 (20.9–29.6)
Education	Low	249	10.6 (9.3–11.8)	47	11.7 (8.6–14.9)	3	0.8 (0.0–1.6)	72	19.1 (15.1–23.0)	35	8.8 (6.0–11.5)	92	22.9 (18.8–27.0)	0	–
	Medium	1549	65.7 (63.6–67.6)	237	59.1 (54.3–63.9)	329	82.7 (78.9–86.4)	222	58.7 (53.8–63.7)	190	47.5 (42.6–52.4)	256	63.7 (59.0–68.4)	315	82.9 (79.1–86.7)
	High	561	23.8 (22.1–25.5)	117	29.2 (24.7–33.6)	66	16.6 (12.9–20.2)	84	22.2 (18.0–26.4)	175	43.8 (38.9–48.6)	54	13.4 (10.1–16.8)	65	17.1 (13.3–20.9)
Current employment status	Employed	1357	57.5 (55.5–59.5)	204	50.9 (46.0–55.8)	240	60.3 (55.5–65.1)	221	58.5 (53.5–63.4)	249	62.3 (57.5–67.0)	244	60.7 (55.9–65.5)	199	52.4 (47.3–57.4)
	Other groups[a]	1002	42.5 (40.9–44.5)	197	49.1 (44.2–54.0)	158	39.7 (34.9–44.5)	157	41.5 (36.6–46.5)	151	37.8 (33.0–42.5)	181	39.3 (34.5–44.1)	181	47.6 (42.6–52.7)
Regular use of non-PFS FS[bc]	No	1536	65.1 (63.2–67.0)	83	20.7 (16.7–24.7)	251	63.1 (58.3–67.8)	311	82.3 (78.4–86.1)	274	68.5 (63.9–73.1)	312	77.6 (73.5–81.7)	305	80.3 (76.3–84.3)

(continued)

Table 3.3 (continued)

Characteristics	Categories	All countries		Finland		Germany		Italy		Romania		Spain		United Kingdom	
		n	% (95 % CI)	n	% (95 % CI)	n	% (95 % CI)	n	% (95 % CI)	n	% (95 % CI)	n	% (95 % CI)	n	% (95 % CI)
	Yes	767	32.5 (30.6–34.4)	306	76.3 (72.1–80.5)	122	30.7 (26.1–35.2)	63	16.7 (12.9–20.4)	112	28.0 (23.6–32.4)	89	22.1 (18.1–26.2)	75	19.7 (15.7–23.7)
	Not sure	56	2.4 (1.8–3.0)	12	3.0 (1.3–4.7)	25	6.3 (3.9–8.7)	4	1.1 (0.1–2.1)	14	3.5 (1.7–5.3)	1	0.3 (0.0–0.7)	0	–
Smoking habit	Never smoker	1100	46.6 (44.6–48.6)	182	45.4 (40.5–50.3)	183	46.0 (41.1–50.9)	181	47.9 (42.8–52.9)	214	53.5 (48.6–58.4)	177	44.0 (39.2–48.9)	163	42.9 (37.9–47.9)
	Former smoker	544	23.1 (21.4–24.8)	129	32.2 (27.6–36.8)	81	20.4 (16.4–24.3)	85	22.5 (18.3–26.7)	57	14.3 (10.8–17.7)	94	23.4 (19.2–27.5)	98	25.8 (21.4–30.2)
	Current smoker	715	30.3 (28.5–32.2)	90	22.4 (18.4–26.5)	134	33.7 (29.0–38.3)	112	29.6 (25.0–34.2)	129	32.3 (27.7–36.8)	131	32.6 (28.0–37.2)	119	31.3 (26.7–36.0)
Self-reported health status	Very good	353	15.0 (13.5–16.4)	81	20.2 (16.3–24.1)	49	12.3 (9.1–15.5)	22	5.8 (3.5–8.2)	80	20.0 (16.1–23.9)	49	12.2 (9.0–15.4)	72	19.0 (15.0–22.9)
	Good	1427	60.5 (58.5–62.5)	225	56.1 (51.3–61.0)	220	55.3 (50.4–60.2)	243	64.3 (59.5–69.1)	245	61.3 (56.5–66.0)	258	64.2 (59.5–68.9)	236	62.1 (57.2–67.0)
	Neither bad nor good	496	21.0 (19.4–22.7)	77	19.2 (15.3–23.1)	111	27.9 (23.5–32.3)	111	29.4 (24.8–34.0)	73	18.3 (14.5–22.0)	81	20.2 (16.2–24.1)	43	11.3 (8.1–14.5)
	Bad	70	3.0 (2.3–3.7)	16	4.0 (2.1–5.9)	18	4.5 (2.5–6.6)	2	0.5 (0.0–1.3)	2	0.5 (0.0–1.2)	14	3.5 (1.7–5.3)	18	4.7 (2.6–6.9)
	Very bad	13	0.6 (0.3–0.9)	2	0.5 (0.0 – 1.2)	0	–	0	–	0	–	0	–	11	2.9 (1.2–4.6)
CAM[d] usage	Yes	947	40.1 (38.2–42.1)	223	55.6 (50.7–60.5)	204	51.3 (46.3–56.2)	96	25.4 (21.0–29.8)	77	19.3 (15.4–23.1)	319	79.4 (75.4–83.3)	28	7.4 (4.7–10.0)
	No	1412	59.9 (57.9–61.8)	178	44.4 (39.5–49.3)	194	48.7 (43.8–53.7)	282	74.6 (70.2–79.0)	323	80.8 (76.9–84.6)	83	20.7 (16.7–24.6)	352	92.6 (90.0–95.3)
Alcohol consumption	0 ≤ 1 times/day	1398	59.3 (57.3–61.3)	281	70.1 (65.6–74.6)	245	61.6 (56.8–66.3)	116	30.7 (26.0–35.3)	232	58.0 (53.2–62.8)	291	72.4 (68.0–76.8)	233	61.3 (56.4–66.2)
	≥ 1 times/day	296	12.6 (11.2–13.9)	13	3.2 (1.5–5.0)	27	6.8 (4.3–9.3)	156	41.3 (36.3–46.2)	9	2.3 (0.8–3.7)	46	11.4 (8.3–14.6)	45	11.8 (8.6—15.1)

	Not sure	614	26.0 (24.3–27.8)	107	26.7 (22.4–31.0)	126	31.7 (27.1–36.2)	106	28.0 (23.5–32.6)	159	39.8 (35.0–44.6)	65	16.2 (12.6–19.8)	102	26.8 (22.4–31.3)
BMI[e] categories	Underweight	69	2.9 (2.4–3.6)	9	2.2 (0.8–3.7)	4	1.0 (0.0–2.0)	12	3.2 (1.4–4.9)	20	5.0 (2.9–7.1)	6	1.5 (.3–2.7)	18	4.7 (2.6–6.9)
	Normal weight	1116	47.3 (45.3–49.3)	188	46.9 (42.0–51.8)	198	49.7 (44.8–54.7)	246	65.1 (60.3–69.9)	184	46.0 (41.1–50.9)	169	42.0 (37.2–46.9)	131	34.5 (29.7–39.3)
	Overweight	818	34.7 (32.8–36.6)	147	36.7 (31.9–41.4)	159	40.0 (35.1–44.8)	98	25.9 (21.5–30.4)	142	35.5 (30.8–40.2)	155	38.6 (33.8–43.3)	117	30.8 (26.1–35.4)
	Obesity	356	15.1 (13.7–16.5)	57	14.2 (10.8–17.6)	37	9.3 (6.4–12.2)	22	5.8 (3.5–8.2)	54	13.5 (10.2–16.9)	72	17.9 (14.2–21.7)	114	30.0 (25.4–34.6)
Physical activity[f]	Low	436	18.5 (16.9–20.1)	53	13.2 (9.9–16.5)	87	21.9 (17.8–25.9)	141	37.3 (32.4–42.2)	5	1.3 (0.2–2.3)	43	10.7 (7.7–13.7)	107	28.2 (23.6–32.7)
	Moderate	909	38.5 (36.6–40.5)	156	38.9 (34.1–43.7)	139	34.9 (30.2–39.6)	191	50.5 (45.5–55.6)	53	13.3 (9.9–16.6)	234	58.2 (53.4–63.0)	136	35.8 (31.0–40.6)
	High	1012	42.9 (40.9–44.9)	192	47.9 (43.0–52.8)	171	43.0 (38.1–47.8)	45	11.9 (8.6–15.2)	342	85.5 (82.1–89.0)	125	31.1 (26.6–35.6)	137	36.1 (31.2–40.9)

[a]Other groups: Unemployed; Housework; Student; Retired; Disabled; and Other

[b]***Question asked:*** Other than PLANT FOOD SUPPLEMENT, have you taken any of the following supplements on a regular basis in the last 12 months? (mark all that apply). ***Possible responses:*** Vitamins (A, B, D, E, etc.); Minerals (e.g. potassium, calcium); Amino acids; Enzymes (e.g. lactase); Prebiotics (e.g. oligosaccharides, fibre); Probiotics (e.g. bifidobacteria, yeasts); Fatty acids (e.g. fish oil); Other

[c]*FS* Food supplements

[d]*CAM* Complementary and Alternative Medicine, including: Acupuncturist; Chiropractor; Homeopath; Herbalist; Massage therapist; Traditional/faith healer; Reflexologist; Recognised treatment i.e. not "alternative"; Esoteric treatment; and "Cannot be classified"

[e]*BMI* Body Mass Index; WHO categories (WHO 2013)

[f]IPAQ categories (Craig et al. 2003)

Table 3.4 PlantLIBRA's PFS consumer survey—characteristics of PFS reported by respondents

	Total	Finland	Germany	Italy	Romania	Spain	United Kingdom
Number of products	1288	213	190	289	196	284	116
Number of botanicals	491	196	191	222	219	218	47
Number of manufacturers	449	69	99	106	61	97	17
Maximum number of ingredients per product	46	23	46	20	39	30	8

Finland (Table 3.3). The proportion of non-consumers varied from 20.7% in Finland to more than 80% in the United Kingdom and Italy. By contrast, in Finland 76.3 % of the individuals were regular consumers of food supplements.

Over half of all respondents (59.5%) reported not having used CAM therapies/treatments in the past year. This is particularly the case in Italy (74.6%), Romania (80.8%) and the United Kingdom (92.6%).

Three quarters of consumers reported their health status as very good or good (75.5%), while 3.6% reported it as bad or very bad and 21.0% as neither bad nor good (Table 3.3). Between countries, more consumers reported their health status as very good or good in Romania (81.3%) and in the United Kingdom (81.1%) than in other countries; though conversely the highest proportion reporting their health status as bad or very bad was also in the United Kingdom (7.6%).

3.5.2 PFS Products Used

Respondents reported a total of 1288 products across the six countries. At individual country level, the highest numbers of different PFS were used in Italy (289) and Spain (284); in the United Kingdom, the number of different PFS was approximately half that of the other countries (Table 3.4). The number of different botanical ingredients was 491, with the maximum number of different botanicals contained in a single product being 46 and present in a German product. The United Kingdom differed from the other countries as the products reported contained a lower number of botanical ingredients (maximum 8).

3.5.3 Botanicals Used

A total of 491 botanicals—used in at least one PFS—were reported across the six participating countries. An overview of all the reported botanicals—clustered by intervals of frequency of intake (number of consumers ranging from 194 to 5)—is shown in Table 3.5. Based on the survey results, the eleven most frequently used

botanicals (numbers of consumers ranging from 194 to 100) in descending order are *Ginkgo biloba* (ginkgo), *Oenothera biennis* (evening primrose), *Cynara scolymus* (artichoke), *Panax ginseng* (ginseng), *Aloe vera* (aloe), *Foeniculum vulgare* (fennel), *Valeriana officinalis* (valerian), *Glycine max* (soybean), *Melissa officinalis* (lemon balm), *Echinacea purpurea* (echinacea) and *Vaccinium myrtillus* (blueberry) (Table 3.5).

Table 3.6 shows the overall unweighted ranking of botanicals, 1–40, according to the number of consumers, in decreasing order. Table 3.6 also shows that when unweighted overall data are stratified by gender, only slight differences between men and women become evident and only *Glycine max* (soybean) was used significantly more by women than by men (Table 3.6).

Cross-country differences emerge when considering the overall top-40 botanicals more frequently present in PFS products in each of the individual six countries (Table 3.7). In the Finnish sample, products containing *Glycine max* (soybean) are the most frequently used, followed by those containing *Echinacea angustifolia and purpurea* (echinacea). German consumers reported *Ginkgo biloba* (ginkgo)*, Cynara scolymus* (artichoke) and *Olea europea* (olive*)* as the most frequently used botanicals; whilst in Romania, *Ginkgo biloba* (ginkgo) was also the ingredient most frequently indicated, followed by *Aloe vera* (aloe) and *Panax ginseng* (ginseng). Amongst Italian consumers, *Aloe vera* (aloe) was the most frequently used botanical, followed by *Foeniculum vulgare* (fennel) and *Valeriana officinalis* (valerian*)*. In Spain, PFS containing *Cynara scolymus* (artichoke) were the most frequently used products, followed by those containing *Valeriana officinalis* (valerian) and *Equisetum arvense* (horsetail). In the United Kingdom, *Oenothera biennis* (evening primrose) was by far the most frequently reported botanical ingredient, followed by *Panax ginseng* (ginseng) and *Hypericum perforatum* (St. John's wort). In addition, there is a great variation in the ranking of consumed botanicals among countries.

3.5.4 Discussion

This section reports the findings from a European multi-country survey of PFS consumers: the PlantLIBRA PFS consumer survey. Data on the usage of PFS at the European level are limited, confined in the main to commercial market data (EAS 2007) as opposed to consumer survey data, as evidenced in the review by Bishop and Lewith (2010), where only 13% of population based consumption studies were in Europe. The European Food Safety Authority (EFSA) has recognised the lack of data in the sector and has published a number of reports addressing related issues (EAS 2009; EFSA 2012).

This is the first survey of consumers of PFS undertaken in Europe. In total 2359 consumers of PFS were recruited in this cross-sectional retrospective survey. Across all countries prevalence of usage is estimated at 18.8%. Vargas-Murga et al. (2011) highlighted that comparable data at European level is difficult to identify when reviewing prevalence data from a selected number of European studies, evaluating

Table 3.5 PlantLIBRA's PFS consumer survey—botanicals used by at least five respondents, ordered by the "n of respondents"

Used by n ≥ 75 respondents		Used by n ≥ 40 ≤ 75 respondents		Used by n ≥ 20 ≤ 40 respondents		Used by n ≥ 5 ≤ 20 respondents	
n	Botanical(s)	n	Botanical(s)	N	Botanical(s)	n	Botanical(s)
194	*Ginkgo biloba; Oenothera biennis*	74	*Glycyrrhiza glabra*	38	*Cichorium intybus; Malus pumila*	19	*Achillea millefolium; Arctium lappa; Centella asiatica; Punica granatum; Raphanus sativus; Pyrus communis*
177	*Cynara scolymus*	72	*Mentha piperita; Paullinia cupana*	37	*Curcuma longa*	18	*Artemisia absinthium; Pollen; Lecithin*
170	*Panax ginseng*	71	*Malpighia glabra*	36	*Ananas comosus*	17	*Betula pubescens; Spirulina spec.; Vegetable charcoal;*
145	*Aloe vera*	70	*Oenothera spec.*	35	*Daucus carota; Glycine spec.*	16	*Origanum majorana; Ruscus aculeatus;Terminalia chebula*
131	*Foeniculum vulgare ssp*	69	*Silybum marianum*	34	*Myristica fragrans*	15	*Citrus paradise; Eschscholzia californica; Medicago sativa; Picea spec.; Vaccinium oxycoccus; Inulin*
128	*Valeriana officinalis*	66	*Citrus limon; Matricaria chamomilla*	33	*Crataegus monogyna; Cucurbita spec.; Dianthus spec.; Monascus purpureus*	14	*Althaea officinalis; Cuminum cyminum; Eryngium planum; Laminaria digitata; Rhamnus purshianus; Trigonella foenum-graecum; Zea mays*
103	*Glycine max ; Melissa officinalis*	64	*Urtica dioica*	32	*Petroselinum crispum; Vaccinium macrocarpon*	13	*Chelidonium majus; Dioscorea villosa; Gossypium spec.; Hyssopus officinalis; Lactuca sativa; Origanum vulgare; Orthosiphon stamineus; Piper nigrum; Theobroma cacao; Trifolium pratense; Uncaria tomentosa; Lycopene; Equisetum spec.; Valeriana spec.*
102	*Echinacea purpurea*	63	*Thymus vulgaris*	31	*Coriandrum sativum; Echinaca spec.; Elettaria cardamomum; Prunus domestica*	12	*Asparagus officinalis; Azadirachta indica; Cassia occidentalis; Eucalyptus globulus; Tagetes erecta; Mentha spec.; Smilax officinalis; Xanthium spinosum*

n	Botanical(s)	n	Botanical(s)	N	Botanical(s)	n	Botanical(s)
100	*Vaccinium myrtillus;*	61	*Salvia officinalis*	30	*Cymbopogon citratus; Rhodiola rosea;*	11	*Abies alba; Artemisia abrotanum; Cetraria islandica; Cinnamomum camphora; Ilex paraguariensis; Laurus nobilis; Nasturtium officinale; Salix alba; Tilia spec.; Fraxinus excelsior; Gentiana asclepiadea; Triticum aestivum*
89	*Camellia sinensis; Zingiber officinale*	60	*Cassia senna; Rosmarinus officinalis*	29	*Calendula officinalis*	10	*Aegle marmelos; Aquilegia spec.; Armoracia rusticana; Brassica oleracea ssp.; Cheilocostus speciosus; Kaempferia galangal; Lepidium meyenii; Pimenta dioica; Populus nigra; Potentilla aurea; Santalum spec.; Sida cordifolia; Terminalia arjuna; Thymus serpyllum; Rubus fruticosus; Carlina acaulis; Centaurium spec.; Ganoderma lucidum; Tamarix gallica; Ceratonia siliqua*
88	*Pimpinella anisum*	59	*Hypericum perforatum; Lavandula angustifolia*	28	*Eleutherococcus senticosus; Fucus vesiculosus; Plantago ovate; Solanum lycopersicum; Spirulina platensis; Saccharomyces cerevisiae*	9	*Aesculus hippocastanum; Aloe ferox; Berberis aristata; Brassica oleracea var. botrytis; Capparis spinosa; Capsicum annuum var. annuum; Hieracium pilosella; Opuntia ficus-indica; Serenoa repens; Solanum nigrum; Tribulus terrestris; Melissa spec.*

(continued)

Table 3.5 (continued)

Used by n ≥ 75 respondents		Used by n ≥ 40 ≤ 75 respondents		Used by n ≥ 20 ≤ 40 respondents		Used by n ≥ 5 ≤ 20 respondents	
n	Botanical(s)	n	Botanical(s)	N	Botanical(s)	n	Botanical(s)
87	*Vitis vinifera*	58	*Carum carvi*	27	*Citrus aurantium*	8	*Allium cepa; Apium graveolens; Boswellia serrate; Coffea spec.; Euterpe oleracea; Fumaria officinalis; Griffonia simplicifolia; Illicium verum; Malva sylvestris; Prunus armeniaca; Raphanus sativus convar. Sativus; Solidago virgaurea; Tamarindus indica; Carotene; ; Garcinia cambogia; Soy lecithin*
81	*Taraxacum officinale*	53	*Ribes nigrum*	26	*Schisandra chinensis; Flavonoids; Syzygium aromaticum*	7	*Acorus calamus; Angelica sinensis; Ascophyllum nodosum; Elymus repens; Ficus carica; Hamamelis virginiana; Phaseolus vulgaris; Prunus persica; Rheum spec.; Lutein; Capsicum annuum; Fraxinus spec.; Chamomile Eng; Violeta tricolor;*
79	*Echinacea angustifolia*	52	*Oryza sativa;*	25	*Angelica archangelica; Beta vulgaris ssp.vulgaris var. conditiva; Citrus sinensis; Juniperus communis; Peumus boldus*	6	*Brassica nigra; Brassica oleracea convar. acephala; Capsicum frutescens; Carthamus tinctorius; Cordyceps sinensis; Dioscorea spec.; Drosera rotundifolia; Echinacea pallida; Emblica officinalis; Fallopia japonica; Hedera spec.; Nigella sativa; Plantago psyllium; Satureja hortensis; Tilia platyphyllos; Hibiscus rosa-sinensis; Cirsium spec.; Fragaria spec.; Viola tricolor; Lavandula spec.; Fructooligosaccharides*

n	Botanical(s)	n	Botanical(s)	N	Botanical(s)	n	Botanical(s)
78	*Allium sativum Passiflora incarnata;*	48	*Hippophae rhamnoides*	23	*Borago officinalis; Gentiana lutea; Helianthus annuus; Ocimum basilicum; Panicum miliaceum; Pinus spec.*	5	*Aloe spec.; Alpinia galanga; Chamaemelum nobile; Coffea arabica; Cola acuminata; Cyamopsis tetragonoloba; Equisetum telmateia; Fagopyrum esculentum; Hibiscus sabdariffa; Pinus pinaster; Pinus sylvestris; Thymus spec.; Undaria pinnatifida; Withania somnifera; Isoflavones; Arecaceae spec.; Fallopia multiflora*
77	*Linum usitatissimum*	46	*Triticum spec.*	22	*Plantago lanceolata; Rhamnus frangula; Vaccinium vitis-idaea*		
76	*Equisetum arvense*	43	*Rosa canina; Cinnamomum spec.*	21	*Carica papaya; Cinnamomum verum; Crataegus spec.; Hordeum vulgare; Polygonum aviculare; Saccharum officinarum; Spinacia oleracea*		
75	*Harpagophytum procumbens; Olea europaea*	42	*Sambucus nigra*	20	*Algae; Avena sativa; Betula spec.; Fiilipendula ulmaria; Humulus lupulus*		

Table 3.6 PlantLIBRA's PFS consumer survey—distribution of the overall top-40 botanicals' reported consumption and the ranking of these botanicals when stratified by gender and age group

	All consumers			Gender						Age group					
Botanicals				Male			Female			18-59 years			≥60 years		
	Rank[a]	n	% (95 % CI)	Rank[b]	N	% (95 % CI)	Rank[b]	n	% (95 % CI)	Rank[b]	n	% (95 % CI)	Rank[b]	n	% (95 % CI)
Ginkgo biloba	1	194	8.2 (7.1–9.3)	1	107	9.4 (7.7–11.0)	3	87	7.1 (5.7–8.6)	2	135	7.7 (6.4–8.9)	1	59	9.9 (7.5–12.3)
Oenothera biennis	2	194	8.2 (7.1–9.3)	3	85	7.5 (5.9–8.9)	1	109	9.0 (7.4–10.5)	1	145	8.2 (6.9–9.5)	2	49	8.2 (6.0–10.4)
Cynara scolymus	3	173	7.3 (6.3–8.4)	5	73	6.4 (5.0–7.8)	2	100	8.2 (6.7–9.7)	4	128	7.3 (6.1–8.4)	4	45	7.6 (5.4–9.6)
Panax ginseng	4	167	7.1 (6.0–8.1)	2	94	8.2 (6.6–9.8)	5	73	6.0 (4.7–7.3)	3	133	7.5 (6.3–8.7)	6	34	5.7 (3.9–7.5)
Aloe vera	5	145	6.2 (5.2–7.1)	4	80	7.0 (5.5–8.5)	7	65	5.3 (4.1–6.6)	5	99	5.6 (4.5–6.7)	3	46	7.7 (5.6–9.8)
Foeniculum vulgare ssp.	6	132	5.6 (4.7–6.5)	7	59	5.2 (3.9–6.4)	4	73	6.0 (4.7–7.3)	6	99	5.6 (4.5–6.7)	7	33	5.6 (3.7–7.3)
Valeriana officinalis	7	125	5.3 (4.4–6.2)	6	62	5.4 (4.1–6.7)	8	63	5.2 (3.9–6.4)	7	97	5.5 (4.4–6.5)	9	28	4.7 (3.0–6.4
Glycine max	8	103	4.4 (3.5–5.2)	24	34	3.0 (2.0–3.9)	6	69	5.7 (4.4–6.9)	10	81	4.6 (3.6–5.5)	14	22	3.7 (2.2–5.2)
Melissa officinalis	9	103	4.4 (3.5–5.2)	8	53	4.7 (3.4–5.8)	10	50	4.1 (3.0–5.2)	9	82	4.7 (3.7–5.6)	17	21	3.5 (2.1–5.0)
Echinacea purpurea	10	102	4.3 (3.5–5.1)	12	43	3.8 (2.7–4.8)	9	59	4.8 (3.6–6.0)	8	83	4.7 (3.7–5.7)	21	19	3.2 (1.8–4.6)
Vaccinium myrtillus	11	100	4.2 (3.4–5.1)	9	53	4.7 (3.4–5.8)	13	47	3.9 (2.8–4.9)	12	71	4.0 (3.1–4.9)	8	29	4.9 (3.1–6.6)
Pimpinella anisum	12	89	3.8 (3.0–4.5)	11	47	4.1 (3.0–5.2)	21	42	3.5 (2.4–4.4)	16	65	3.7 (2.8–4.5)	11	24	4.0 (2.5–5.6)
Zingiber officinale	13	89	3.8 (3.0–4.5)	10	53	4.7 (3.4–5.8)	29	36	3.0 (2.0–3.9)	15	66	3.7 (2.9–4.6)	13	23	3.9 (2.3–5.4)

Botanicals				Male			Female			18-59 years			≥60 years		
	Rank[a]	n	% (95 % CI)	Rank[b]	N	% (95 % CI)	Rank[b]	n	% (95 % CI)	Rank[b]	n	% (95 % CI)	Rank[b]	n	% (95 % CI)
Camellia sinensis	14	87	3.7 (2.9–4.5)	17	39	3.4 (2.4–4.4)	11	48	3.9 (2.9–5.0)	11	72	4.1 (3.2–5.0)	33	15	2.5 (1.3–3.7)
Vitis vinifera	15	87	3.7 (2.9–4.5)	16	41	3.6 (2.5–4.6)	15	46	3.8 (2.7–4.8)	13	71	4.0 (3.1–4.9)	32	16	2.7 (1.4–4.0)
Taraxacum officinale	16	80	3.4 (2.7–4.1)	21	36	3.2 (2.1–4.1)	17	44	3.6 (2.6–4.6)	17	65	3.7 (2.8–4.5)	34	15	2.5 (1.3–3.7)
Echinacea angustifolia	17	79	3.4 (2.6–4.1)	23	34	3.0 (2.0–3.9)	16	45	3.7 (2.6–4.7)	20	60	3.4 (2.6–4.2)	20	19	3.2 (1.8–4.6)
Passiflora incarnata	18	78	3.3 (2.6–4.0)	30	30	2.6 (1.7–3.5)	12	48	3.9 (2.9–5.0)	19	61	3.5 (2.6–4.3)	30	17	2.9 (1.5–4.2)
Linum usitatissimum	19	77	3.3 (2.6–4.0)	13	43	3.8 (2.7–4.8)	33	34	2.8 (1.9–3.7)	22	56	3.2 (2.4–4.0)	16	21	3.5 (2.1–5.0)
Equisetum arvense	20	76	3.2 (2.5–3.9)	19	37	3.2 (2.2–4.2)	23	39	3.2 (2.2–4.2)	23	55	3.1 (2.3–3.9)	15	21	3.5 (2.1–5.0)
Allium sativum	21	75	3.2 (2.5–3.9)	28	32	2.8 (1.9–3.7)	18	43	3.5 (2.5–4.5)	29	50	2.8 (2.1–3.6)	10	25	4.2 (2.6–5.8)
Harpagophytum procumbens	22	75	3.2 (2.5–3.9)	18	39	3.4 (2.4–4.4)	26	36	3.0 (2.0–3.9)	40	40	2.3 (1.6–2.9)	5	35	5.9 (4.0–7.7)
Olea europaea	23	75	3.2 (2.5–3.9)	27	33	2.9 (1.9–3.8)	20	42	3.5 (2.4–4.4)	24	55	3.1 (2.3–3.9)	19	20	3.4 (1.9–4.8)
Glycyrrhiza glabra	24	74	3.1 (2.4–3.8)	26	33	2.9 (1.9–3.8)	22	41	3.4 (2.4–4.4)	25	54	3.1 (2.3–3.8)	18	20	3.4 (1.9–4.8)
Mentha piperita	25	72	3.1 (2.4–3.8)	20	36	3.2 (2.1–4.1)	27	36	3.0 (2.0–3.9)	27	53	3.0 (2.2–3.8)	22	19	3.2 (1.8–4.6)

(continued)

Table 3.6 (continued)

	All consumers			Gender						Age group					
Botanicals				Male			Female			18-59 years			≥60 years		
	Rank[a]	n	% (95 % CI)	Rank[b]	N	% (95 % CI)	Rank[b]	n	% (95 % CI)	Rank[b]	n	% (95 % CI)	Rank[b]	n	% (95 % CI)
Paullinia cupana	26	72	3.1 (2.4–3.8)	14	43	3.8 (2.7–4.8)	38	29	2.4 (1.5–3.2)	14	66	3.7 (2.9–4.6)	74	6	1.0 (0.2–1.8)
Malpighia glabra	27	71	3.0 (2.3–3.7)	15	41	3.6 (2.5–4.6)	37	30	2.5 (1.6–3.3)	18	61	3.5 (2.6–4.3)	51	10	1.7 (0.7–2.7)
Oenothera spec	28	70	3.0 (2.3–3.7)	41	23	2.0 (1.2–2.8)	14	47	3.9 (2.8–4.9)	21	59	3.3 (2.5–4.2)	47	11	1.9 (0.8–2.9)
Silybum marianum	29	69	2.9 (2.2–3.6)	25	34	3.0 (2.0–3.9)	30	35	2.9 (1.9–3.8)	32	46	2.6 (1.9–3.3)	12	23	3.9 (2.3–5.4)
Matricaria chamomilla	30	67	2.8 (2.2–3.5)	34	29	2.5 (1.6–3.4)	25	38	3.1 (2.1–4.1)	26	54	3.1 (2.3–3.8)	38	13	2.2 (1.0–3.3)
Citrus limon	31	66	2.8 (2.1–3.5)	37	24	2.1 (1.3–2.9)	19	42	3.5 (2.4–4.4)	30	48	2.7 (2.0–3.5)	25	18	3.0 (1.7–4.4)
Urtica dioica	32	64	2.7 (2.1–3.4)	31	30	2.6 (1.7–3.5)	34	34	2.8 (1.9–3.7)	28	51	2.9 (2.1–3.7)	37	13	2.2 (1.0–3.3)
Thymus vulgaris	33	63	2.7 (2.0–3.3)	36	28	2.5 (1.6–3.3)	31	35	2.9 (1.9–3.8)	33	44	2.5 (1.8–3.2)	24	19	3.2 (1.8–4.6)
Salvia officinalis	34	61	2.6 (2.0–3.2)	32	22	1.9 (1.1–2.7)	35	39	3.2 (2.2–4.2)	34	43	2.4 (1.7–3.1)	29	18	3.0 (1.7–4.4)
Cassia senna	35	60	2.5 (1.9–3.2)	43	29	2.5 (1.6–3.4)	24	31	2.6 (1.7–3.4)	37	43	2.4 (1.7–3.1)	28	17	2.9 (1.5–4.2)
Rosmarinus officinalis	36	60	2.5 (1.9–3.2)	38	24	2.1 (1.3–2.9)	28	36	3.0 (2.0–3.9)	39	41	2.3 (1.6–3.0)	23	19	3.2 (1.8–4.6)
Carum carvi	37	59	2.5 (1.9–3.1)	22	35	3.1 (2.1–4.0)	43	24	2.0 (1.2–2.7)	31	46	2.6 (1.9–3.3)	36	13	2.2 (1.0–3.3)

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Botanicals				Male			Female			18-59 years			≥60 years		
	Rank[a]	n	% (95 % CI)	Rank[b]	N	% (95 % CI)	Rank[b]	n	% (95 % CI)	Rank[b]	n	% (95 % CI)	Rank[b]	n	% (95 % CI)
Hypericum perforatum	38	59	2.5 (1.9–3.1)	29	31	2.7 (1.8–3.6)	39	28	2.3 (1.5–3.1)	35	43	2.4 (1.7–3.1)	31	16	2.7 (1.4–4.0)
Lavandula angustifolia	39	57	2.4 (1.8–3.0)	40	23	2.0 (1.2–2.8)	32	34	2.8 (1.9–3.7)	36	43	2.4 (1.7–3.1)	35	14	2.4 (1.1–3.5)
Ribes nigrum	40	53	2.3 (1.7–2.8)	42	22	1.9 (1.1–2.7)	36	31	2.6 (1.7–3.4)	38	41	2.3 (1.6–3.0)	41	12	2.0 (0.9–3.1)

[a]Products ordered according to the consumer distribution of the overall top-40 used botanicals (unweighted ranking)
[b]Ranks show the shifts of the botanicals in the position of the overall 1–40 unweighted ranking when stratified by gender and age group

Table 3.7 PlantLIBRA's PFS consumer survey—ranking of the overall top-40 botanicals' reported consumption when stratified by country

Botanicals	Finland			Germany			Italy			Romania			Spain			United Kingdom		
	Rank[a]	n	% (95 % CI)	Rank[a]	n	% (95 % CI)	Rank[a]	n	% (95 % CI)	Rank[a]	n	% (95 % CI)	Rank[1]	n	% (95 % CI)	Rank[a]	n	% (95 % CI)
Ginkgo biloba		0	–	1	50	12.6 (9.3–15.8)	12	17	4.5 (2.4–6.6)	1	105	26.3 (21.9–30.6)	27	11	2.7 (1.1–4.3)	11	11	2.9 (1.2–4.6)
Oenothera biennis		0	–	22	15	3.8 (1.9–5.6)	174	1	0.3 (0.0–0.8)	164	1	0.3 (0.0–0.7)	20	13	3.2 (1.5–5.0)	1	164	43.2 (38.2–48.1)
Cynara scolymus	53	12	3.0 (1.3–4.7)	2	47	11.8 (8.6–15.0)	10	20	5.3 (3.0–7.6)	7	27	6.8 (4.3–9.2)	1	67	16.7 (13.0–20.3)		0	–
Panax ginseng	42	16	4.0 (2.1–5.9)	7	26	6.5 (4.1–9.0)	4	28	7.4 (4.8–10.1)	3	41	10.3 (7.3–13.2)	16	15	3.7 (1.9–5.6)	2	41	10.8 (7.7–13.9)
Aloe vera	172	1	0.3 (0.0–0.7)	25	12	3.0 (1.3–4.7)	1	44	11.6 (8.4–14.9)	2	47	11.8 (8.6–14.9)	37	8	2.0 (0.6–3.4)	4	33	8.7 (5.9–11.5)
Foeniculum vulgare ssp.	31	21	5.2 (3.1–7.4)	11	20	5.0 (2.9–7.2)	2	29	7.7 (5.0–10.4)	8	27	6.8 (4.3–9.2)	4	34	8.5 (5.7–11.2)	33	1	0.3 (0.0–0.8)
Valeriana officinalis	192	1	0.3 (0.0–0.7)	19	16	4.0 (2.1–6.0)	3	29	7.7 (5.0–10.4)	43	11	2.8 (1.2–4.4)	2	51	12.7 (9.4–15.9)	6	17	4.5 (2.4–6.6)
Glycine max	1	73	18.2 (14.4–22.0)	6	27	6.8 (4.3–9.3)	161	1	0.3 (0.0–0.8)		0	–	114	2	0.5 (0.0–1.2)		0	–
Melissa officinalis	14	39	9.7 (6.8–12.6)	12	20	5.0 (2.9–7.2)	7	25	6.6 (4.1–9.1)	74	5	1.3 (0.2–2.3)	18	14	3.5 (1.7–5.3)		0	–
Echinacea purpurea	3	55	13.7 (10.3–17.1)		0	–	59	5	1.3 (0.2–2.5)	13	24	6.0 (3.7–8.3)	70	4	1.0 (0.0–2.0)	7	14	3.7 (1.8–5.6)
Vaccinium myrtillus	23	30	7.5 (4.9–10.1)	30	12	3.0 (1.3–4.7)	5	28	7.4 (4.8–10.1)	15	20	5.0 (2.9–7.1)	43	8	2.0 (0.6–3.4)	26	2	0.5 (0.0–1.3)
Pimpinella anisum	16	36	9.0 (6.2–11.8)	28	12	3.0 (1.3–4.7)	38	8	2.1 (0.7–3.6)	21	15	3.8 (1.9–5.6)	11	18	4.5 (2.5–6.5)		0	–
Zingiber officinale	13	41	10.2 (7.3–13.2)	36	11	2.8 (1.2–4.4)	67	5	1.3 (0.2–2.5)	4	30	7.5 (4.9–10.1)	131	2	0.5 (0.0–1.2)		0	–
Camellia sinensis	28	23	5.7 (3.5–8.0)	16	16	4.0 (2.1–6.0)	22	12	3.2 (1.4–4.9)	47	10	2.5 (1.0–4.0)	6	26	6.5 (4.1–8.9)		0	–

	Rank[a]	n	% (95 % CI)	Rank[a]	n	% (95 % CI)	Rank[a]	n	% (95 % CI)	Rank[a]	n	% (95 % CI)	Rank[1]	n	% (95 % CI)	Rank[a]	n	% (95 % CI)
Vitis vinifera	34	20	5.0 (2.9–7.1)	5	28	7.0 (4.5–9.6)	28	11	2.9 (1.2–4.6)	127	2	0.5 (0.0–1.2)	12	18	4.5 (2.5–6.5)	13	8	2.1 (0.7–3.6)
Taraxacum officinale	65	10	2.5 (1.0–4.0)	52	10	2.5 (1.0–4.1)	9	21	5.6 (3.2–7.9)	24	15	3.8 (1.9–5.6)	8	24	6.0 (3.7–8.3)		0	–
Echinacea angustifolia	2	55	13.7 (10.3–17.1)		0	–	48	6	1.6 (0.3–2.9)	117	2	0.5 (0.0–1.2)	31	10	2.5 (1.0–4.0)	15	6	1.6 (0.3–2.8)
Passiflora incarnata	75	8	2.0 (0.6–3.4)	62	7	1.8 (0.5–3.1)	6	26	6.9 (4.3–9.4)	65	7	1.8 (0.5–3.0)	5	30	7.5 (4.9–10.0)		0	–
Linum usitatissimum	24	28	7.0 (4.5–9.5)	27	12	3.0 (1.3–4.7)	95	3	0.8 (0.0–1.7)	14	24	6.0 (3.7–8.3)	73	4	1.0 (0.0–2.0)	16	6	1.6 (0.3–2.8)
Equisetum arvense	26	26	6.5 (4.1–8.9)	153	1	0.3 (0.0–0.7)	60	5	1.3 (0.2–2.5)	82	4	1.0 (0.0–2.0)	3	40	10.0 (7.0–12.9)		0	–
Allium sativum	27	25	6.2 (3.9–8.6)	92	3	0.8 (0.0–1.6)	69	4	1.1 (0.0–2.1)	64	7	1.8 (0.5–3.0)	7	24	6.0 (3.7–8.3)	10	12	3.2 (1.4–4.9)
Harpagophytum procumbens		0	–	9	21	5.3 (3.1–7.5)	20	13	3.4 (1.6–5.3)	55	9	2.3 (0.8–3.7)	40	8	2.0 (0.6–3.4)	5	24	6.3 (3.9–8.8)
Olea europaea	30	22	5.5 (3.3–7.7)	3	40	10.1 (7.1–13.0)		0	–	84	4	1.0 (0.0–2.0)	42	8	2.0 (0.6–3.4)	36	1	0.3 (0.0–0.8)
Glycyrrhiza glabra	47	14	3.5 (1.7–5.3)	18	16	4.0 (2.1–6.0)	17	14	3.7 (1.8–5.6)	10	26	6.5 (4.1–8.9)	71	4	1.0 (0.0–2.0)		0	–
Mentha piperita	4	47	11.7 (8.6–14.9)	24	14	3.5 (1.7–5.3)	78	4	1.1 (0.0–2.1)	75	5	1.3 (0.2–2.3)	119	2	0.5 (0.0–1.2)		0	–
Paullinia cupana	130	4	1.0 (0.0–2.0)	10	21	5.3 (3.1–7.5)	8	23	6.1 (3.7–8.5)	76	5	1.3 (0.2–2.3)	14	16	4.0 (2.1–5.9)	21	3	0.8 (0.0–1.7)
Malpighia glabra	12	41	10.2 (7.3–13.2)	21	15	3.8 (1.9–5.6)	18	14	3.7 (1.8–5.6)		0	–	169	1	0.3 (0.0–0.7)		0	–
Oenothera spec	10	43	10.7 (7.7–13.8)		0	–		0	–		0	–	10	20	5.0 (2.9–7.1)	14	7	1.8 (0.5–3.2)

(continued)

Table 3.7 (continued)

Botanicals	Finland			Germany			Italy			Romania			Spain			United Kingdom		
	Rank[a]	n	% (95 % CI)	Rank[a]	n	% (95 % CI)	Rank[a]	n	% (95 % CI)	Rank[a]	n	% (95 % CI)	Rank[1]	n	% (95 % CI)	Rank[a]	n	% (95 % CI)
Silybum marianum	190	1	0.3 (0.0–0.7)	35	11	2.8 (1.2–4.4)	15	15	4.0 (2.0–5.9)	23	15	3.8 (1.9–5.6)	19	14	3.5 (1.7–5.3)	9	13	3.4 (1.6–5.3)
Matricaria chamomilla	66	10	2.5 (1.0–4.0)	38	11	2.8 (1.2–4.4)	35	9	2.4 (0.8–3.9)	20	16	4.0 (2.1–5.9)	9	21	5.2 (3.1–7.4)		0	–
Citrus limon	7	43	10.7 (7.7–13.8)	112	2	0.5 (0.0–1.2)	29	10	2.7 (1.0–4.3)	146	1	0.3 (0.0–0.7)	30	10	2.5 (1.0–4.0)		0	–
Urtica dioica	9	43	10.7 (7.7–13.8)	53	10	2.5 (1.0–4.1)	133	2	0.5 (0.0–1.3)	89	4	1.0 (0.0–2.0)	66	5	1.2 (0.2–2.3)		0	–
Thymus vulgaris	6	47	11.7 (8.6–14.9)	177	1	0.3 (0.0–0.7)	66	5	1.3 (0.2–2.5)	87	4	1.0 (0.0–2.0)	53	6	1.5 (0.3–2.7)		0	–
Salvia officinalis	8	43	10.7 (7.7–13.8)	80	5	1.3 (0.2–2.4)	82	4	1.1 (0.0–2.1)	66	7	1.8 (0.5–3.0)	124	2	0.5 (0.0–1.2)		0	–
Cassia senna		0	–		0	–	11	19	5.0 (2.8–7.2)	11	25	6.3 (3.9–8.6)	22	12	3.0 (1.3–4.7)	17	4	1.1 (0.0–2.1)
Rosmarinus officinalis	64	10	2.5 (1.0–4.0)	34	11	2.8 (1.2–4.4)	129	2	0.5 (0.0–1.3)	12	25	6.3 (3.9–8.6)	25	12	3.0 (1.3–4.7)		0	–
Carum carvi		0	–	8	23	5.8 (3.5–8.1)	33	9	2.4 (0.8–3.9)	9	26	6.5 (4.1–8.9)	149	1	0.3 (0.0–0.7)		0	–
Hypericum perforatum		0	–	157	1	0.3 (0.0–0.7)	34	9	2.4 (0.8–3.9)	56	9	2.3 (0.8–3.7)	63	5	1.2 (0.2–2.3)	3	35	9.2 (6.3–12.1)
Lavandula angustifolia	17	34	8.5 (5.8–11.2)	161	1	0.3 (0.0–0.7)		0	–	60	8	2.0 (0.6–3.4)	32	10	2.5 (1.0–4.0)	19	4	1.1 (0.0–2.1)
Ribes nigrum	20	32	8.0 (5.3–10.6)	172	1	0.3 (0.0–0.7)	44	7	1.9 (0.5–3.2)	176	1	0.3 (0.0–0.7)	24	12	3.0 (1.3–4.7)		0	–

[a]Ranks show the shifts of the botanicals in the position of the overall 1–40 unweighted ranking when stratified by country

PFS or CAM usage, with values ranging from 0.8% to 70%. All studies were based on nationally representative samples but the definition of use of supplements varied widely, in some cases being self-defined by the participant and not distinguishing between PFS and HMP. The use of dietary supplements in a European population was measured in the European Prospective Investigation into Cancer and Nutrition (EPIC) study (Skeie et al. 2009). Usage was measured by completion of a standardised 24-h dietary recall and included all dietary supplements that met the EU Directive 2002/46/EC. Results indicated significant differences in overall dietary supplement use between countries with herbs/plant-based supplements representing 8-17% of the products used across the ten countries.

The prevalence rate reported here can be compared to rates from surveys conducted in the United States, where data on usage of dietary supplements, including herbal supplements, is collected more routinely. It is similar to the rate reported in the 2002 and 2007 National Health Interview Surveys (NHIS), 18.9% and 17.9% respectively (Wu et al. 2011); higher than the rates of both the Eisenberg's survey (Eisenberg et al. 1998) and the Slone survey (Kaufman et al. 2002), with 14% and 12.1% respectively; and lower than the 2002 Health and Diet Survey (42%) (Timbo et al. 2006) or the 1999 Kaiser Permanent Medical Care Program of Northern California (KPMCP), with a prevalence of 28.3% (Schaffer et al. 2003). These differences in prevalence across studies may in part be due to the distinct selected population samples, survey methodologies (i.e. sampling methods, data collection techniques) or definitions of usage, as well as possible variations in health beliefs and health behaviour of the different populations of study (Vargas-Murga et al. 2011; Schaffer et al. 2003).

Survey respondents were recruited to set quotas for both age and gender to reflect characteristics previously reported for dietary supplement users. Age and gender are significant determinants of the consumption of dietary supplements in general and in botanical products in particular. Previous studies on the use of dietary supplements or other herbal-related use show a higher consumption among women as compared to men (Menniti-Ippolito et al. 2002; NCHS 2009; Schaffer et al. 2003; Messerer et al. 2001; Nilsson et al. 2001; Nielsen et al. 2005; Thomas et al. 2001) and a higher consumption among older adults as compared to younger adults (Schaffer et al. 2003; Foote et al. 2003; Radimer et al. 2004; Kelly et al. 2005; Bailey et al. 2013).

Other characteristics of dietary supplements users that have been reported previously in the literature include having higher educational attainment and socioeconomic status (Schaffer et al. 2003; Rock 2007; Block et al. 2007), being less likely to smoke (Harrison et al. 2004; Bailey et al. 2013; Touvier et al. 2009), being more physically active (Harrison et al. 2004; Foote et al. 203; Bailey et al. 2013). Bailey et al. also reported a moderate alcohol consumption (1 drink per day) among dietary supplement users as compared to nonusers. In contrast, a study by Rovira et al. (2013) in a southern European population found no differences in lifestyle factors such as physical activity, smoking, and alcohol consumption between dietary supplement users and non-users. The PlantLIBRA survey population consists exclusively of PFS consumers, but their responses to a series of questions on health-related lifestyle factors reflect some of the characteristics mentioned above. The majority of PFS consumers perceived their health status to be "very good or good", reflecting

results reported in a number of studies on dietary supplement users (Bailey et al. 2013) and CAM and dietary supplement users (Schaffer et al. 2003), where the answer "very good or excellent" has been reported for self-reported health status.

A wide variety of botanicals (491) was used in PFS consumed by the respondents in this survey. Overall raw data show that the most frequently (n > 100) used botanicals in descending order are *Ginkgo biloba* (ginkgo), *Oenothera biennis* (evening primrose), *Cynara scolymus* (artichoke), *Panax ginseng* (ginseng), *Aloe vera, Foeniculum vulgare* (fennel), *Valeriana officinalis* (valeriana), *Glycine max* (soybean), *Melissa officinalis* (lemon balm), *Echinacea purpurea* (echinacea) *and Vaccinium myrtillus* (blueberry). These results reflect some commercial data, which reported that ginkgo followed by echinacea, garlic and ginseng were the four most commercially important botanicals in the combined markets of seventeen EC Member States. In this data, echinacea and ginkgo were part of the composition of products registered as medicines (EAS 2007; Vargas-Murga et al. 2011), which were excluded from the PlantLIBRA survey. Similarly, the US Food and Drug Administration 2002 Health and Diet Survey, also a 12-month retrospective study, reported the same four herbs/botanicals/or other nonvitamin-nonmineral dietary supplements being the most used by its adult population—although in the following order: echinacea, garlic, ginkgo and ginseng (the latter including tea) (Timbo et al. 2006). Schaffer et al. also reported echinacea as the most consumed botanical in the Californian 1999 KPMCP survey, followed by ginkgo (Schaffer et al. 2003). Differences between countries are more evident; the top list of botanicals contained in PFS for each single country complies little with the ranking of the overall data. As mentioned earlier, data were not weighted by country population size because of the study methodology which included very similar country-sample sizes of PFS consumers only, therefore caution is needed when drawing conclusions from these results at the overall 6-country level. Overall data merely describes the collected pooled data from all 6 countries. However, if the overall ranking data were to be weighted by the population size—for example the 1–5 ranking data—the positions of the botanicals would have been only slightly altered, with *Oenothera biennis* (evening primrose) being the most consumed one, followed by *Cynara scolymus* (artichoke) *Ginkgo biloba* (ginkgo), *Panax ginseng* (ginseng) and *Aloe vera* (aloe).

The results of the survey highlight clear differences between countries in terms of the botanicals used by consumers as PFS. This may reflect the fact that the current legal and regulatory framework for botanicals has a major influence on the nature of the local PFS markets. The EU Directive 2002/46/EC does not provide a clear definition of what is encompassed by the term 'other substance with a nutritional or physiological effect', although it is generally accepted that botanicals and their extracts fall into this category. Current legislation varies across Europe, with significant differences in the botanical species permitted in PFS. These issues were highlighted in a recent review of the regulations applicable to PFS in the European Union by Silano et al. (2011). They provide examples of the different national approaches for the use of selected botanicals in food supplements in the EU Member States.

To illustrate the above complexity, in Germany, food supplements are regulated by the German Regulation on Food Supplements (Verordnung über Nahrungsergänzungsmittel 2004) and the German Law on Food and Feed (Lebensmittel 2013). Positive lists are available for minerals and vitamins. Food supplements have to be registered with the Federal Office of Consumer Protection and Food Safety (BVL 2010). The BVL maintains a list of plants which are either classified as a food or a medicinal product, and which is neither considered complete nor legally binding (BVL 2010). Data on the intake of PFS in Germany is limited and, despite food supplement intake being recorded in recent health and nutrition surveys (Finger et al. 2013; Bundesinstitut für Risikobewertung 2013; Max Rubner Institut 2008), no specific data was published on PFS intake. The results from the PlantLIBRA consumer survey do not include *Valeriana officinalis* in the German top list of botanicals used in PFS, whereas 1852 medicinal products containing Valerian exist on the market (Lebensmittel 2013). The absence of *Valeriana officinalis* in the German list of botanicals can be explained by its dominant presence as a HMP in the German market.

The results of this survey represent some of the first data on the usage of PFS at European level, thus addressing the existing deficit of such data by collecting retrospective data directly from consumers in six European countries. The benefits of the data collection instrument used in this study included that it was relatively straightforward to administer, did not alter habitual usage patterns and allowed the classification of individuals into categories of usage. However, the results must be considered in the light of their limitations. The sample population comprises exclusively of PFS consumers, recruited to meet very specific inclusion criteria and hence no comparisons can be made with the general population. Future studies should seek to compare users and non-users of PFS.

Further limitations relate to the retrospective nature of the data being collected. In many cases respondents needed to rely on memory to report usage of products in the preceding 12 months. Where products are available for inspection at data collection, there is a need for careful recording of product details to ensure accurate coding. The lack of a comprehensive product database containing reliable ingredient information meant a bespoke database needed to be created.

3.5.4.1 Recommendations

Future studies should seek to collect prospective data. Prospective dietary intake surveys offer an ideal opportunity to collect data on supplement use in conjunction with data on food and beverages. Care needs to be taken to collect sufficiently detailed information about ingredients and amounts consumed. This research encourages further research that implements future surveys/studies to overcome the bottlenecks in PFS risk and benefit assessments at the European level.

3.6 Food Supplements Containing Botanicals in Health and Disease

Plant food supplements (PFS) usage and recommendations of use are not fully evidence based, but rather are based on tradition and epidemiological data showing no adverse effects (Silano et al. 2011). Trends data indicate that PFS consumption is increasing, not only in the USA but also in European countries (Vargas-Murga et al. 2011). As such, communication and information related to PFS should be as evidence based as possible. This section presents a systematic review conducted with the purpose of analysing the uses that PFS have in gastrointestinal health and disease (laxative, carminative and hepatoprotective effects). The plants selected for this study were *Cassia senna, Buckthorne, Artichoke, German Chamomile, Milk thistle, Lemon Balm, Fennel, Anise, Boldo* and *Desert Indianwheat.*

3.6.1 Methodology

A preliminary Pubmed search identified the ten most relevant PFS in relation to gastrointestinal uses (constipation, dyspepsia and liver protection or hepatoprotection). The selection of the plants to be reviewed was based on the number of publications found (including studies in vitro and in vivo) and PlantLibra partners' knowledge suggestions. Accordingly, the botanicals selected for inclusion in the review as being the most adequate for gastrointestinal functioning were *CassiaSenna (Cassia angustifolia Vahl./Cassia senna L.), Buckthorne (Rhamnus purshianus D.C.), Artichoke (Cynara scolymus L.), German Chamomile (Matricaria recutita L. *), Milk thistle (Sylibum marianum Gaertner *), Lemon Balm (Melissa officinalis L. *), Fennel (Foeniculum vulgare Miller *), Anise (Pimpinella anisum L.), Boldo (Peumus boldus Molina *), Desert Indianwheat (Plantago ovata Forsk)*. Two of them are stimulants (containing anthracenic derivates from *Cassia senna* and *cascara*) as well as bulk forming (containing fibre and mucilags from *Plantago ovata*). *Pimpinella anisum*, *Matricaria recutita*, *Foeniculum vulgare* and *Melissa officinalis* are considered carminative plants, while *Cynara scolymus*, *Peumus boldus* and *Sylibum marianum* play a beneficial role as hepatic plants.

Search strategy:
Electronic searches were carried out on EMBASE, MEDLINE, SciFinder Scholar and Cochrane library, from January 1970 to December 2010. An update of the search was conducted including the information published from January 2011 to July 2013.

The key words and search strategy conducted followed the sequence: (1) Scientific name, common name and other synonymous; (2) fortified food* or enriched food* or fortification; (3) control* stud* or Random* control* stud* or control* trial* or Random* control* trial* or clinical* stud* or Random*

clinical* stud* or clinical* trial* or Random* clinical* trial* or clinical* control* trial* or Random* clinical* control* trial* or clinical* control* stud* Random* clinical* control* stud* or RCT or human intervention* or human intervention* stud* or human intervention* trial*; (4) #1 not #2; (5) #4 and #3. The search strategy was adapted to each data base methodology. Only studies published in English and with an abstract were included in the analysis. A two stage study selection was applied, the first one based on abstracts and a second one based on full papers. Three researchers conducted the initial screening and two researchers identified relevant papers based on full papers. Disagreement in the study eligibility was resolved by consensus between the three researchers responsible for the initial screening. The abstracts obtained from the initial search strategy were reviewed to identify those that had affirmative answers to the following questions: Does the paper address a targeted PFS? Does the paper address the gastrointestinal health area? Is it a human intervention study? Is the intervention a randomized controlled trial (RCT)? Is the botanical under study prepared as a supplement/food/extract (and not as a food fortificant)?

Whenever the above assumptions were not met, the abstract was excluded from the analysis. The results of the initial search were combined in Refworks and thus duplicates were identified and removed. Data extraction was conducted in a database (MS Access). A second researcher checked 10% of the data extraction forms. Papers were stored in a reference manager (Endnote X1.0.3).

The quality of the studies included in the analysis was evaluated by identifying the randomization procedures as described in the publication.

3.6.2 Results

The search strategy identified 554 references, 137 of them were duplicates and thus, they were removed. The remaining references were considered for applying the selection criteria. Three hundred and forty-one studies were excluded due to the following reasons: no information about the type of preparation, post marketing surveillance information, experimental studies (in vivo and in vitro studies), validation studies, studies evaluating herbal teas, studies evaluating clinical effects of the PFS. The remaining 76 citations were potentially relevant and retrieved for assessment. A final selection of 36 studies remained for the analysis. An updated search conducted in August 2013 provided 50 publications (eight of them were duplications). Only one study dealing with *Sylibum marianum* supplements was included in the analysis.

No studies were identified for the other plants.

Only 19 of the publications gave information about the sequence generation, which indicated a low risk of bias in eight of the studies (Higgins and Green 2011). Table 3.8 shows the main results.

Table 3.8 Plants, participants, treatment and main results in the studies evaluating PFS containing *Plantago ovata, Cassia Senna, Sylibum marianum, Cynara scolymus* and *Foeniculum vulgare*

Plant/references	Participants (gender/age). Health related condition	Treatment	Main results
Plantago ovata			
Marteau et al. (1994)	7 (2 women, 5 men; 21–35 years). Healthy adults	Ispaghula (Spagulax®), 18 g/day × 15 days vs. Placebo both with 5 g PEG 4000®, as faecal recovery marker	Ispaghula lower hydrogen rectal excretion ($p < 0.05$), higher number of stools ($p < 0.05$), faecal wet ($p < 0.05$), dry ($p < 0.05$) and water weight ($p < 0.05$), higher concentration of neutral sugars in the faecal content ($p < 0.05$), higher faecal concentrations and total output of acetate ($p < 0.05$), propionate ($p < 0.05$) and total short chain fatty acids ($p < 0.05$), higher outputs of butyrate and valerate (but not higher faecal concentration). Equal transit time and symptoms and tolerance
Ritchie and Truelove (1979)	96 (74 women, 16–69 years) Irritable bowel syndrome	Lorazepam (Ativan® 1 mg twice/day) + Hyoscine butylbromide (Buscopan® 10 mg × 4/day) + Ispaghula derivative (Fybogel®, 3.5 g/sachet, one sachet twice/day) vs. a combination of a dummy preparation for every treatment in the following way: ABF, ABf, aBF, AbF, Abf, aBf, abF and abf (being capitals letters the real treatment and lower letters the dummy preparation) × 4 weeks	Ispaghula husk given alone provided an improvement in the symptomatology ($p < 0.05$)

Ritchie and Truelove (1980)	96 (71 women, 14–82 years) Irritable bowel syndrome	Ispaghula (Fybogel®, 3.5 g/sachet, one sachet twice/day) + Lorazepam (Ativan® 1 mg, twice/day) + Hyoscine butylbromide (Buscopan®, 10 mg, 4/day) vs. Ispaghula + motival + mebeverine vs. Ispaghula + motival + hyoscine vs. Ispaghula + lorazepam + mebeverine vs. Bran (two tablespoons, about 20 g/ day) + Lorazepam + Hyoscine butylbromide vs. Bran + motival + mebeverine vs. Bran + motival + hyoscine vs. Bran + Lorazepam + mebeverine	Ispaghula provided more improvement in the symptomatology than bran ($p < 0.05$), which was maintained over the 3-month trial. The best combination was Ispaghula + Motival + mebeverine ($p < 0.05$)
Prior and Whorwell (1987)	57 (72 women, 8 men, 18–63 years) Irritable bowel syndrome	Ispaghula husk (Regulan®, 3.6 g refined active mucilloid-56% ispaghula) vs. Placebo ×3/day × 3 months	Ispaghula husk provided an improvement in constipation (number of days with no bowel actions ($p = 0.026$) and overall assessment of treatment success ($p = 0.02$)). Higher improvement in transit time ($p = 0.001$). Equal decrease in abdominal pain or bloating
Eogan et al. (2007)	147 women (median age 31 years Ispaghula and lactulose, 29 years Ispaghula alone) Primary repair of obstetric anal sphincter injury	Ispaghula (Fybogel® 1 sachet/d) + lactulose (10 mL thrice/day × 3d and a dose sufficient to maintain a soft stool up to 10d) vs. Lactulose (10 mL thrice/day × 3d and a dose sufficient to maintain a soft stool up to 10d) both × 10 days	Equal results in postoperative bowel movement and length of postoperative stay, equal pain to first bowel action, perineal pain and overall satisfaction. Ispaghula higher postnatal faecal incontinence ($p = 0.02$)
Kecmanovic et al. (2006)	98 (median age 48.5 years Plantago ovata, 49.9 years control). Grade III or IV hemorrhoids who required surgery	Plantago ovata (3.26 g) ×2/day vs. Glyrecin oil × 20 days	Plantago better efficiency in relieve of pain at first bowel movement after surgery ($p < 0.001$) and at 10 days after surgery ($p < 0.001$) (but no difference after 20 days), better global pain score, shorter hospital stay ($p < 0.001$), lower tenesmus at 20 days ($p < 0.01$)

(continued)

Table 3.8 (continued)

Plant/references	Participants (gender/age). Health related condition	Treatment	Main results
Fernandez-Banares et al. (1999)	61 (mean age 46 years Plantago ovata, 43.7 years Mesalamina, 39.7 years Plantago ovata and Mesalamina). Ulcerative colitis in remission for at least 3 months	Plantago ovata (Cenat® 10 g/sachet) ×2/day vs. Mesalamine (Clavesal® 500 mg/tablet) vs. Plantago ovata (Cenat® 10 g/sachet ×2/day) + Mesalamine (Clavesal® 500 mg/tablet) for 12 months (evaluation every 3 months)	Plantago equal efficiency in probability of maintained remission over 12 months analysis (additionally, no difference was found after adjusting for baseline imbalance in some prognostic variable). Equal side effects
Ornstein et al. (1981)	58 (36 women, 22 men; median age 64 years) Uncomplicated symptomatic diverticular disease	Ispaghula (Fybogel®) 2 sachets/day + 8 placebo biscuits/day vs. 8 bran biscuits (Energen®) + 2 placebo sachets Ispaghula vs. Placebo (for both treatments) × 16 weeks each treatment	Ispaghula higher improvements in stool consistency ($p < 0.001$). Equal results in symptoms score (except for flatus with higher frequency with Ispaghula; $p < 0.05$)
Perez-Miranda et al. (1996)	50 (42% women; mean age 48 years) Bleeding internal hemorrhoids	Plantago ovata (11.6 g/day) vs. Placebo × 40 days	Plantago produced a decrease in the number of bleeding episodes ($p < 0.001$), the number of congested haemorrhoidal cushions ($p < 0.01$). Equal results in the degree of prolapse
Webster et al. (1978)	53 (37% women; age range 23–71 years) Symptomatic hemorrhoids	Ispaghula husk (7 g/day) vs. Placebo (Weetbix®) × 6 weeks	Significant benefit on symptoms ($p < 0.025$), easy of defecation ($p < 0.001$) and general bowel habit ($p < 0.01$). Equal on prolapse, bleeding and pruritus
Hart and Dobb (1988)	68 (25 women, 43 men; mean age Ispaghula group: 47.1 years vs. 48.5 years in the placebo group) Intensive Care Unit receiving enteral feeding	Ispaghula (Fybogel®) 1/day vs. Placebo (Weetbix®, a wheatbased breakfast cereal) for up to 18 days	Equal results in the diarrhoea score
Ziai et al. (2005)	36 (35–70 years) Type II diabetes	Plantago ovata (Diamed®, Psyllium (5.1 g)) vs. Placebo, ×2/day × 8 weeks	Plantago better in decreasing fasting plasma glucose and HbA1c levels ($p < 0.05$). No effects on levels of total Cholesterol, LDL-C or tryglicerides. Plantago also improved tolerance to metformin and reduced flushing ($p = 0.002$).

Cassia Senna			
Sykes 1996	25 (19 women, 6 men; median age 33 years) Opioid induced constipation	Lactulose 74–11 mL (6.7 g/10 mL) vs. Senna 4–8 tablets (7.5 mg total sennosides/tablet) vs. Codanthrusate 37 capsules (Danthron 50 mg + Docusate sodium 60 mg). The dosage was self administered according to the bowel function and with increasing doses of loperamide (from 3 to 17 days of duration)	Equal efficacy on ease of defecation or bowel function rate. Senna higher side effects (abdominal pain) ($p < 0.05$)
Agra et al. (1998)	91 (mean age 66.1 years (lactulose group), 69.8 years (senna group) Terminal cancer patients with constipation	Lactulose 15 mL (10 g) up to 60 mL (60 g) vs. Senna 0.4 mL (12 mg) up to 1.6 mL (48 g) for 27 days	Equal efficacy in defecation free intervals or days with defecations. Equal side effects
Perkin (1977)	20 (children under 15 years) Constipation for 3 months	Lactulose (10–15 mL/day) × 1 week vs. Senna (10–20 mL/day) per 1 week and 1 additional week of no treatment	Equal efficacy in the number of stools passed. Lactulose better efficacy in the consistency of stools. Senna higher side effects ($p < 0.001$)
Ramesh et al. (1998)	36 (15–70 years) Cancer patients with constipation	Senna (Sofsena®, 60 mg purified extract, 12 mg senna glucosides as calcium salt) in 3 steps (2 tablets at night/4 tablets at night/2 tablets morning and 4 tablets at night) vs. Misrakasnehan sol (ayurvedic preparation, 15 plants) in 3 steps (2.5 mL/5 mL/10 mL) administered with 30 mL warm milk or water, given in the morning. Duration of 14 days	Equal efficacy in the number of satisfactory bowel movements and side effects
MacLennan and Pooler (1974–1975)	50 (36 women, 14 men; median age 78 years in the Senna group and 79 years in the Sodium picosulphate group) Living in long stay hospitals with constipation	Senna (sennosides A + B, 7.5 mg) ×2 tablets at night (increments by 1 tablet) vs. Sodium picosulphate 20 mL (10 mg) (increments by 5 mL) × 2 weeks	Senna lower number of bowel actions, less loose or unformed bowel motions ($p < 0.001$). Equal side effects

(continued)

Table 3.8 (continued)

Plant/references	Participants (gender/age). Health related condition	Treatment	Main results
Marlett et al. (1987)	42 individuals Constipation (≤3 bowel movements in 1 week)	Psyllium (7.2 g/day) vs. Senna (1.5 mg) + Psyllium, (6.5 mg)/day	Senna higher defecation frequency and stool weight.
Sondheimer and Gervaise (1982)	37 (3–12 years). Chronic functional constipation	Senna (Senokot® a dose sufficient to produce one bowel movement daily and then the dose was maintained for 3 months) vs. Mineral oil (twice a day a dose sufficient to produce loose stool and leakage of oil per rectum, afterwards a 1.5–5 cc/Kg/day dose was maintained for 3 months)	Mineral oil better in number of daily bowel movements ($p < 0.05$), less involuntary fecal soiling ($p < 0.05$), fewer and later relapses ($p < 0.05$) and greater ease of weaning for medication
Shelton (1980)	471 women Immediate postpartum puerperium	Senna (Senokot® with 7 mg sennosides A + B) 4 tablets/day until appearance of a satisfactory bowel action vs. Placebo	Senna provided more successful rates (time of the first spontaneous normal bowel action) than placebo ($p < 0.001$)
Lewis et al. (1997)	36 women (mean age 35 ± 9 years) Premenopausal volunteers	Senna (Senokot®) vs. Wheat bran vs. Loperamide (Imodium®) × 2 menstrual cycles (cross over design with a two menstrual cycles of washout)	Senna produced a decrease in whole gut transit time ($p < 0.001$), an increase in defecatory frequency ($p = 0.002$), stool form score ($p < 0.001$), and stool output ($p = 0.001$). No values are provided to compare the effect between treatment groups.
Lewis and Heaton (1997)	13 (age range 23–58 years) Healthy	Senna (Senokot®) vs. Wheat bran (Prewetts®) vs. Loperamide (Imodium®) × 9 days with two weeks to four washout period	Senna produced a decrease in whole gut transit time ($p = 0.004$) and an in the frequency of defecation ($p = 0.005$) and stool form score ($p = 0.005$). No values are provided to compare the effect between treatment groups
Lewis and Cochrane (2007)	14 (median age 28 years) Healthy	Senna (7.5 mg) + Sodium sulphate vs. Sodium sulphate × 14 days with a 2 weeks washout period	Senna decreased the whole gut transit time and oral faecal transit time compared to the placebo agent (non significant)

Sylibum marianum			
El-Kamary et al. (2009)	105 (81.9% men, mean age 29,8 years) Acute hepatitis	Silymarin (Legalon 140®) ×3/day vs. Vitamin placebo × 4 weeks	Silymarin better at reducing symptoms of impaired biliary excretion (dark urine ($p = 0.013$), jaundice ($p = 0.02$), scleral icterus ($p = 0.043$) and at reducing indirect bilirubin values at day 56 ($p = 0.012$) or subjective symptoms (fatigue ($p = 0.06$), malaise ($p = 0.045$) or anorexia ($p = 0.061$)) at 8 weeks. Equal results at reducing AST, ALT or direct bilirubin. Equal adverse effects
Gordon et al. (2006)	17 (9 women, 15 men; age range 18–65 years) Chronic hepatitis C	Silybum marianum (80% Sylimarin, Warwick®) (600 mg) vs. Silybum marianum (1200 mg) vs. Placebo × 12 weeks with a 4 weeks washout interval	Equal results on serum hepatitis C virus, ALT (alanine transaminase), bilirubin, GGT (g-glutamyl transferase), ALP (alkaline phosphatase) or quality of life tests (Short Form 36 and State-Trait Anxiety Inventory Strait-Anxiety). Equal adverse effects
Tanamly et al. (2004)	141 (65.5% men, mean age 44.1 years) Chronic hepatitis C	Silymarin (Legalon 140®, 124.5 mg/capsule) vs. Vitamin placebo × 12 months	Equal results on serum anti-hepatitis C virus and hepatitis C virus, ALT (alanine transaminase), serum hepatic fibrosis markers or quality of life tests (Short Form 36). Equal tolerance
Fried et al. (2012)	154 (71% men, median age 54 years) Chronic Hepatitis C with no response to previous Interferon based therapy	Silymarin 420 mg (Legalon 140®) vs. silymarin 700 mg (Legalon 140®) vs. Placebo ×24 weeks or matching placebo gelatin capsules administered 3 times daily for 24 weeks	Equal results in achieving serum ALT level of 45 U/L or less or attainment of at least 50% decline of serum ALT level to less than 65 U/L or change in serum ALT and serum HCV RNA. Equal results in quality of life test (Short Form 36), chronic liver disease health-related quality-of-life assessments (Chronic Liver Disease Questionnaire), or in depression scores (Center for Epidemiologic Studies–Depression)

(continued)

Table 3.8 (continued)

Plant/references	Participants (gender/age). Health related condition	Treatment	Main results
Fried et al. (2012)	154 (71% men, median age 54 years) Chronic Hepatitis C with no response to previous Interferon based therapy	Silymarin 420 mg (Legalon 140®) vs. silymarin 700 mg (Legalon 140®) vs. Placebo ×24 weeks or matching placebo gelatin capsules administered 3 times daily for 24 weeks	Equal results in achieving serum ALT level of 45 U/L or less or attainment of at least 50% decline of serum ALT level to less than 65 U/L or change in serum ALT and serum HCV RNA. Equal results in quality of life test (Short Form 36), chronic liver disease health-related quality-of-life assessments (Chronic Liver Disease Questionnaire), or in depression scores (Center for Epidemiologic Studies–Depression)
Hashemi et al. (2009)	100 (43 women, 57 men; age range 20–50 years) Non alcoholic steatohepatitis	Silymarin (Livergol®, 140 mg silymarin/capsule) vs. placebo × 2/day × 6 months	Silymarin better at reducing levels of ALT (alanine transaminase) ($p = 0.001$), AST (aspartate aminotransferase) ($p = 0.006$). Equal effect on levels of glucose metabolism, dyslipidemia or BMI
Ferenci et al. (1989)	170 (26 women, 61 men; mean age 57 years). Cirrhosis (alcoholic and non alcoholic)	Silymarin (140 mg silymarin/capsule) vs. placebo × 3/day × 2 years	Patients in the Silymarin group had lower mortality rates after 2 years of treatment ($p = 0.07$). Also, after 4 years of treatment the cumulative survival was higher in the Sylimarin group ($p = 0.036$) No differences in hepatic functional tests
Ladas et al. (2010)	49 children (58% men, mean age 8.7 years Sylimarin and 7 years placebo) Acute lymphoblastic leukemia and at least grade 2 hepatic toxicity	Silibinin 5.1 mg/Kg/day in the form of Milk thistle (Siliphos®, 80 mg silibinin) vs. placebo ×28 days	Silibinin better at reducing AST at day 56 ($p = 0.05$). No changes at day 28 on AST or ALT levels. No differences in side effect levels
Gharagozloo et al. (2009)	59 (24 women, 35 men; mean age 19.5 years) β-thalasemia major	Silymarin (Legalon®, 80% silymarin, 140 mg/capsule) ×3/day + desferrioxamine vs. Placebo + desferrioxamine × 3 months	Silymarin better efficiency in reducing levels of ALP (alkaline phosphatase) ($p < 0.05$). No changes of ALT (alanine transaminase), AST (aspartate aminotransferase), bilirubin. Equal effect on levels of glucose metabolism, dyslipidemia or BMI.

Huseini et al. (2006)	51 (32 women, 19 men; mean age 53.5 years) Type II diabetes	Silymarin (200 mg, provided by the Institute of Medicinal Plants, Iran, ×3/day) vs. Placebo (×3 day) × 4 months	Silymarin produced a decreased in the fasting glucose levels ($p < 0.001$), HbA1c levels $p < 0.001$), and also reduced the levels of total cholesterol ($p = 0.0001$), LDL-cholesterol ($p = 0.005$), triglyceride ($p = 0.004$), AST ($p = 0.008$) and ALT ($p = 0.0001$)
Hutchinson et al. (2010)	10 (1 woman, 9 men; age range 43–69 years). Hereditary hemochromatosis homozygous for the C282Y mutation with grade 4 siderosis and fibrosis (n 7) or cirrhosis (n 3) on liver biopsy	200 ml water vs. 200 ml water and silybin in the form of Legalon Forte® 140 mg vs. 200 mL black tea infusion containing 170 mg polyphenols expressed as gallic acid equivalents before a meal containing 478 kcals, 22 g fat, 124 mg vitamin C and 13.9 mg non-haem iron (3.9 mg endogenous and 10 mg added in the form of FeCl3 solution)	Silibinin reduced the iron absorption from a single meal compared to water and tea ($p < 0.05$)
Cynara scolymus			
Holtmann et al. (2003)	247 (244 women, 154 men; age range 18–75 years) Dyspepsia	Artichoke leaf extract (HeparSL forte® (dried herb:extract ratio: 3.8–5.5:1) 320 mg) × 2 × 3 times/day vs. Placebo × 6 weeks	Artichoke leaf extract improved the self-rated intensity of dyspeptic symptoms ($p = 0.007$), the proportion of patients with complete resolution of symptoms, ameliorate the intensity of symptoms: fullness, flatulence, early satiety, nausea, vomiting and epigastric pain (all of them, $p < 0.05$), improved health related quality of life ($p < 0.01$)
Englisch et al. (2000)	143 (96 women, 47 men) Total cholesterol >7.3 mmol/L (>280 mg/dL)	Artichoke dry extract (Valverde® (drug/extract ratio 25–35: 1, aquous extract, CY450) as coated tablets containing 450 mg extract ×4/day ×6 weeks vs. Placebo	Artichoke produced a superior improvement in total cholesterol and LDL-cholesterol from baseline to the end of treatment ($p = 0.0001$) than the control group. No effect on HDL-cholesterol or triglycerides
Pittler et al. (2003)	15 (age range 18–65 years) Healthy	Artichoke leaf extract Ll120 (320 mg) × 3 capsules/before and after alcohol intake vs. Placebo	No effect on the score for hangover symptoms assessed by a self-reported questionnaire. POMS (Profile of Mood States) score or PAST-BAT (Processing Speed-Accuracy Test Battery) score

(continued)

Table 3.8 (continued)

Plant/references	Participants (gender/age). Health related condition	Treatment	Main results
Foeniculum vulgare			
Alexandrovich et al. (2003)	121 (66 women, 55 men; 2–12 weeks old) Colic	Water emulsion of 0.1% fennel seed oil + 0.4% polysorbate-80 vs. Placebo (0.4% polysorbate) (up to 4/day, maximum 12 mL/kg/day) × 7 days	Fennel better at relieving colic symptoms (decrease of cumulative crying time to <9 h/week) in 65% infants compared to 24% in the control group ($p < 0.01$)

3.6.3 Discussion

This systematic review summarises the evidence for the beneficial effects of the ten most common digestive plants, of which *Plantago ovata, Cassia Senna and Sylibum marianum* were the main plants found in the review. The discussion about whether the selected PFS are suitable for gastrointestinal uses is limited by the heterogeneity of the studies, in terms not only of the pathology under study, treatment applied (dosage and duration), study population, outcomes, etc., but also in terms of the study design. Although being RCTs, few studies inform about the strategy followed to randomize and allocate individuals, blinding, etc.

3.6.3.1 Plantago ovata

Plantago ovata (also known as Psyllium Husk) obtained from *Plantago ovata* Forssk. [*P. ispaghula* Roxb.] (*Plantaginaceae*) is particularly rich in alimentary fibres and mucilages. Its mucilage content is higher than that of other Plantago species. The active ingredient of *Plantago ovata* consists of water-soluble fibre. D-xylose, L-arabinose, rhamnose and D-galacturonic acid are the main polysaccharide fraction components.

Plantago ovata absorbs about 40 times its own weight of water, increases stool weight and enhances bowel movements. Its use as a bulk forming evacuator has been advocated in the treatment of constipation under different conditions (Gilani et al. 1998; Ford et al. 2008; Alonso-Coello et al. 2005).

The beneficial effects of *Plantago ovata* was assessed in 12 studies. Three of them were conducted specifically in individuals with irritable bowel syndrome, as bulking agents are often recommended as part of the initial treatment. These three studies were also included in a recent meta-analysis that evaluated the effect of fibre, antispasmodics, and peppermint oil in the treatment of irritable bowel syndrome (Franz 1993). The authors found that, taking into account only the studies with the highest quality (in terms of study design and analysis), *Plantago ovata* was not statistically effective in treating irritable bowel syndrome. Nevertheless, the authors of the meta-analysis concluded that *Plantago ovata*, together with antispasmodics (preferably hyoscine as first line treatment) and peppermint oil could be considered as a recommended therapy for irritable bowel syndrome.

The laxative effect of *Plantago ovata* compared to glycerin oil or placebo was evaluated in three studies conducted in individuals with haemorrhoids (suffering either grade III or IV haemorrhoids, bleeding internal haemorrhoids or symptomatic haemorrhoids). In agreement with the review by Alonso-Coello et al. (2005), the studies showed an improvement in the treatment of symptomatic haemorrhoids (although without showing a beneficial effect on the degree of prolapse, pruritus or bleeding).

3.6.3.2 Cassia senna

The leaves and fruits of two species of *Cassia senna*: *Cassia senna L. [C. acutifolia* Delile], known as Alexandrian or Khartoum senna and *Cassia angustifolia* Vahl, known as Tinnevelly senna, or a mixture of these two, belong to the *Fabaceae* family. The main active constituents are sennosides A and B, which are rhein-dianthrone diglycosides. Smaller amounts of other dianthrone diglycosides, monoanthraquinone glycosides and aglyka are also present (Kolts et al. 1993). A recent review has evaluated scientific literature and experts' opinion, pharmacology, folklore and the history of senna (Staumont et al. 1988).

Cassia senna is used as a laxative for the treatment of constipation. The anthraquinone derivatives are activated by colonic bacteria and have a direct effect on the intestinal mucosa by increasing the rate of colonic motility, enhancing colonic transit, and inhibiting water and electrolyte secretion (Wilkins and Hardcastle 1970; Morales et al. 2009; Leung et al. 2011). Although there has been intense debate as to the potential risk of colonic carcinoma when anthraquinones are taken at higher doses than the recommended and when used chronically, the evidence does not support such a relationship (Flora et al. 1998).

The results of the studies included for the review indicate that *Cassia senna* does not produce additional advantages over other laxative treatments in terms of improving benefits or causing lower side effects. As stated recently, the recommendation for treating chronic constipation indicates that *Cassia senna* and other stimulants are in the third line of pharmacological recommendations (with a bulk-forming agent such as *psyllium* or bran being the first choice and a stool softener/osmotic agent such as lactulose, PEG, or docusate the second choice). Pharmacological agents are recommended when dietary fibre, exercise, and fluids do not improve the symptoms (Wagner et al. 1974).

3.6.3.3 Sylibum marianum

Fruits and seeds of *Sylibum marianum* (L.) Gaertner (or milk thistle plant), belonging to *Asteraceae* family (a family of Angiosperms that include daisies, asters and sunflowers), are widely used for "liver support". *Sylibum marianum* preparations can be used as a supportive treatment of acute or chronic hepatitis and cirrhosis induced by alcohol, drugs or toxins, because of its antioxidant, anti-inflammatory and iron chelating properties (Polyak et al. 2013). The beneficial effect of *Sylibum marianum* is attributed to its main bioactive compounds flavonolignans, collectively known as silymarin. Silymarin complex is composed of four isomers: silybin, isosilybin, silychristin and silydianin (Lattanzio et al. 2009). Most of the studies included in the review reported no benefits on improving liver enzymes in individuals with hepatitis C, in accordance with a review conducted by Polyak et al. (2013).

3.6.3.4 Cynara scolymus

The leaves of *Cynara scolymus L. [Cynara cardunculus* L.*] (Asteraceae)*, also known as globe artichoke, are traditionally used for the symptomatic relief of digestive disorders such as dyspepsia, bloating and flatulence. Its major constituents are phenolic acids (chlorogenic acid, cynarin, cynaragenin, cynarapicrin, and caffeic acid); bitter sesquiterpene lactones (cynaropicrin); flavonoids (scolymoside, cynaroside and cynarotrioside) and phytosterols (Karlsen et al. 1969).

The three studies selected for the review reported the effects on two types of digestive disorders (dyspepsia and hangover symptoms) and on blood lipid levels. Regarding the role of *Cynara scolymus* on improving blood cholesterol levels, a recent review (based on the evaluation of three randomized controlled trials including 262 patients) suggested a modest positive effect of the PFS on blood cholesterol levels, an effect that is not compelling enough to recommend it as a treatment option for hypercholesterolaemia (Miraldi 1999).

3.6.3.5 Foeniculum vulgare

The seeds of *Foeniculum vulgare* Miller subsp. vulgare var. dulce (Miller) Thellung (*Apiaceae*), known as sweet fennel, have traditionally been used in the symptomatic treatment of mild, spasmodic gastro-intestinal complaints including bloating and flatulence. The beneficial effect of fennel seed is attributed to its volatile oil, which contains anethole, fenchone and estragole (Lattanzio et al. 2009; Wider et al. 2013).

The sole study found, showed that standardized fennel seed oil emulsion was effective in reducing the intensity of infantile colic.

3.6.4 Conclusions

The studies with comparable data showed that *Plantago ovata* can be of use as to alleviate the symptoms of irritable bowel syndrome (if combined with antispasmodics) and haemorrhoids in adults. No evidence was found to support the use of *Cassia senna* or *Sylibum marianum* for gastrointestinal uses. The heterogeneity of the studies reviewed, in terms of herbal preparations, posology, duration of use, population under study and outcomes of interest, do not allow for the drawing of other definite conclusion about the PFS uses for gastrointestinal health. Further adequately designed RCTs addressing specific gastrointestinal conditions, age group populations and evaluating concrete treatment dosage and duration are needed to elucidate the efficacy of PFS on gastrointestinal health.

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Chapter 4
Benefits: Tradition of Use, Experimental Models and Human Studies to Support Health Claims of Botanicals

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Abstract Making health claims for botanical and plant food supplements (PFS) requires serious investigation and a collection of scientific evidence. The present chapter summarizes different aspects that should be considered for the evaluation of PFS benefits. Well-designed translational in vitro methods combined with human studies provide the best predictive information about their efficacy and safety. In vitro studies should rely on the most predictable cellular model to investigate the molecular mechanisms underlying the biological effect, based on approved standard operating protocols. Studies in the scientific literature generally do not consider the metabolic conversion of PFS and their active principles, as well as the chemical preparation of the extracts. To obtain the highest relevance for health claims, human studies should always describe inclusion criteria, group size, characterization of the intervention material, the control, blinding, duration of intervention and the reporting of study events. Furthermore, suitable use of in vivo validated biomarkers must be combined with large intervention studies to support health benefit of PFS.

Keywords Botanicals • Benefit • Human intervention studies • Metabolism • Biomarkers

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4.1 Traditional Use as Source of Botanicals with Biological Activity

Botanicals, which are basis of many plant food supplements (PFS), constitute the major part of worldwide and historically founded traditional medicines (TM) used to cure diseases and maintain health. A recently published trilogy called "The Art and Science of Traditional Medicine", published by Science/AAAS (2015a, b, 2014) reveals many research aspects and examples of TM including the complexity of efficacy and safety testing of botanicals. It also exemplifies currently available innovative options to ensure and improve quality of TM (from Traditional Chinese Medicine, to Ayurveda and classic Greek, Roman and Celts' medicines). The WHO, who in view of their Traditional Medicine Strategy 2014–2023 triggered this trilogy, aims to promote TM as a worldwide affordable health care option. In addition, because the use of TM, including botanicals and plant food supplements (PFS), are increasing in popularity, the WHO favours instalment of better regulations on the use of PFS. In the editorial of this trilogy the WHO asks attention for three key objectives when considering TM, that is: (1) to build a knowledge base for active management of traditional medicines through national policies, aimed at understanding and recognizing the potential of TM and at global harmonization and knowledge generation; (2) to strengthen quality assurance, safety, proper use and effectiveness of TM by regulating TM products, practices and practitioners; and (3) to promote universal health coverage by integrating TM services into health care service delivery and self-health care.

Safety and efficacy evaluations of Western medicines of the last five centuries have largely relied on animal testing, mostly using rodents. The last 20–30 years, however, animal test-free options have become more in use, partly driven by societal concerns about animal use. But more importantly scientists are becoming increasingly aware of the fact that well-designed translational in vitro methods combined with human studies provide better predictive information about efficacy and safety of a new health care products, including PFS.

This chapter aims to review aspects of interest and attention, when considering the use of a traditional approach for testing efficacy of PFS as a type of TM.

4.2 Design of Molecular, Cellular and Human Intervention Studies for Assessing the Potential Health Benefits of Botanicals

Biological activities of certain PFS have been assessed in human intervention studies and several systematic reviews have been published from the PlantLIBRA project (Di Lorenzo et al. 2013a; Dell'Agli et al. 2013). These assessments of benefits in human intervention studies are considered the strongest level of evidence for efficacy. However, when assessing new PFS, or determining the mechanism of

action of the PFS or its pure components, in vitro assays are conducted initially prior to an expensive commitment to an intervention study. In vitro investigation could provide new relevant information to approach the following human studies; in addition, in vitro evidence play a key role when the molecular target has to be identified, providing useful information on the molecular target modulated by the active principles occurring in PFS.

4.2.1 What is Necessary and Desirable for Optimum Design of In Vitro Assays?

In recent decades, strenuous efforts have been made to study the beneficial and safety effects of PFS ingredients using in vitro studies; however, most of them have only a limited predictive value. In this section we will try to elucidate what is necessary and desirable to design a suitable in vitro assays for the purpose of efficacy and safety evaluation of PFS.

Many reports have been published where an ingredient of a PFS, as extract or a purified component, have been tested using in vitro assays. When an effect is observed, conclusions are then drawn on the biological activity. However, before in vitro testing can be considered of any human relevance the following points need to be at least considered: (1) the chemical preparation (i.e. extraction) and characteristics of the PFS; (2) the status of validation and (3) translational value of the in vitro assay (including the parameters measured); (4) absorption, metabolism, distribution and excretion (ADME) of the compounds and active principles occurring in PFS.

It is evident that to provide reproducible biological effects the quality of the prepared samples should be of guaranteed and constant composition. The validation status of the in vitro assays used should imply robust, reproducible outcomes. Preferably, in vitro assays should be based on approved standard operating protocols and comply with intra- and inter-laboratory transferability. Moreover, concentrations used in in vitro tests but also parameters should be relevant to the situation in man (based on for instance kinetic data and in vitro-in vivo translation).

When assessing and designing in vitro approaches to test potential benefits of PFS, it is crucial to address aspects of ADME of the potentially active components. However, metabolism of PFS is not always taken into account in in vitro testing and in particular the importance of metabolic conversion by microbiota is generally neglected. The reason for this is that the role of microbiota has only recently become more clear. Another important reason is the difficulty to include microbiota, which are personalized, directly into in vitro models. So, similar to safety/efficacy testing of food ingredients, a meaningful strategy for PFS testing should therefore include careful evaluation of the composition of PFS in relation to ADME and of the possible usage by the human population (see also Blaauboer et al. 2016 for extended review on considerations for food ingredients).

False indications of efficacy or safety can arise from improperly designed in vitro experiments, and can lead to disappointing results when using the data to design human intervention studies. All above points are considered in the following paragraphs.

4.2.1.1 Sample Preparation

Before performing in vitro assays with PFS, it is important to provide quality control and check sample variability, which is pivotal for reproducibility and standardisation of biological effects. This implies that before using difficult and expensive approaches, it is necessary to have a robust control of plant mixture preparation (batch-to-batch variability), as stated for the applied in vitro assays (see above). Each study performed on PFS ingredients should adequately address the identity of the plant material, the part of the plant used, the kind and the conditions applied to prepare the extract. For an unequivocal identification, the plant material should be compared to a drug standard and/or reliable information sources by a qualified person, in general a botanist. A sample of plant material should be kept at a botanical laboratory as reference standard.

Extraction is the first and most important step in recovery and purification of active ingredients from plant raw materials. The parameters that significantly affect extraction are type of solvent and volume, pressure, temperature, and extraction time. The extraction solvent must be able to solubilise all target compounds, and decrease co-extraction of matrix or undesirable components. Therefore polarity of the solvent should be close to that of the target compounds. Non-polar solvents such as hexane, pentane or a combination of non-polar with medium-polarity solvents, such as pentane/dichloromethane or cyclohexane/ethyl acetate, have frequently been used to obtain lipophilic compounds-enriched extracts. On the contrary, more polar solvents, such as ethanol, methanol, or water have been employed in the case of polar and hydrophilic compounds. Mixtures of low- and high-polar solvents generally provide more efficient extractions than individual solvents, when the target compounds have a wide range of polarity.

In any case, safety must be assessed initially with regard to content of solvents. The presence of residues of solvent must be carefully addressed according to the current legislation, implying that whenever possible water as solvent is preferred. The rules dictated by European Commission concerning the limit of solvents in foodstuffs or food ingredients, including botanicals, should be taken into consideration carefully to ensure the safety of samples. Directive 2009/32/EC reports guidelines related to the use of solvents and focus on their limits in foodstuffs and food ingredients. For some solvents (e.g. ethanol, ethyl acetate, carbon dioxide, acetone) the Directive specifies only that the solvents must be used under "conditions of good manufacturing practice" meaning that the extraction procedure should result in the removal of all, or most of the solvent residues from the food ingredients. Inevitably, the presence of residues in the final product may be unintentional and technically unavoidable. For other solvents, the conditions of use and the maximum residue

limits are clearly established. For example, methyl acetate, used specifically for decaffeination of coffee or tea, must remain under the limit of 20 mg/kg in the final product. Methanol must be below 10 mg/kg, while dichloromethane should not exceed 2 and 5 mg/kg in the roasted coffee and tea, respectively (Directive 2009).

Temperature is also important in allowing an efficient extraction, considering that high values result in an increased diffusivity of the solvent into the matrix core and thus in enhanced extraction. The optimal temperature usually ranges between 20 and 60°C. Using higher temperatures, several chemical interactions will start such as a reduction of the solvent viscosity and decrease of surface tension of the solvent. However, optimal temperature is linked to thermo-sensitivity/stability of the active compounds. Although several studies have reported improvement of extraction efficiency due to enhanced temperature (Ju and Howard 2003; Jun 2013), optimal temperature strictly depends on the raw material. Plants containing essential oils or compounds highly sensitive to temperature, for example *Valeriana officinalis* L. and *Matricaria recutita* L., should not exceed 35 °C to avoid decrease of the volatile components or degradation of the active principles.

The pressure is another important parameter relating to the extraction optimization particularly when ultrahigh pressure extraction (UPE) is used. High pressure increases the solvent strength and the solubility of polar compounds. Furthermore, high-pressure treatment can increase the rate of mass transfer, enhance solvent penetration into the cells by disrupting the intercellular vacuoles, cellular walls and impairing hydrophobic bonds in the cell membrane leading to a high permeability.

The extraction time and the number of repeated extractions are related parameters, which also have a strong influence on extraction efficiency. The extraction time should be long enough to ensure contact between the bioactive ingredients and the solvent (Xi et al. 2009). The long exposure to the solvent allows the matrix to get soaked, thus improving penetration of solvent into the matrix allowing better interaction between solvent and target compounds. These details are rarely included in scientific papers, although some journals are now requiring the authors to give details about their test materials.

4.2.1.2 Validation of the In vitro Assay

The first consideration is the type of cell model employed. Often this will be dictated by the target tissue, and common examples are Caco-2 cells for examining effects on the intestine and on absorption/metabolism, HepG2 as a model for hepatocytes, and HUVEC cells as a model for the endothelium lining the blood vessels walls. In vitro assays using cell lines, although less representative when compared to in vivo models, could be reliable and predictive of the molecular mechanisms underlying the biological activity of plant extracts. The upcoming availability of complex culture techniques, including stem cell cultures, co-cultures, tissue slices, either or not combined with fluidics may help to decrease the gap between in vivo and in vitro, and in addition provide new mechanistically important knowledge.

However, the use of phytocomplexes in in vitro assays is often hampered by the complexity of the molecular mixtures, with many different molecules participating to the overall effect, either positively or negatively, and not necessarily targeting the same pathway or even cell. In addition, complex mixtures of compounds, like PFS, may also by themselves interfere producing false positives in a variety of in vitro assays. As an example, the application of the tetrazolium assay (MTT test) in a screening system for natural products to detect their influence on cell viability demands precautions. It is recommended to perform a pre-screening in a cell-free system to examine their intrinsic reductive (anti-oxidant) potential before any cell culture experiment is performed. In fact, some natural compounds may reduce the MTT salts to blue formazan by a cell-independent chemical reaction. When reduction occurs, adequate washing procedures of cells after treatment should be implemented to avoid false positive results. In addition, more than one test evaluating cell viability should be included, such as measurement of lactate dehydrogenase and trypan blue assay. As another example, inhibitors of succinate dehydrogenase could strongly influence MTT assays giving misleading results.

Thus, the development, application and validation of reliable in vitro methods for evaluating benefit assessment should be carefully considered and preferably use read out parameters that can be directly translated to a relevant human situation. It is not likely that one assay will suffice to fully predict safety of efficacy of PFS, but rather a smart strategy should be followed.

In general, primary cultures (from humans or animals although humans are preferred) are considered the most predictive, as primary cells mainly retain the characteristics of the starting tissues. However, the isolation of appropriate cells from primary cultures can be difficult since the cell population is heterogeneous; in addition, primary cells have a limited lifetime. The recently developed stem cell-derived culture methods (e.g. so called organoids, spheroids etc.) may provide new options for testing of PFS as well. These stem cell-derived cultures have the advantage that they are in principle long-lasting, and can be derived from specific individuals or patients allowing personalized evaluation of efficacy and safety. An interesting example are the intestinal organoids, which in contrast to for instance Caco-2 cell lines contain not only enterocytes, but also e.g. Goblet cells and endocrine cells. This model has recently been shown to allow evaluation of the GLP1 stimulating effect of rebaudioside, which could not be done in cell-lines lacking endocrine gut cells (van der Wielen 2016). Other organoid systems of relevance to PFS are under development, such as for liver and skin. Of note, these innovative new options need similar optimization (for purpose) and validation as the classical tumor-derived cell lines. This will be a huge challenge, considering the increased complexity of these innovative culture techniques.

Several cell systems (cell lines, organoids, etc) can be useful to study the effect of PFS on cellular target, and are efficiently used to investigate the mechanism of action of a target compound. After choosing the more appropriate cellular model, characterization should be carried out. To evaluate complete differentiation of Caco-2 cells from colonocytes to enterocytes, expression of genes typically occurring in enterocytes and absent in colonocytes, such as α1-antitripsin (α1-AT), sucrose isomaltase

(SI), apolipoprotein C3 (APOC3), and apolipoprotein A1 (APOA1), should be checked. This is important to correctly monitor differentiation of the cell line.

Several in vitro assays mimicking *Helicobacter pylori* (H.p.) gastric infection have been developed, including cytokine secretion and mRNA level measurements. After H.p infection gastric epithelial cells show higher levels of cytokines release including TNFα and IL-8; the latter is a potent neutrophil-activating chemokine that plays a central role in gastric diseases (Crabtree et al. 1995). According to the literature, the most representative in vitro assays include human epithelial adenocarcinoma cells AGS and MKN-1 cell lines that are able to release high amount of IL-8. Since TNFα and IL-1β are released by immune cells during H.p. infection, thus increasing IL-8 secretion by epithelial gastric cells, these stimuli are advisable to study the effect of PFS on gastric inflammation, whereas exogenous stimuli, such as phorbol myristate acetate (PMA) should be avoided or used only as tool to investigate molecular mechanisms.

The use of appropriate positive and negative controls should be considered. For example, to study the effect of PFS on the pro-inflammatory transcription factor NF-κB, which is involved in the downstream signalling cascades of inflammatory conditions, well-known NF-κB inhibitors such as parthenolide (from *Tanacetum parthenium* L.) and curcumin (from *Curcuma longa* L.) at concentrations reasonably low (micromolar order) are typically used as reference compounds (Li et al. 2016; Mishra et al. 2015; Xu et al. 1997; Dell'Agli et al. 2009; Hehner et al. 1999; Pozarowski et al. 2003).

4.2.1.3 Take into Account Metabolism with Respect to the Target Organ

Health effects from plant-derived products are often attributed to their polyphenol content. However, better understanding of bioavailability of polyphenols suggests caution in interpretation of the physiological relevance of such findings. Indeed, it has become clear that the bioavailability of polyphenols, as they occur in our diet, is highly variable between individuals and generally too low to explain direct biological effects of the parent compound in vivo. According to these evidences, it has been pointed out that bio-converted forms of polyphenols, conjugated forms of intact polyphenols resulting from phases I and II metabolism, may probably have more physiological importance than their native free form present in the diet (Scalbert and Williamson 2000; Stockley et al. 2012).

To design in vitro assays capable to predict at least partially the in vivo situation, metabolism of the target compounds should be considered. This implies that, if the target organ is the gut, metabolite formed by interaction with the digestive enzymes and microbiota should be taken into account. In vitro simulated gastrointestinal digestion should be considered a reliable and cheap method to obtain information about transformation of PFS ingredients in the gastrointestinal tract. However, very limited information on the changes made by human intestinal microbiota is available. An intriguing set of data from incubation experiments using faecal samples of ten different individuals shows that production of various metabolites of polyphenols

(from black tea and red wine/grape fruit juice) depends on individualized microbiota composition. For extensive reviews on this topic we refer to (Possemiers et al. 2011; Saad et al. 2012; Laparra and Sanz 2010; Kemperman et al. 2010; van Dorsten et al. 2012).

Recently, an in vitro protocol to evaluate the effects of simulated gastrointestinal digestion on the anti-inflammatory activity of *Vitis vinifera* extract (Sangiovanni et al. 2015) was assessed, showing that the biological effect on IL-8 release by human gastric epithelial cells was maintained after gastric digestion; in contrast, the effect after intestinal digestion was dramatically decreased due to the extensive degradation (up to 70%) of the active components (mainly flavonols and anthocyanins). The protocol did not take into consideration the effect of intestinal microbiota, thus suggesting that mechanisms in addition to bacterial biotransformation are important in decreasing the biological activity of PFS ingredients.

Metabolic Steps: Chemical Changes

The biological activity of anthocyanins for example can be studied at the gastric level since the pH of the stomach (1–2) ensures that these compounds are maintained as the flavylium cation, which is the most stable form. The stability of anthocyanins under gastric conditions has been confirmed by in vitro studies (Perez-Vicente et al. 2002; Possemiers et al. 2011). Conversely, the neutral pH of the small and large intestines makes anthocyanins much less stable, and these molecules are converted into a variety of metabolites (McDougall et al. 2005). The aglycones are unstable at neutral pH and rapidly degrade to their corresponding phenolic acids and aldehydes through cleavage of the C-ring. Similar results were obtained after incubation of free and acylated anthocyanins with human faecal microbiota (McGhie and Walton 2007; Aura et al. 2005). It has been proposed that the decrease of anthocyanin concentration after pancreatin bile salt digestion (as a simulation of small intestine digestion) could be partially explained by the transformation of the flavylium cation to the chalcone at neutral pH (Perez-Vicente et al. 2002).

Metabolic Steps: Hydrolysis of Attached Sugars or Organic Acids

Most of the phytochemicals in plants and foodstuffs are found naturally in glycosylated forms with a large variety of sugar moieties, namely glucose, galactose, rhamnose, arabinose, xylose and glucuronic acid, or attached to organic acids such as quinic acid (Fleschhut et al. 2006). Undoubtedly, it is very important to understand the absorption process of these compounds since the forms and degree of phytochemicals available at the target sites after oral administration will drastically affect their biological activities in the human body.

Quercetin is a common component of many PFS, including green tea (*Camellia sinensis* Kuntze), and *Ginkgo biloba* L. A considerable amount of evidence has accrued on the mechanisms of absorption of quercetin, a component of many foods

and PFS, and this will be used here as an example. There are now two enzymes that are known to aid the deglycosylation of quercetin glucosides, cytosolic broad-specificity β-glucosidase (CBG) and lactase phlorizin hydrolase (LPH), which is located on the luminal side of the brush border in the small intestine epithelial cell (Nemeth et al. 2004). Other types of glycosides, such as rhamnosides, are not cleaved by either LPH or CBG, and not absorbed in the upper part of the small intestine. They usually pass through the small intestine in the intact form to reach the large intestine where they are either hydrolysed, absorbed, metabolised and/or partially degraded by the colonic microflora (Seeram et al. 2008). This results in poor absorption and high inter-individual variations due to the smaller absorptive surface in the colon and its dependency on the activity of microflora present in the colon (Cerda et al. 2004).

Metabolic Steps: Conjugation

After conversion to the corresponding aglycone, the quercetin molecule is very active in in vitro assays. However, the next step of metabolism is conjugation, with methyl, sulphate or glucuronide groups. This can have a substantial impact on the biological activities for one or more of the following reasons: (1) a compound as conjugate can no longer enter the cell to exert a biological activity; (2) conjugation affects the ability to interact with a molecular target; (3) compound as conjugate is actively effluxed from the cell.

All of these changes affect the biological activity, and in this way, greatly modify any risk benefit analysis based on in vitro data.

Generally, flavonols are hydrophobic enough to be able to passively diffuse across membranes. Conjugation changes the hydrophobicity and hence the distribution coefficient (logP). This is illustrated by a study where the distribution of quercetin was compared to conjugates from the medium into PC-12 cells.

Since conjugates are too hydrophilic to passively diffuse across membranes, then they are taken into cells via transporter proteins (Proctor et al. 2016). OATs and OATPs are the major transporters responsible for the uptake of organic anions in the liver. Human hepatocytes have very high expression of OATP1B1 and OATP1B3 on the basolateral membrane as well as moderate expression of OATP1A2, OATP4C1, OATP2B1, OAT2, OAT4 and OAT7. In HepG2 cells (a human hepatocyte-derived cell line), OATP4C1 and OAT4, were involved in the uptake of quercetin-3′-*O*-sulfate (Wong et al. 2011b) while in human embryonic kidney 293H cells over-expressing OAT1, there was an increased uptake of the sulfated conjugates genistein-4′-O-sulfate and quercetin-3′-O-sulfate. When OAT3 was over-expressed in the same model, an enhanced uptake of glucuronide conjugates, such as daidzein-7-O-glucuronide, genistein-7-O-glucuronide, and quercetin-3′-O-glucuronide was seen (Wong et al. 2011a) suggesting that OAT1 and OAT3 were responsible for basolateral uptake of flavonoid conjugates in the kidney. Similarly, phenolic acid sulfates were efficiently transported by OAT1, and to a lesser extent, by OAT3, while glucuronides were good substrates of OAT3 (Wong et al. 2011a). Since kid-

ney is one of the main excretion route of bioactive compounds, interaction with OATs limits systemic availability of the studied flavonoids. Since cells possess a variety of transporter proteins, the use of specific inhibitors for each of them could be highly useful to investigate the molecular mechanisms underlying absorption of pure natural compounds.

Conjugation Affects the Ability to Interact with a Molecular Target

Plant secondary metabolites, xenobiotics, drugs, toxins and a variety of nutrients are metabolised by the body into compounds that can be excreted through the bile, urine or faeces. These metabolic steps most commonly include conjugation with methyl, acetyl, sulphate, glucuronides, glycine or glutathione moieties. Once conjugated, the properties of the original molecule are changed, including distribution, excretion and biological properties. In general, compounds are less active after conjugation, but for a limited number of drugs, these changes can maintain or increase efficacy: codeine, for example, is metabolised to codeine-6-glucuronide and this conjugate is fully active in analgesia (Vree et al. 2000). Ezetimibe is a lipid-lowering drug that selectively inhibits the intestinal absorption of cholesterol. It is conjugated in the liver to form a glucuronide which is much more active than the parent drug (Patel 2004). In addition, glucuronidation of morphine at the three and six positions leads to potentially higher activity than the parent drug (Frances et al. 1992). For most drugs, however, conjugation leads to lower activity.

Advances in understanding of metabolism of polyphenols and other phytochemicals present in PFS have revealed the nature of the chemical species present in plasma and urine. This information can be exploited to allow the appropriate in vitro experiments to be designed. Many reports have indicated that conjugation affects the ability of a bioactive compound to interact with a molecular target, such as an enzyme or receptor. This has been reviewed previously (Williamson et al. 2005). This will not be extensively repeated here, but a good example is the inhibition of xanthine oxidase, an enzyme that produces superoxide during its catalytic reaction (Day et al. 2000). For quercetin glucuronides, the Ki for the inhibition of xanthine oxidase by quercetin glucuronides followed the order $4' \rightarrow 3' \rightarrow 7 \rightarrow 3$-, with quercetin-4′-glucuronide a particularly potent inhibitor (Ki = 0.25 mM) comparable to quercetin aglycone. This indicates that the position of conjugation is also an important factor in determining the biological activity. In some reported papers, quercetin exhibits more activity compared to its conjugates. This is to be expected for the reasons stated above, but only limited studies have considered conjugates compared to those that have considered aglycones. In differentiated PC-12 cells, quercetin-3-O-glucuronide shows dose-dependent reduction of 13-hydroperoxy octadecadienoic acid-induced reactive oxygen species induction with ~30% reduction at 1 mM. Quercetin is slightly more active (~40% reduction) (Shirai et al. 2006).

In HUVEC cells, inflammation induces over-expression of VCAM-1 and ICAM-1, key molecules involved in monocyte recruitment during the early stages

of atherogenesis. In this system, quercetin was protective and modulated MCP-1 gene expression, whereas quercetin-3′-*O*-sulfate, quercetin-3-*O*-glucuronide and isorhamnetin-3-*O*-glucuronide were either much less effective or ineffective (Tribolo et al. 2008). Some reports demonstrate that quercetin conjugates have more activity than, or are similar, to quercetin aglycone. For example, quercetin-3-*O*-glucuronide, but not quercetin nor quercetin-3′-*O*-sulfate, significantly decreased *N*-formyl-methionyl-leucyl-phenylalanine-evoked calcium influx in human neutrophils (Suri et al. 2008). Quercetin-3-*O*-glucuronide was equally effective as quercetin at inhibiting Cu^{2+} or 2,2′-azobis(2-amidino-propane) dihydrochloride-induced human LDL-cholesterol oxidation (Thilakarathna et al. 2013).

Compound as Conjugate is Actively Effluxed from the Cell

The extent to which the polyphenols and their metabolites accumulate within tissues is frequently limited not only by their ability to enter cells but also by their tendency to leave. This may arise from active efflux mechanisms present in the plasma membrane. Intestinal ABC transporters that have been related to flavonoid transport include P-glycoprotein (P-gp/MDR1/ABCB1), multidrug resistance proteins (MRPs/ABCCs), and breast cancer resistance protein (BCRP/ABCG2), localized in the apical membrane. BCRP is responsible for the apical efflux of 7-O-glucuronide and 7-O-sulfate metabolites of hesperetin in Caco-2 cells (Brand et al. 2011). The same transport systems involved in the metabolic fate of polyphenol metabolites are shared by drugs used for treatments of various important diseases. The principle that drug efficacy is affected by transporter distribution has been shown for some compounds, supporting the hypothesis for polyphenols. For example, statins are used widely in CVD therapy, and target 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase enzyme in the liver. Therefore, it might be expected that reduced hepatic uptake by OATP1B1 would be accompanied by reduced efficacy, as statins would not reach enough concentration in the liver to inhibit the enzyme effectively. The same principle applies for polyphenols. Both drugs and polyphenols are conjugated by comparable systems. The cholesterol-lowering drug ezetimibe appears for more than 80% as its glucuronidated form in human plasma, and MRP2 and P-gp have been associated with its disposition in humans. Similarly, the absorption and potency of many anticancer agents is restricted by P-gp and other ABC efflux transporters. Hesperidin and other polyphenols inhibit the efflux of the P-gp substrate rhodamine 123 more efficiently than verapamil, a standard P-gp inhibitor (El-Readi et al. 2010).

Metabolic Steps: Changes through Colonic Microbiota

Many compounds, which are not absorbed in the small intestine, pass to the colon, where gut microbiota can carry out an extensive range of biotransformations. Ellagitannins (ET) are extensively metabolised by gut microbiota. ETs are

quite stable under the physiological conditions of the stomach. The acid conditions (pH 1.8–2.0) and the gastric enzymes do not hydrolyse the native ETs to ellagic acid, and no degradation of ETs has been observed (Quideau 2009; Fumagalli et al. 2016). The stomach seems to be a location for the absorption of free EA, but ETs are not absorbed (Quideau 2009). ETs from pomegranate release EA in the gut, and this compound is poorly absorbed in the small intestine; conversely, ellagic acid is largely metabolized by human gut microflora in the intestinal lumen into urolithins, such as urolithins A and B, and urolithin-8-methyl ether (Seeram et al. 2004; Seeram et al. 2008). These metabolites reach relevant plasma concentrations (3–5 μM) after pomegranate juice consumption (Colombo et al. 2013). The absorbed metabolites are conjugated with glucuronic acid and/or methylated to give ether derivatives (Cerda et al. 2004). According to these studies, it appears to be mandatory to test the effect of urolithins when ETs are the target compounds and the presence in the blood is an essential requisite for the effect on target organ.

Isoflavones occurring mainly in soy products are suggested to protect against human chronic diseases such as coronary heart disease, atherosclerosis and diabetes (Franke et al. 2014).

The human metabolism of isoflavones is complex and involves both mammalian and gut microbial processes. They are present almost exclusively as glycosides in most commercially available soya products; however, their bioavailability requires hydrolysis of the sugar moiety by intestinal beta-glycosidases. Only a small amount of free aglycone has been detected in blood, demonstrating that the rate of conjugation is high. Isoflavones are extensively metabolised to equol and O-desmethylangolensin by gut bacteria. In human subjects, there is large inter-individual variation in the metabolism of isoflavones, particularly in the production of the gut bacterial metabolite equol.

Bearberry (*Arctostaphylos uva ursi* L.) leaves and preparations made of them are traditionally used to treat urinary tract infections. In patients suffering from urinary infections, arbutin is converting to the active principle hydroquinone, which exerts the antimicrobial activity. Therefore, the total amount of hydroquinone equivalents in urine is crucial for the therapeutic activity. It has been demonstrated that the major metabolite of arbutin is hydroquinone glucuronide, accounting for around 70% of total metabolites. The glucuronide form appears within 4 h after the absorption of the prodrug (Schindler et al. 2002).

Lignans are found in a wide range of food daily consumed. The most frequently studied dietary lignans, such as secoisolariciresinol diglucoside, secoisolariciresinol and matairesinol are converted into the enterolignans enterodiol and enterolactone by human bacterial microflora; these metabolites are efficiently absorbed, conjugated and are present in consistent amount in the blood. Thus, studies regarding the effect of lignans should take into consideration the in vivo transformation to the corresponding metabolites (Clavel et al. 2006a, b).

4.2.2 How In vitro Assays Can be Used to Predict In vivo Benefits Effects?

On the basis of the information presented in the previous paragraphs, it is clear that the scientific value of an in vitro assay depends on its capability to predict in vivo conditions. Indeed, considering the high costs associated with the development of human studies, it is important to identify and select in vitro parameters that will be affected by a PFS treatment and predictive of a beneficial effect in vivo. i.e. serve as predictive biomarker. The literature reports several in vitro and in vivo studies performed to assess the benefits of different plant extracts in some physiological/pathological conditions. In a recent critical review, evaluating methods on in vitro and clinical approaches for benefit assessment of PFS on inflammatory conditions in Metabolic Syndrome (MS) and diabetes (Di Lorenzo et al. 2013b), we report that IL-6 and TNF-α are two pro-inflammatory parameters that can be affected both in in vitro and in vivo systems by treatment with PFS ingredients; therefore, they should be carefully considered in future studies when PFS beneficial effects on MS are studied. The review by Di Lorenzo et al. (2013b) reports the two inflammatory targets changing in MS and diabetes after different PFS treatments and measured both by in vitro and in vivo assays.

4.2.3 Human Intervention Studies to Assess Potential Benefits of Botanicals

While human, animal and in vitro studies can all be used to produce data for the substantiation of health claims for PFS, it is acknowledged that data from human studies has the highest relevance for such claims. Data from studies in humans addressing the relationship between the consumption of the food/constituent and the claimed effect will be required for substantiation of a health claim according to EFSA guidelines. However, the level of quality of studies measuring the effect of PFS in human subjects can vary greatly. Within the context of initiatives aiming at introducing evidence-based guidelines or recommendations, the quality of human studies has been often ranked with experimental intervention studies (randomized and randomized controlled) providing the strongest evidence of an effect, followed by quasi-experimental intervention studies (non-randomized controlled and non-randomized non-controlled), and observational studies (cohort, case-control, cross-sectional and other studies). Such categorization is reflected in EFSA documents (EFSA Panel on Dietetic Products 2011) and implicitly supports the choice of studies with the highest quality and strength of evidence.

Considering the specificities of PFS (low intrinsic potency, extensive biotransformation, slow onset and sustained activity, health promoting vs. therapeutic activity etc.), one of the fundamental questions concerning the design of clinical studies to be used in the assessment of health-promoting activities of PFS is

represented by the type of clinical study presenting the best compromise between quality and relevance to the specific effects of PFS. While a number of initiatives have resulted in some guidance and identification of critical aspects of study designs for the evaluation of health benefits of PFS (Aggett et al. 2005; Gallagher et al. 2011; Welch et al. 2011), many differences still exist between studies performed on PFS, with a variable impact on the quality of the studies.

The first and foremost difference between clinical studies of medicines and PFS is at the level of the target population considered. Whilst the beneficial effects of medicines are exclusively tested in a diseased population, PFS primary target population is represented by the healthy population. Within this healthy population (possibly including subpopulations of individuals at different positions on the therapeutic continuum), the objective of the studies should be to demonstrate a relationship between the consumption of the PFS and a sustainable effect of a relevant magnitude obtained in conditions of relevance (environment, daily assumption of PFS) to the use of the product in real life. Within this context, real-life outpatient studies of at least 4–8 weeks-duration with adequate monitoring insuring the compliance of participants with the dietary requirements and supplementations are strongly indicated and representative of the target health promoting effect to be demonstrated for the PFS.

In the interest of providing the best possible quality data and minimize any possible bias due to experimental demographic or interventional differences double-blind randomized controlled trials should be still considered the gold standard in the study of PFS, and adapted to the specificities mentioned earlier. Specific aspects of the study design should be carefully adapted to the use of a supplement-type of intervention: (a) inclusion criteria (b) group size (c) the characterization of the interventional material (d) the control (e) the blinding (f) the duration of intervention (g) the reporting of study events.

Inclusion Criteria: this is a critical factor in determining the homogeneity and the relevance of the study population. Particular attention should be devoted to the selection of participants so as to exclude pathologies or treatments potentially biasing the results of the evaluation of PFS. Participants should also be representative of subgroups of the healthy population at different levels of progression along the continuum leading to health deterioration and disease. The identification of critical biomarkers and the identification of a relevant validating diagnostic system to differentiate between health and diseased individuals are crucial in this context.

Group Size: group size should be justified based on the required statistical potency needed to detect a physiologically relevant effect in the target population. Considering the healthy status of the target population, expected variations of key biomarkers in the studies are likely to be of a smaller magnitude compared to those observed in the study of herbal medicines or therapeutic intervention. Study groups should therefore be adequately sized according to validated statistical procedures in order to avoid type II errors.

Control: studies should include a valid control taking into account the specificities of the group on active treatment. While the presence of dietary requirements/food diary can help controlling the effect of the PFS versus effects due to differences in

habits between the study groups, the complementation of PFS intervention with dietary intervention can also better control for the placebo effect inherent with the participation of subjects to a study.

Blinding: studies should be ideally double blind to minimize biases in the assessment of the effect of PFS. Considering the good health status of the target population and the known safety profile of most botanical ingredients used in PFS, limitations to the use of blinding protocols are limited. Blinding could be unethical whenever the treatment with the PFS is used as an alternative to a validated therapeutic intervention thus potentially exposing patients to therapeutic failure. Indeed, in a previous review of studies performed in the cardiovascular area, a greater proportion of open studies in PFS interventions in diseased population were found (Meoni et al. 2013). However, a practical limitation to blinding procedures could be represented by the organoleptic properties of the PFS administered in a liquid or tea form, as these may be particularly difficult to match within the placebo. The possible effect of all these factors should be adequately considered in planning the clinical study and described in the study report or publication.

Duration: the duration of the studies assessing the effects of PFS should be sufficient to measure slow-onset activities of the botanical ingredients and possibly demonstrate that the health promoting effect is not transient in nature. Typically, and in line with most guidance documents from regulatory agencies studies should be at least 4 weeks in duration and possibly extend to 8 weeks to demonstrate that the effect is sustainable. Longer durations could be necessary for chronic conditions or in particularly significant deviations from normal physiological function.

4.2.3.1 Study Endpoints, Processes and the Role of 'Omics'-Based Technologies

In consideration of the fact that the study population for studies looking at the health promoting effect of food supplement is the healthy population, study endpoints will be significantly different from similar studies of the medicinal properties of plants. While studies in diseased or high-risk populations making use of dietary or pharmacological interventions are allowed under the condition of extrapolation justification to a healthy population, the range in biomarker values within the population and between baseline and endpoint are expected to be narrower in studies of PFS versus studies of therapeutic interventions. Biomarkers can be broadly defined as biological indicators of normal biological or pathogenic processes or responses to different kinds of intervention (Winklhofer-Roob and Roob 2013). Biomarkers should tell something about one or more processes, and they will often consist of clusters of compounds combined with physiological parameters, image data or even subjective information like pain scores, feelings of satiety etc. Instead, there is a demand for biomarkers, which should be indicative for body responses to PFS in terms of "normal physiological functioning" or "reduction of risk for disease".

Given the complex, slow and subtle effects of PFSs as mentioned before, and the importance of demonstrating enhanced function or risk reduction, the application of

clinical endpoints as used in clinical studies of novel pharmaceutical drugs may be limited. However, physiological or risk reduction biomarkers that can be placed on a thoroughly characterized disease continuum could be common between studies of medicinal interventions and PFS. One such example is the use of lipidemia and lipid function biomarkers such as total cholesterol, HDL and LDL for the estimation of cardiovascular risk. In this case, the same biomarkers will be used to assess the effects of PFS in the normal or slightly hypercholesteremic population as well as in the high-risk population subject to the use of ipocholesterolemic drugs (Meoni et al. 2013) will almost always be impossible in intervention studies with PFSs.

Use of disease risk factors: in epidemiology, risk factors are variables associated with an increased risk of a specific disease. The relation between the variable and the disease can be causal (i.e. the variable measured is directly linked to the aetiopathology of the disease) or purely correlational (i.e. the variable is indicative of another process or factor directly associated with the induction of the disease). Many medical guidelines use risk factors to identify subgroups of the general population with an increase probability of developing the disease and therefore where lifestyle and/or pharmacological interventions could prevent the onset of the disease and more serious threats to the health of the individual (i.e. a truly health maintaining effect). Validated risk biomarkers exist in several diseases and especially in those conditions where a continuum can be mapped between health and disease such as cardiovascular diseases (Borjesson et al. 2011) or diabetes (Paulweber et al. 2010; Lindstrom and Tuomilehto 2003). Of particular interest, a condition known as Metabolic Syndrome has been defined based on the presence of a cluster of multiple risk factors for cardiovascular and metabolic diseases (central obesity, high fasting plasma glucose, high triglycerides, low HDL-cholesterol and high blood pressure) (Liese et al. 1998; Lakka et al. 2002).

In all these conditions, biomarkers such as Body-Mass Index (BMI), abdominal fat distribution (waist-to-hip ratio), total triglycerides, High Density Lipoproteins, Low-Density Lipoproteins, blood pressure, glucose metabolism can effectively map the transition of individuals from an absence of risk compared to the general population, to moderate and high risk, to declared metabolic or cardiovascular disease. Interestingly, most of these biomarkers are already widely used in the performance of clinical studies and are included in EFSA guidelines on the scientific requirements for health claims related to different health areas. A summary of claimed effects, which are considered beneficial physiological effects as well as of studies/outcome measures considered to be appropriate for the substantiation of health claims in EFSA guidance documents, is provided in Table 4.1.

Use of Omics Data: postgenomic technologies such as transcriptomics, proteomics, and metabolomics have become important in this respect, since they take a more holistic perspective compared to traditional biomarkers currently included in clinical guidelines. Nutrigenomic refers to the branch of nutrition and food research applying new profiling techniques for transcripts, proteins and metabolites to better understand the interplay of the genome with its nutritional environment (Wittwer et al. 2011). Within this context, nutrigenomic-based biomarkers represent a set of information consisting mainly of quantitative levels of gene-expression and/or pro-

Table 4.1 Different approaches in the approval of health claims

Effect described in THMP monographs and accepted as indications for THMP	Effects considered by EFSA to be beneficial for health
For symptoms of temporary fatigue and sensation of weakness	Reduction of mental fatigue is a beneficial physiological effect
For the symptomatic relief of digestive disorders such as dyspepsia with a sensation of fullness, bloating and flatulence	Reducing gastro-intestinal discomfort is considered a beneficial physiological effect
For relief of mild symptoms of mental stress	Alleviation of psychological stress is a beneficial physiological effect
To aid sleep	Maintenance or improvement of one or more aspects of sleep is a beneficial physiological effect
To relieve symptoms of discomfort and heaviness of legs related to minor venous circulatory disturbances	Maintenance of elasticity and strength of the venous walls is a beneficial physiological effect. Improvement of endothelium-dependent vasodilation may be a beneficial physiological effect
For the prophylaxis of migraine headaches after serious conditions have been excluded by a medical doctor	Relief from stress-induced headache is a beneficial physiological effect
For the relief of menopausal complaints such as hot flushes and profuse sweating	Reduction of menopausal discomfort is a beneficial physiological effect
For symptomatic treatment of minor spasm associated with menstrual periods	Reduction of menstrual discomfort is a beneficial physiological effect
For the relief of minor symptoms in the days before menstruation (premenstrual syndrome)	Reduction in the severity of symptoms related to the premenstrual syndrome is a beneficial physiological effect

teins and/or metabolites that can be measured in a reproducible affordable way, expressing a health-benefit, either a reduction of disease-risk or a physiological/nutritional benefit. Advances in analytical techniques in transcriptomics (gene-expression microarrays, sequencing technologies), proteomics (combination of 2D gel analysis or other separation techniques and Mass Spectrometry or other hyphenated techniques; Shotgun approaches) and metabolomics (NMR, Mass spectrometry) (for a review see Garcia-Canas et al. 2010; Astle et al. 2007) coupled with increased capacity and sophistication of bioinformatics tools have greatly impacted the capacity of the nutrigenomic approach to elucidate the health-promoting activities of PFS. Measuring and integrating process parameters has also made much progress thanks to the developments in IT and statistics. An interesting example of such an approach has been described by Bakker et al. (Bakker et al. 2010) who used a 3-D model to analyse health effects of a nutrient mixture along an inflammatory, metabolic and oxidative stress axis which were all built up from several biochemical markers. However, many techniques remain mainly descriptive, and a crucial requirement for such an approach is that there needs to be scientific consensus on

the issues whether certain changes in physiological processes are indicators for health improvement (Wittwer et al. 2011). Surely, the issue of biomarker validation is crucial to the whole construction of a common assessment paradigm for health benefits for PFS and deserves further consideration.

4.2.3.2 Use of Biomarkers of Normal Physiological Functioning and Challenge Tests

Based on the resilience concept there is an increasing interest in methods to challenge homeostasis and to measure disturbance and restoration of homeostasis as target resultant. Actually this principle is not that new, since for example the oral glucose tolerance test to diagnose sub-clinical diabetes is already known for years. Several new experimental models have recently been set-up intended to measure health and well-being. The kinetic response to a homeostatic perturbation is hypothesized to be a more sensitive measure for detecting effects of nutritional interventions. Moreover, comprehensive multi-parametric ("omics") analysis measured under conditions of physiological stress may identify key parameters that are more adequate to describe healthy and compromised conditions when compared to current biomarkers, which are typically assessed during steady state and regarded as markers of disease. A limitation of these approaches is that there will be no challenge tests that are indicative of general health. Instead these tests will be indicative to define specifically health condition. Another prerequisite is that there should be consensus and acceptance by regulatory authorities regarding the value of these tests in terms of claim support. Ideally test should be standardised to such an extent that they can become part of guidelines for applicants.

Overall, the main issue concerning the use of biomarkers to measure progression along the disease continuum or resilience against different kinds of challenges to homeostasis is the degree of validation of their predictive value. While adequate studies should prove a direct relation between PFS use and biomarker changes, the critical aspect lies in the translation of a biomarker to a physiologically relevant process. The relation between variations in biomarker values and the risk of disease/loss of resilience should be properly assessed according to the same standards as those used for the identification of risk factors for a specific disease (see for example Mandrekar and Sargent 2010) so as to establish an unequivocal relation between the biomarker and the risk of disease/loss of resilience. Only in these conditions the effect of the PFS on the biomarker can be identified as a potential health risk or benefit. As an example, lipoproteins are currently probably the most assessed risk factors for cardiovascular disease, and oxidative modification of lipid particles appears to influence their atherogenic potential (Greenland et al. 2010). In comparison, the relation between plasma antioxidant capacity and cardiovascular disease has not been unequivocally proven to be associated with cardiovascular disease through the analysis of prospective or retrospective data (Wang et al. 2013).

The increasingly accessible use of molecular biology, analytical and -omics technologies has effectively magnified the impact of this problem, as many potential

biomarker candidates are easily accessible and monitorable in vivo following the use of specific dietary interventions. However, the proper validation of these biomarkers rely on large interventional study programs that are not necessarily endeavoured within the context of public health initiatives or therapeutic interventions (as disease biomarkers would be favoured within these contexts) and beyond the scope of the development of novel PFS by private companies. It is therefore to be anticipated that the bottleneck in the effective use of novel biomarkers describing the progression from health to disease and the possible health benefit of plant food supplements will be represented from the appropriate validation of these biomarkers. Obviously, regulatory agencies should encourage this process by clearly outlining the required validation criteria for biomarkers to be used in studies supporting health benefits and by publishing more exhaustive and up-to date lists of acceptable biomarkers for the different physiological systems supporting health. A critical role of public research is also anticipated in the identification and validation of novel biomarkers for health benefits evaluation in Plant Food Supplements.

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Chapter 5
The Other Face of the Moon: Side Effects, Interactions and Molecules of Concerns

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Abstract Botanicals and Plant Food Supplements (PFS) have received an increasing interest in the last decades. Although these products are intended to improve physiological functions, concerns about their safety have been raised. To collect new information about the risk associated with PFS consumption, different activities were performed during the EU project PlantLIBRA: (1) a critical review of the adverse effects described in published case reports and human clinical studies; (2) a multicentre retrospective study involving the European Poison Centres; (3) the assessment of adverse effects self-reported by people participating to the PlantLIBRA PFS consumer survey. The results were integrated with recent data on adverse effects collected by the Pavia Poison Centre, ANSES and FDA. According to PlantLIBRA results and the new collected data, *Valeriana officinalis* and *Camellia sinensis* are the plants most frequently involved in adverse effects in Europe. Data from FDA showed that *Silybum marianum* and *Serenoa repens* are the most cited in US. Although most case reports showed minor symptomatology, some severe events occurred, including fatalities. Symptoms involved mainly liver, gastrointestinal and nervous systems. Generally speaking, the high variability found in the quality of

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reports determined a significant reduction of the number of cases assessable, since the causality between a specific botanical (or its derivative) and the adverse effect was not always scientifically supported.

Keywords Botanicals • Plant Food Supplements • Adverse effects • Phytovigilance • European Poison Centres • ANSES • FDA

5.1 Introduction

In the last years, the consumption of dietary supplements, including those containing botanicals (Plant Food Supplements—PFS), has considerably increased both in US and EU (Egan et al. 2011). The high request of these products by consumers has been associated with different factors including: (1) a growing skepticism in conventional drugs together with a higher demand and interest for alternative products and medicines; (2) the consumer's perception that "natural" means "healthy" and, as a consequence, botanicals are always safe; (3) an increasing tendency to buy these products, which are commercialized as food and do not require any medical prescription (Egan et al. 2011; Vargas-Murga et al. 2011).

However, considering the very high number of producers and the thousands of food supplements on the European market, concerns have been raised about their quality, efficacy and safety. While efficacy is partially supported by data coming from the "tradition of use", more difficult is the collection of data allowing a risk and benefit assessment. Among the problems faced in collecting suitable data, there is the fact that the international legislation on botanicals is not totally harmonized: the same botanical can be included as an ingredient both in food supplements and in products of traditional medicine. Furthermore, PFS are not only present in shops but can be sold by parallel markets, such as gyms, complementary–alternative medicine practitioners, and online, increasing the risk for uncertainty in their quality (Ekor 2014).

Different factors play a role in the occurrence of adverse effects to botanicals: quality of the raw material, presence of contaminants, extraction procedures, misidentifications of the plant ingredients, adulterations, counterfeits. In addition, consumer's age and gender, inappropriate use of PFS, genetic factors and concomitant physiological or pathological conditions may be further elements in the induction of side effects.

The quality of raw material depends also on the presence of specific natural toxic compounds and extrinsic factors, like environmental contaminants and the incorrect use of good agricultural practices. The combination of all these factors makes complex the routinely quality control of these products (ILSI 2003; WHO 2004).

For the univocal identification of a botanical, scientific community suggests avoiding the common names, in favour of the binomial Latin names. For example, using the common name, heliotrope (*Heliotropium europaeum*), which contains

hepatotoxic pyrrolizidine alkaloids, could be confused with garden heliotrope (*Valerian officinalis*) (Ekor 2014). The use of common names can also contribute to the misidentification of species with consequent adverse reactions (De Smet 2002). A sadly famous example was the confusion between *Stephania tetranda* and *Aristolochia fangchi*; both are commonly known as "fang ji" and *S. tetranda* was accidentally substituted with *A. fangchi*, responsible for several nephrotoxic effects (Taneku et al. 2016). Intoxications can be also due to confusion between similar species, or to the use of the wrong part of the plant. For example, the leaves of *Symphytum officinale* (comfrey), used to prepare infusions, contain much lower concentration of pyrrolizidine alkaloids than the roots (Betz et al. 1994).

Furthermore, the phytochemical profile of a botanical can change significantly according to the usual biological variability or as a consequence of the technological processes used. Since different kinds of extraction procedures can be applied to raw materials, significant changes in the amounts of active components can be obtained, with affection not only of safety but also of physiological effects. Among others, some factors have been identified as a source of variability for botanical preparations: the solvent used for extraction, the temperature, the extraction time, the age of the plant, the time from harvesting, etc.

Another important issue is the possibility of interactions between different active compounds (conventional drugs or other natural compounds) taken in association; it could result in both reducing and increasing the plasmatic concentration of one or more active compounds. For example, grapefruit juice contains an inhibitor of cytochrome P450 enzymes (Fuhr 1998), which are responsible for metabolizing many drugs. In case of metabolic inhibition, a significant increase of the plasmatic level of some drugs (e.g. statins) can be observed with a parallel worsening of known side effects (Bibi 2008). Cases of harmful interactions between botanical products and conventional drugs have been described; St. John's Wort (*Hypericum perforatum*) produced adverse effects when associated with anti-depressants drugs, belonging to selective serotonin-specific reuptake inhibitors class (Di Carlo et al. 2001).

TCMs are treatments commonly advocated for a wide range of conditions in many Eastern countries, and which have also become popular in the West, (Ernst and White 2000). TCMs are usually complex mixtures of several (often 20 or more) different medicinal plants. TCMs are usually prescribed by therapists or marketed as dietary supplements thus avoiding usual standards as long as no medical claims are made. The toxicity of TCMs has been repeatedly reviewed (Ernst 2002a), but other safety issues are often neglected.

Contamination and adulteration are other factors responsible for adverse effects in humans. Different cases of PFS adulteration have been described, where PFS were added with raw material having lower quality and/or conventional drugs (or their analogues) for enhancing the profit and/or for increasing the biological effect (Wheatley and Spink 2013). Pharmaceutical adulterants include drugs active on the central nervous system, appetite reducers, steroid hormones and phosphodiesterase type-5 enzyme (PDE-5) inhibitors to improve sexual performances (see also Chap. 12).

There are considerable concerns about the prevalence of adulteration in products coming from Eastern Countries (most of them belonging to Traditional Chinese Medicine, TCM). A study performed by researchers in Taiwan showed that 24% of tested samples were contaminated with at least one conventional pharmacological compound (Li et al. 2008; Dunnick and Nyska 2013). These products (usually a complex mixtures of several different medicinal plants) enter in the European market as dietary supplements, to avoid the legislative procedures required for marketing medicines. Moreover, the addition of conventional drugs could convince the consumer that the adultered product is more efficient in comparison with other PFS present on the market. Other safety issues are related to contamination with heavy metals (Kim et al. 2014; Chan and Critchley 1994; Ernst 2002b; Ernst and Coon 2001); lead has been often implicated, but mercury, cadmium, arsenic, copper and thallium have been also found in TCMs. Several clinical consequences were evidenced, particularly in children. Symptoms were mainly associated with skin, gastrointestinal and nervous systems, and hematologic apparatus.

The presence of heavy metals in TCMs could be explained by intentional addition or as a part of the preparation. For example, mercury is used for a variety of indications (e.g. tranquillizer, antiepileptic agent, sedative, etc.) as mercury sulphide or chloride, under the term of cinnabaris or calomelas, respectively (Koh and Woo 2000).

Finally, some considerations should be done about consumer's related factors, which can affect the risk for adverse effects. Among them, one of the most important is the age; in fact, old people are at higher risk for adverse effects due to their chronic diseases, the associated consumption of conventional drugs, and the decreased metabolic detoxification pathways.

Other evidences suggest that ethnicity could the responsible for side effects to active compounds due to the known polymorphisms in genes encoding for drug metabolizing enzymes (such as cytochrome P-450) (Bing et al. 2003; Dybing et al. 2002).

5.2 Review of Scientific Papers Describing Adverse Effects to Botanicals Consumed as Food or Food Supplements (PFS)

Several authors reported adverse effects to PFS, even though most of them were case reports, where specific acute adverse effects occurred or in reviews focused on a specific clinical field (Restani et al. 2016).

An important document on botanical safety assessment has been provided by the experts of the Scientific Committee (SC) of EFSA (European Food Safety Authority), who prepared in 2004 a document on botanicals and botanical preparations widely used in food supplements. The "compendium" raised concerns about quality and safety issues, and underlined the need for a better characterization of the products on the market (EFSA 2012).

Considering the need for additional studies on PFS-related adverse effects and the critical points raised by international committees, novel approaches were adopted during the EU project PlantLIBRA:

(1) A systematic review of the data on adverse effects due to PFS/botanical ingredients as such, or for their misidentification and interactions with pharmaceutical drugs. Assessment of causality was performed according to the WHO guidelines (WHO 2004);
(2) A multicentre retrospective study, involving European Poison Centres, that documented cases of adverse effects due to intake of PFS or plants consumed as food in the period 2006–2010;
(3) Adverse effects self-reported by participants to the European PlantLIBRA consumer Survey (2011–2012) were collected and evaluated.

After the end of EU Project, new data on adverse effects were collected by the Pavia Poison Centre - National Toxicology Information Centre (PPC) and from two among the most important public bodies for food safety: the Nutrivigilance Service of the French Agency for Food, Environmental and Occupational Health and Safety (ANSES) and the Center for Food Safety and Applied Nutrition's (CFSAN's) Adverse Event Reporting System (CAERS) of FDA (US Food and Drug Administration).

5.2.1 Botanical Ingredients Involved in Adverse Effects Described in the Scientific Literature

A review of adverse effects described in the scientific literature (case reports and clinical studies) was performed during the EU project PlantLIBRA; papers reporting events due to the plant as such, interactions or misidentification, were extracted and selected (Di Lorenzo et al. 2015a). Data were collected for 66 botanicals, since they were considered among the most frequently used ingredients in EU food supplements (Table 5.1). All papers were evaluated for causality according to the WHO guidelines and classified as "certain, probable, possible, and unclassified events" (WHO 2004).

The papers describing adverse effects were 492; they were due to 39 out of 66 botanicals included in the study. Of the total papers, 81.7% (402) reported cases due to the botanical, as such or as an ingredient of PFS, and 18.1% (89) to interactions with conventional drugs. One case was due to a misidentification of *Passiflora incarnata.* Most events were associated with 14 plants (343 papers), which were evaluated in further details. In particular, 41.4% of these papers were associated with *Glycine max* (91 papers) and *Glycyrrhiza glabra* (51 papers) (Fig. 5.1); of them 63.7% and 74.5%, respectively, were classified as certain or probable. Figure 5.2 illustrates the distribution of papers describing adverse effects due to interactions; 32 out of 83 were considered certain or probable.

Table 5.1 Plants included in the literature review during the EU Project PlantLIBRA

Abies alba Mill.	*Cynara scolymus* L.	*Ocimum basilicum* L.
Aesculus hippocastanum L.	*Echinacea pallida* (Nutt.)	*Olea europaea* L.
Aloe ferox Mill.	*Echinacea purpurea* (L.) Moench	*Panax ginseng* C.A. Meyer
Artemisia abrotanum L.	*Epimedium brevicornum* Maxim/*sagittatum*	*Passiflora incarnata* L.
Artemisia dracunculus L.	*Eschscholzia californica* Cham.	*Pelargonium sidoides* DC.
Borago officinalis L.	*Foeniculum vulgare* Mill.	*Peumus boldus* Molina
Boswellia serrata Roxb. ex Colebr.	*Ginkgo biloba* L.	*Pimpinella anisum* L.
Calendula officinalis L.	*Glycine max* (L.) Merr.	*Plantago lanceolata* L.
Camellia sinensis (L.) Kuntze	*Glycyrrhiza glabra* L.	*Plantago ovata* Forssk
Carica papaya L.	*Grindelia robusta* Nutt.	*Pseudowintera colorata* (Raoul) Dandy
Carum carvi L.	*Harpagophytum procumbens* (Burch.) DC.	*Rhamnus purshiana* DC.
Cassia angustifolia M. Vahl/*Cassia senna* L.	*Helichrysum italicum* (Roth) G. Don	*Salvia hispanica* L./ *columbariae* Benth.
Cassia obtusifolia L./*Cassia tora* L	*Heliotropium* spp.	*Serenoa repens* (W. Bartram) Small.
Chrysanthemum balsamita (*L*) Baill	*Hibiscus sabdariffa* L.	*Serenoa serrulata* (Michx.) Hook f.
Cichorium intybus L.	*Hippophae rhamnoides* L.	*Silybum marianum* (L.) Gaertn.
Cimicifuga racemosa (L.) Nutt.	*Humulus lupulus* L.	*Taraxacum officinale* (L.) Weber
Cinnamomum verum J. Presl (*Cinnamomum zeylanicum*)	*Hypericum perforatum* L.	*Thymus serpyllum* L.
Citrus aurantium L.	*Lavandula angustifolia* Mill.	*Trifolium pratense* L.
Citrus limon (L) Burm.	*Lycium barbatum* L.	*Vaccinium myrtillus* L.
Citrus sinensis (L.) Osbeck	*Matricaria chamomilla* L.	*Valeriana officinalis* L.
Crataegus monogyna Jacq.	*Melissa officinalis* L.	*Vitex agnus castus* L.
Cuminum cyminum L.	*Myrtus communis* L.	*Vitis vinifera* L.

The 91 papers reporting adverse effects to *Glycine max* were mainly associated with allergic reactions and hormone-like activities of isoflavones (Aaronov et al. 2008; Kwack et al. 2009). Regarding licorice (*Glycyrrhiza glabra*), glycyrrhetic acid was responsible for hypokalemia and hypertension, as well as for hypertension caused by interactions with conventional drugs, such as diuretics and oral contraceptives (Leitolf et al. 2010). Other botanicals involved in a certain number of cases were *Camellia sinensis* (34 due to the plant as such and 9 to interactions) and *Ginkgo biloba* (28 papers). The former was involved mainly in hepatotoxic effects, with some case of severe entity (Federico et al. 2007; Gloro et al. 2005; Fong et al. 2010). Catechins and, in particular, epigallocatechin-3-gallate (EGCG) were suggested as

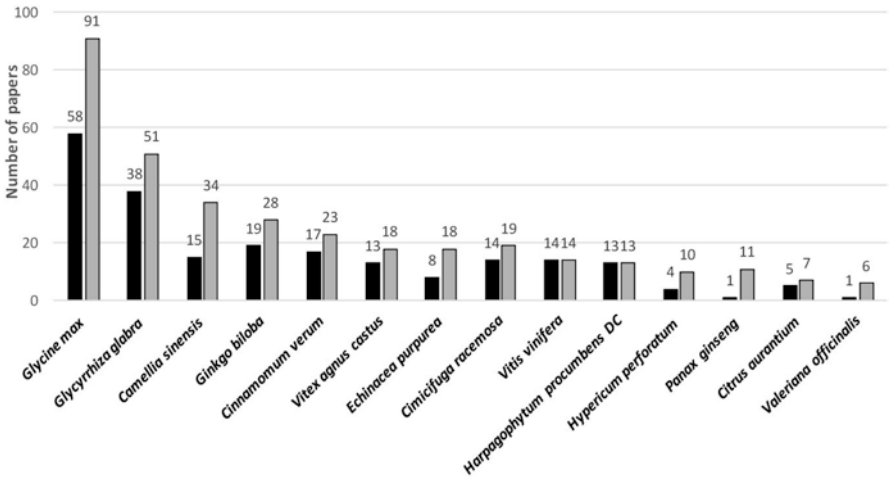

Fig. 5.1 Papers reporting adverse effects for the 14 botanicals having more than 10 significant associations; total number (*grey*) and cases belonging to the highest classes of causality (*black*)

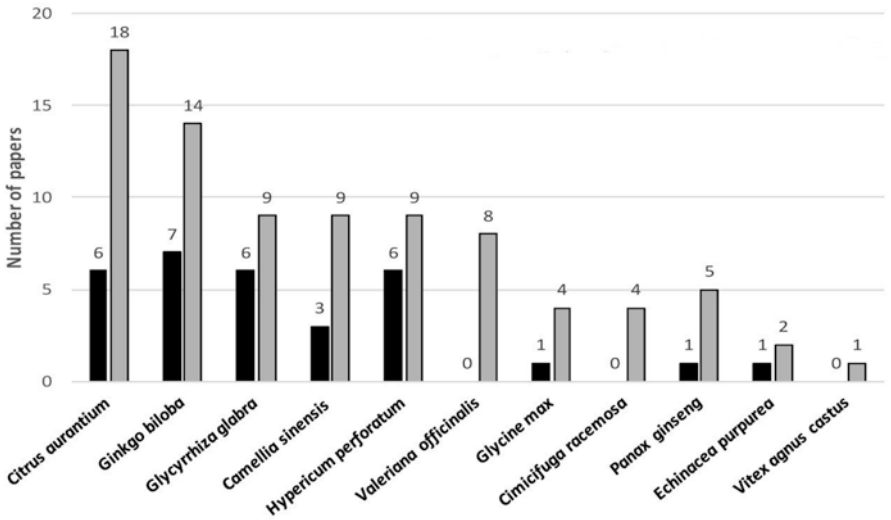

Fig. 5.2 Cases reporting adverse effects due to interactions with conventional drugs. Total number (*grey*) and cases belonging to the highest classes of causality (*black*)

the main responsible for liver toxicity (Di Lorenzo et al. 2015a), even though adulteration could not be always excluded. The adverse effect occurrence and the degree of causal relationship was critically associated with:

1. The type of tea: green tea is more involved than black tea;

2. Extraction/preparation as shown by the higher number of events observed with PFS containing hydro-alcoholic extracts rich in hydrophilic and lipophilic compounds; less numerous were the cases when tea was consumed as infusion or PFS containing aqueous preparations, where only hydrophilic compounds were present (Gloro et al. 2005; Vial et al. 2003).

Nine papers reported interactions with statins (with an increase of their plasmatic concentrations and their side effects, such as rhabdomyolysis), and warfarin, where the pharmacological effect was reduced by the presence in green tea of vitamin K (Mazzanti et al. 2009; Werba et al. 2008). *Ginkgo biloba* is at the fourth position for direct adverse effects (28 papers) and at the second one for interactions (14 papers). Ginkgolides were considered responsible for adverse effects involving coagulation process, the most usual symptom caused by this plant, taken both alone and in association with anticoagulants (Miller and Freeman 2002; Xia and Fang 2007). In particular, ginkgo was involved in a fatal case (breakthrough seizure) due to the induction of CYP2C19, which caused the drastic reduction of plasmatic levels of the anticonvulsants phenytoin and valproic acid in an epileptic patient (Kupiec and Raj 2005). Among the cases due to interaction with drugs, 18 out of 83 papers (22%) were associated with *Citrus aurantium* (6 were classified as "certain/probable", 11 as "possible"). Normally used in PFS for body weight control, the most frequent adverse reaction due to *Citrus aurantium* is the affection of cardiovascular system with hypertension, tachycardia and ventricular extrasystoles. These effects are mainly due to the presence of the active amines synephrine and octopamine (Haller et al. 2005). It is important to underline that *Citrus aurantium* extracts are often in combination with other compounds, such as caffeine and phenylethylamine, with additional stimulant effects on cardiovascular system.

Despite the long period of time searched in the selected databases (from the inception of scientific databases to 2014), the number of adverse effects collected was relatively low, and severe clinical outcomes were quite rare. This review did not discriminate cases due to botanicals taken as PFS or as traditional medicines, since the aim of the work was the identification of symptoms associated with specific botanicals, having enough documented causality. These data were useful in the second part of research done during the EU project PlantLIBRA.

5.3 Data on Adverse Effects Collected by Poisons Centres

Poison Centres and phytovigilance services were identified as an important source of data on adverse effects. Several data are here collected and discussed to improve the body of knowledge in the field of possible risks associated with the consumption of botanicals.

5.3.1 *Data Collected During the Retrospective Study of the EU Project PlantLIBRA*

A multicentre retrospective study was performed during the EU project PlantLIBRA, with the involvement of several European Poison Centres (30 contacted) and the Sao Paulo Poisons Centre (Brazil, one of the extra-European country partner of the project). They collected cases of adverse effects involving adults and children (≤ 16 years old) where the intake of PFS or plants consumed as food were considered the reason of hospitalization. The period of case collection was established from 2006 to 2010 (Lüde et al. 2016).

Eight Poisons Centres provided a total of 75 cases (Finland 9, France 31, Germany 4, Italy 13, Serbia 4, Sweden 5, Switzerland 5 and Brazil 4), involving mainly adults (91%, age 16-92) and only 9% of children (age 2–15).

According to the Poison Severity Score (PSS) (Persson et al. 1998), most cases (70) showed mild clinical symptoms and only 5 were considered severe. In 57 cases (76%) responsible of the adverse effect was a PFS and in 18 (24%) a plant consumed as food. The involved PFS contained only one ingredient in 30 cases and more than one component in 27. PFS containing more than one ingredient were more frequently associated with moderate and severe clinical outcomes (33.3%) compared to mono-ingredient PFS (10%). The top-15 plants involved in at least three adverse effects/each and the severity of the associated signs are reported in Table 5.2.

Generally speaking, adverse effects occurred more frequently after consumption of the plant as PFS than as food; moreover, some plants were involved in severe cases: three of them were caused by a multi-ingredient PFS and had positive outcomes (Lüde et al. 2016). Causality assessment was evaluated "certain" in two cases (positive rechallenge): the first involved a mono-ingredient PFS (*Glycine max*—soybean powder diluted in one glass of soybean milk) taken by a 57 years old male for 9 days; the patient developed angioedema after the last exposure. The second case involved a 40 years old man, who developed a transient ischemic attack few hours after the intake of four tablets of a multi-ingredient PFS (containing *A. sativa*, *C. sinensis, Capsicum sp., Carum carvi, Citrus aurantium, Coleus forsklolii, Dioscorea villosa, Glycine max, Glycyrrhiza glabra, Ilex paraguanensis, Lepidium meyenii, Panax ginseng, Paullinia cupana, Rhodiola rosea, Turnera diffusa*).

Although valerian was the plant with the highest number of adverse effects (drowsiness and somnolence were the main symptoms), the severity of clinical manifestation was evaluated as "minor" (Table 5.2). On the contrary, more severe symptoms were shown after consuming plants less frequently involved in adverse effects.

Gastrointestinal symptoms and allergic reactions were the most frequent clinical manifestations caused by the ingestion of a single plant (mainly as food), while several organs were involved when PFS contained more than one botanical. Generally speaking, gastrointestinal and nervous systems were involved in 52 and 41 cases, respectively.

Table 5.2 The top-15 plants involved in adverse effects collected during the Poisons Centres retrospective study of the EU project PlantLIBRA

Plant/ingredient	Total	Food	PSF	Severity		
				Minor	Moderate	Severe
Valeriana officinalis L.	23	1	22	23	–	–
Camellia sinensis L.	10	1	9	6	3	1
Melissa officinalis	7	–	7	7	–	–
Mentha x piperita	7	–	7	6	–	1
Passiflora incarnata	7	1	6	7	–	–
Paullinia cupana	7	–	7	4	1	2
Glycyrrhiza glabra L.	6	5	1	1	4	1
Ilex paraguariensis	6		6	4	1	1
Panax ginseng C.A.Mey.	5	–	5	3	1	1
Citrus aurantium L.	4	–	4	3	–	1
Cynara scolymus L.	4	4	–	3	–	1
Dioscorea villosa L.	4	–	4	–	4	–
Allium ursinum L.	3	3	–	3	–	–
Carum carvi L.	3	–	3	1	2	–
Taraxacum officinale L.	3	2	1	1	2	–
Total	99[a]	17	82	72	18	9

[a]Total cases were 57 but in several cases the PFS was a multi-ingredient product so that the total counts are higher

It is important to note that a causal relationship between the intake of a plant and the adverse effects could not always be clearly demonstrated due to the insufficient information (e.g. ingested dose and duration of intake), the presence of several ingredients in the PFS involved and the lack of precise information about the composition.

Although data reported by Poisons Centres show an important contribution to the definition of adverse effects to botanicals, some limitations must be hypothesized; in fact, the real number of adverse effect can be underestimated for different reasons, such as the kind of adverse effects (e.g. delayed effects or symptoms not recognized) or the attitude of the patient, who is unconscious or does not declare the use of PFS to the physicians (Lüde et al. 2016).

5.3.2 *Adverse Effects of Plant Food Supplements and Plants Consumed as Food: A Five-Year Survey at Pavia Poisons Centre*

After the end of EU Project PlantLIBRA, a further effort was done in collecting new cases (2011–2015) of adverse effects due to PFS.

This new study has been performed at the Pavia Poisons Center—National Toxicology Information Centre (PPC), a hospital based unit, where clinical toxicologists (medical doctors) advise on, and assist for diagnosis and management of poisoning (telephone consultation) the physicians requiring specialistic support from the emergency departments and intensive care units all over Italy. For all cases, a computerized medical record containing detailed information on the agents involved, the clinical picture at admission and during hospital stay, the laboratory investigations and the toxicological analysis, treatments, clinical follow-up, and outcome is registered. The medical records are then stored in a database, which can be searched by fields or by keywords.

For the present study, all medical records reporting the word "supplement" or "plant" as the involved "agent" were retrospectively reviewed over a 5-year period (2011–2015).

A data collection sheet was used to extract selected information on patients: the included cases were assessed for age, sex, plant food supplements or plant ingested as food and modality of ingestion (voluntary/accidental), clinical manifestations that caused hospital admission and treatments. A senior toxicologist retrospectively reviewed the medical records of patients; data were divided in two groups: the first included adverse effects due to PFS and the second the adverse effects due to plants consumed as food. Each year, poisoning due to food supplements account for about 1.5–2% of activity of the Poisons Centre of Pavia; the same percentage of consultation is required for problems due to plants consumed as food.

5.3.2.1 Adverse Effects to Plant Food Supplements

For this part of the study, exclusion criteria were: patients aged under 15 years, asymptomatic patients, cases involving vitamins, melatonin or voluntary ingestion of plant food supplement in association with drugs (Table 5.3).

Of the total 556 cases involving PFS, 64 were included in the study; the mean age of patients was 38 ± 15.7 years, 37 (58%) were females and 27 (42%) males. Most

Table 5.3 Cases screened and included in the study by Pavia Poisons Centre after application of the inclusion/exclusion criteria (*PPS* Poison Severity Score)[a]

Year	Total cases	Unknown composition/ co-somministration of drugs/pediatric drugs	Supplements not containing botanicals	Asymptomatic patients	Included cases
2011	65	30	9	15	11
2012	125	52	20	38	15
2013	133	42	44	31	16
2014	121	31	48	30	12
2015	112	28	51	23	10
Total	556	183	172	137	64

[a]From Persson et al. (1998)

cases (44/64) showed minor/moderate signs and symptoms and only one case was considered of severe grade, according to PSS (Persson et al. 1998): administration of antidotes or gastrointestinal decontamination were required in 2% and 20% of cases, respectively. The plants involved in adverse effects were 229. Considering the high number of botanicals involved, Table 5.4 lists only plants involved in at least three cases.

PFS involved in adverse effects were mainly "multi-ingredients" (47%), while "mono" PFS represented 17%. *Camellia sinensis* and *Valeriana officinalis* were the plants more frequently responsible for adverse effects, confirming data discussed in the Sect. 5.3.1. Other plants frequently involved were *Paullinia cupana, Capsicum annuum* and *Panax ginseng*. Although included frequently as ingredients of PFS (Di Lorenzo et al. 2015a; Restani et al. 2016; Wu et al. 2011), *Ginkgo biloba, Allium sativum* and *Serenoa repens* were rarely involved in the cases collected in this study (only one case for each plant). Data collected by the retrospective study of PlantLIBRA indicated gastrointestinal and nervous systems as the main targets of adverse effects; in this study, the cardiovascular system (n = 24), gastrointestinal system (n = 23), nervous system (n = 13), neuromuscular apparatus (n = 7) and skin/mucosa (n = 4) were the most involved. Other sign and symptoms (n = 23) were not associable with a specific organ, and included weakness, metabolic acidosis, headache, dizziness, and blood chemistry abnormalities. As

Table 5.4 Plants associated with at least three cases of adverse effects collected by the Pavia Poisons Centre in 2011–2015

Plant		
Latin name	Common name	N. of cases
Camellia sinensis	Green tea	12
Valeriana officinalis	Valerian	11
Paullinia cupana	Guaranà	10
Capsicum annuum	Pepper	8
Panax ginseng	Ginseng	7
Cola acuminata	Cola nut	5
Citrus aurantium	Bitter orange	5
Piper nigrum	Black pepper	5
Crataegus monogyna	Common hawthorne	5
Melissa officinalis	Lemon balm	4
Zingiber officinalis	Ginger	4
Citrus paradisi	Grapefruit	4
Ribes nigrum	European blackcurrant	4
Griffonia simplicifolia	Griffonia	3
Uncaria tomentosa	Cat's claw	3
Rosa canina	Dog rose	3
Theobroma cacao	Cocoa tree	3
Coffea arabica	Arabica coffee	3
Taraxacum officinale	Common dandelion	3
Mentha x piperita	Peppermint	3
Cassia angustifolia	Alexandrian senna	3

regarding the causality assessment, the association was considered "certain" in 43% of cases and possible in 45%.

5.3.2.2 Adverse Effects Due to Plants Consumed as Food

In order to evaluate adverse effects due to plants consumed as food, patients aged less than 6 years (accidental ingestion), patients for which the age was not reported and cases of voluntary ingestion of plants with suicidal purpose were excluded.

The number of included cases was 498, but excluding those cases where a specific plant was not recognized (n = 60), the total selected cases were 225 (Table 5.5). The mean age of patients was 43±21.9 years; 46% were females and 53% males. Plants consumed as food and the associated adverse effects are listed in Table 5.6.

It is important to underline that most cases showed a benign clinical course and patients developed minor sign and symptoms (42%). However, three deaths occurred: two patients (husband and wife) died after ingestion of *Colchicum autumnale* (a misidentification of wild onions); a patient died after ingestion of wild saffron (possible misidentification with *Colchicum autumnale*). Several severe cases of *Colchicum* poisoning are described in the literature, generally caused by misidentification with similar plants (e.g. *Aliium ursinum*). The poisoning is due to colchicine, and, according to published data, doses greater than 0.8 mg/kg are generally fatal (Brvar et al. 2004).

Plants responsible for adverse effects were mainly consumed as whole plants (34%); leaves were ingested in 20% of cases followed by berries (18%), roots/bulbs (10%), seeds (9%) and flowers (8%). The most frequently affected organ/system was

Table 5.5 Plants misidentified and consumed as food, involved in at least ten cases

Plant		
Latin name	Common name	N. of cases
Mandragora officinarum	Mandrake	28
Datura stramonium	Jimsonweed	21
Laburnum anagyroides	Golden Chain Tree	19
Wisteria sinensis	Wisteria	17
Nerium oleander	Oleander	17
Borago officinalis	Borage	16
Plants with anticholinergic effects (unknown)	–	16
Atropa belladonna	Belladonna	15
Sambucus nigra	Elderberry	13
Asparagus acutifolius	Wild asparagus	11
Conium maculatum	Hemlock	11
Lupinus spp.	Lupin	11
Colchicum autumnale	Meadow saffron	10
Daphne mezereum	Daphne	10
Narcissus spp.	Narcisus	10
	Total	225

Table 5.6 Signs and symptoms observed in consumers after the intake of the listed botanicals consumed as food (Pavia Poisons Centre)

			Nervous system					Skin/ mucosa	Neuro-muscolar	Cardiovasular system		GI tract	
	Other	Xerostomia	Drawsiness coma	Agitation	Confusion	Allucinations	Midriasis	Urticaria/ hyperemia/ sweating	Paresthesia, astenia, myalgia	Tachycardia	Other CD symptoms	GI symptoms	Tot
Mandragora officinarum	1	9	1	10	8	6	17	–	1	12	2	2	**69**
Datura stramonium	3	6	–	9	6	14	13	1	–	8	–	1	**61**
Laburnum anagyoides	–	1	–	1	–	–	–	2	1	–	–	11	**16**
Wisteria sinensis	–	–	–	–	–	–	–	–	–	–	–	12	**12**
Nerium oleander	2	–	–	–	–	–	–	2	2	–	1	5	**12**
Borago officinalis	–	2	–	8	4	4	8	4	–	5	1	6	**42**
PAE[a]	–	3	–	3	4	10	5	–	–	13	8	3	**49**
Atropa belladonna	1	9	1	2	3	3	6	–		3	–	9	**37**
Sambucus nigra	–	–	–	–	–	–	–	–	1	–	–	12	**13**
Asparagus acutifolius	2	1	–	–	–	–	–	–	2	1	–	6	**12**
Conium maculatum	4	–	–	–	–	–	–	–	3	–	–	1	**8**
Lupinus spp.	1	7	–	1	–		3	–	–	2	–	3	**17**

Colchicum autumnale	–	–	–		1	–	–	–	–	–	4	8	**13**
Daphne mezereum	–	–	–	–	–	–	–	–	–	–	–	4	**4**
Narcissus spp.	–	–	–	–	–	–	–	–	–	–	3	8	**11**
Unknown	4	6		3	7	8	3	3	1	3	1	26	**65**
Total	**18**	**44**	**2**	**37**	**33**	**45**	**55**	**12**	**11**	**47**	**20**	**117**	–

[a]Plants with anticolinergic effects

the gastrointestinal tract (41% of cases), which required decontamination in 72% of patients. Nervous system and cardiovascular system were affected in 29% and 16% of cases, respectively, followed by mydriasis (13%), skin/mucosa (4%) and neuromuscular apparatus (3%). Xerostomia and other symptoms (dizziness, dyspnea, tremors) represented 9% of symptomatology. An overview of the reported sign and symptoms in relation to the plants mostly involved in adverse effects is reported in Table 5.6.

Apart from *Dafne mezereum* and *Wisteria sinensis*, adverse effects involved more than one organ/organ system. The most frequent target systems were the gastrointestinal tract (n = 117), the nervous system (n = 115) and the cardiovascular system (n = 67). Excluding the "unknown" plants, gastrointestinal symptoms were recorded after ingestion of *Wisteria sinensis*, *Sambucus nigra* and *Laburnum anagyroides.* Similar effects are reported in the literature (Barceloux 2008), where it is described how the ingestion of the seeds of *Laburnum anagyroides* could be fatal in children (more rarely in adults), due to the presence of cystisine, a pyridine-like alkaloid responsible for symptoms similar to those of mild nicotine toxicity (Musshoff and Madea 2009). The most frequent signs and symptoms involving nervous and cardiovascular systems were due to compounds having anticholinergic properties. In particular, *Mandragora officinarum* and *Datura stramonium* were responsible for agitation, confusion, hallucinations, mydriasis, xerostomia, tachycardia caused by their content in tropanic alkaloids. Anticholinergic effects of these plants are well known and several cases of poisoning are described in the scientific literature (Tsiligianni et al. 2009). In this study, *Borago officinalis* was involved in 16 cases with symptoms associated with nervous system, cardiovascular system and skin/mucosa. Considering that these symptoms are very similar to those reported for intake of *Mandragora* and *Datura*, poisoning due to misidentification with other plants containing tropanic alkaloids is always possible (Amini et al. 2012).

At the time of first call, the causes of intoxication were ascertained in 59%, and considered possible in 30% of cases. In the other cases, the exposure was evaluated as "probably not related to the appearance of signs and symptoms" (7%) and "surely not related to exposure" (2%).

In conclusion, in this study cases were separated according to the use as food or as ingredients of PFS. Most adverse effects were caused by the consumption of plants as food (n = 225), where anticholinergic effects were the most commonly observed symptoms. The most severe cases (death) occurred after ingestion of plants as food, due to misidentification. Cases involving a PFS were 62, with milder symptoms. The limits of this study are comparable to those observed in the PlantLIBRA retrospective study: the difficulty in collecting the complete case data, in identifying the plant ingested (for food), the dose and period of intake (for PFS), in confirming the quality of PFS involved when ingredients are numerous, etc.

5.4 Data from PlantLIBRA Consumers' Survey

Another source of data on adverse effects due to PFS was the self-reported cases collected during the PlantLIBRA Consumers' Survey (2011–2012). The survey enrolled 2359 adults from Finland, Germany, Italy, Romania, Spain and UK

(Garcia-Alvarez et al. 2014), who completed a questionnaire on PFS usage. Consumers participating to the survey were asked on adverse effects with two questions: (1) *Have you experienced any adverse effects while taking this product?* (2) *If yes, which one?*

Of the 2359 consumers enrolled, 82 people (3.5%) reported 87 adverse effects. In the six involved countries, the percentage of consumers complaining adverse effects were: Finland 5.7%; Germany 5.5%; Italy 1.3%; Romania 1.8%; Spain 6%; UK 0.3%.

The causality assessment was not possible considering that adverse effects were self-reported by consumers; however, the symptomatology reported was evaluated on the basis of the reviewed scientific literature and data collected from Poisons Centres during the EU Project PlantLIBRA.

The evaluation was also based on other data regarding each consumer, who reported the adverse effect: age and gender, the identification of botanicals contained in PFS (label); daily dose and period of intake, reason of use; the congruence between the known physiological activity of the botanical/s and the expected effect; the general health status of the consumer; the reported intake of conventional drugs or other food supplements.

Considering all parameters listed above, 53 out of 87 cases were classified as possible and 4 as probable; 30 cases were evaluated as unlikely/unassessable.

The total number of botanical ingredients involved in adverse effects was 72; in 66 cases the PFS involved was mono-ingredient, in 28 cases PFS contained a mixture of 2–3 ingredients and in 50 ≥4 ingredients. Considering the frequency of adverse effects reported, 14 botanicals were mainly responsible for adverse effects (68 cases) (Fig. 5.3); forty botanicals were cited only once and most of them were (80%) in association with other ingredients.

Valeriana officinalis, Camellia sinensis, Ginkgo biloba and *Paullinia cupana* were associated with 27 cases of adverse effects, which are detailed in Table 5.7;

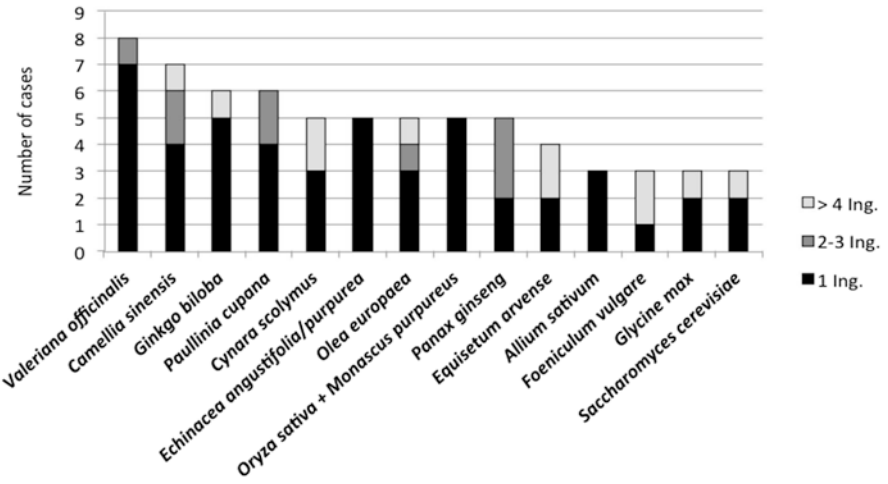

Fig. 5.3 Number of botanical ingredients contained in PFS associated with the adverse effects. *Ing* = Ingredient

Table 5.7 Adverse effects self-reported by consumers participating to the PlantLIBRA consumers' survey; cases associated with *Valeriana officinalis, Camellia sinensis, Ginkgo biloba* and *Paullinia cupana* (from Restani et al. 2016)

Age/ gender	Botanical/s[a]	Dose and period	Reasons for use	Present or past main health problems	Conventional drugs + FS	Adverse effects	Causality
31/M	*Matricaria chamomilla*, *Melissa officinalis*, *Valeriana officinalis*	3/d × 6m	Sleeping and mood problems	Migraine, peptic ulcer, sleep disorders	None	Dizziness	Possible (Mayo Clinic 2010)
36M	*Valeriana officinalis*	4/w × 5m	Sleeping, relaxing, mood	None	None	Insomnia	Unlikely but described (Bauer 2016)
61/F	*Valeriana officinalis*	2/d × 12m	Sleeping, relaxing, mood	HCHO, heart disease, muscles, joint/bone pain, cataract	Alprazolam, Simvastatin	Constipation	Possible—abdominal cramps have been described (EMA 2010)
72/F	*Valeriana officinalis*	1/d × 8m	Sleeping, memory, relaxing	Cancer, joint/bone pain	None	Migraine	Possible (Bauer 2016)
49/F	*Valeriana officinalis*	1/d × 3m	Relaxing	HCHO; hypertension, migraine, allergy, anxiety	No drug + SI, VM	Flatulence	Possible—abdominal cramps have been described (EMA 2010)
42/M	*Valeriana officinalis*	2/d × 4m	Sleeping, relaxing	Liver disease, gallbladder inflammation	None	Insomnia	Unlikely but described (Bauer 2016)
30/M	*Valeriana officinalis*	2/d × 12m	Sleeping	Allergy	None	Migraine	Possible (Bauer 2016)

55/F	*Camellia sinensis*, *Panax ginseng*	1/d × 2w	Tonic	Migraine	Antihistaminics, Corticosteroids, Ibuprofen, Roxithromycin + FO, PO, VM	Gastric problems	Possible worsening of gastric side effects of anti-inflammatory drugs
35/M	*Camellia sinensis*	2/d × 2m	Immunity, body weight, tonic, HCHO	HCHO	No drug + Vitamin D, AA, FO	Insomnia and nausea	Possible for the content in caffeine (EMA 2013)
24/M	*Camellia sinensis*	1/d × 2m	Immunity, body weight, tonic, antioxidant	None	No drug + E, FO, PO, VM	Nausea	Possible (NCCIH 2012)
64/F	*Camellia sinensis*, *Crataegus* spp., *Olea europaea* (olive oil), *Viscum album*	Unknown					Possible (olive oil)
57/F	*Camellia sinensis*, *Paullinia cupana*	2/d × 2m	Body weight, digestion, energy/tonic	None	No drug	Insomnia	Probable due to the content in caffeine (Bailey et al. 2014)
50/M	*Camellia sinensis*	1/d × 2w	Immunity	HCHO, diabetes, migraine	None	Diarrhoea, gastric problems (nausea)	Unlikely

(continued)

Table 5.7 (continued)

Age/ gender	Botanical/s[a]	Dose and period	Reasons for use	Present or past main health problems	Conventional drugs + FS	Adverse effects	Causality
49/M	*Camellia sinensis*	1/d × 2w	Immunity	Migraine, ulcer	None	Diarrhoea, gastric problems (nausea)	Unlikely
47/M	*Auricularia auricula-judae* [fungus], *Coffea arabica*, *Fallopia japonica/Polygonum cuspidatum*, *Ginkgo biloba*, *Panicum miliaceum*, *Polyporus umbellatus* [fungus], *Saccharomyces cerevisiae* [yeast], *Serenoa repens*, *Trigonella foenum-graecum*, *Ziziphus jujuba*	2/d × 12m	Hair/skin, energy	HCHO	None	Discomfort	Unassessable due to the presence of several ingredients
65/M	*Ginkgo biloba*	4/w × 12m	Memory	HCHO	Iron supplementation, V	Insomnia	Possible (Salehi 2010)
66/F	*Ginkgo biloba*	5/w × 6m	Memory	None	None	Constipation	Possible (Mayo Clinic 2013)
69/M	*Ginkgo biloba*	2/d × ?	Joints/bones, blood circulation	Diabetes, heart disease, hypertension, liver disease, stroke, gallbladder inflammation/stones	Acenocumarole, Captopril, Trimetazidine	Insomnia	Possible (EHealthMe 2016)

21/F	*Ginkgo biloba*	1/d x[b]	Memory	None	No drug + Polyphenols	Dizziness	Possible (EMA 2014)
19/M	*Ginkgo biloba*	2/d × 2w	Memory	Hypertension	Captopril	Insomnia	Possible (eHealthMe 2016)
23/M	*Paullinia cupana*	1/d × 2m	Energy	None	None	Diarrhoea	Unlikely
19/M	*Paullinia cupana*	2/w × 3w	Energy, urinary tract	None	None	Constipation	Unlikely
36/F	*Panax ginseng*, *Paullinia cupana*	1/d × 3m	Energy/tonic	None	Birth-control pill	Tachycardia	Probable (EMA 2012)
50/F	*Paullinia cupana*	1/d × 2w	Energy/tonic	Hypertension, anxiety, depression	Fluoxetine	Tachycardia	Probable (EMA 2012)
35/M	*Paullinia cupana*	1d × 5m	Energy/tonic, mood	Heart disease	None	Dizziness	Possible (Smith 2010)

AA supplement containing amino acids, *FO* fish oil, *E* enzymes, *HCHO* hypercholesterolemia, *M* Supplement containing minerals, *PE* Prebiotics, *PO* Probiotic, *SI* Soy isoflavones, *V* Supplement containing vitamins; *VM* Supplement containing vitamins and minerals, *d* day, *m* month, *w* week

[a]According to: for plants US Department of Agriculture (plants.usda.gov); for algae www.algaebase.org; for fungi www.indexfungorum.org

[b]Unknown

in most of them, these botanicals were the only ingredient in PFS involved and were consumed in a period of time ranging between 2 weeks and 12 months. Causality assessment was generally defined as “possible”. These plants were also involved in some cases of drug interaction: *Ginkgo biloba,* in association with drugs for cardiovascular diseases caused the development of insomnia and dizziness, while tachycardia was the main symptom of the association of *Paullinia cupana* with fluoxetine, iron or oral contraceptives. As observed in the Poison Centres study described in Sect. 5.3.1, gastrointestinal and nervous system were the main targets, being involved in the 60% and 17% of the reported adverse effects, respectively.

5.5 Data on Adverse Effects to Botanicals Collected by the French Agency for Food, Environmental and Occupational Health and Safety (ANSES)

Data on adverse effects due to botanicals are collected by the French Agency for Food, Environmental and Occupational Health & Safety (ANSES). Starting from 2010, ANSES launched a nutritional vigilance program for novel foods, fortified foods, food supplements and foodstuffs intended for specific diets. A pilot phase, limited to food supplements, has been undertaken in 2009. Online-forms for reporting adverse effects, available at ANSES Internet site, are filled in by health professionals (doctors, pharmacists, dietitians, etc.). Declarations are then analyzed by the nutritional risk assessment unit (to determine the seriousness of the incident, the product’s composition, concordance with previous declarations, etc.) and are then submitted to a technical working group for more thorough analysis, in order to determine to what extent the product is responsible for the occurrence of the adverse effect. In this study, the collection of data on adverse effects due to botanicals was performed from 2009 to 2016. A causality assessment was performed for each PFS.

Data were collected for 192 plants responsible for 273 cases involving 190 females (69.6%) and 83 males (30.4%). The patients were 255 adults (from 17 to 93 years old) with mean age of 51.0 ± 17.4 years, and 18 children (from newborn to 15 years old), with a mean age of 5.0 ± 6.1 years. PFS containing one botanical were involved in 92 cases, and multi-ingredient PFS in 181. Table 5.8 lists the plants involved in at least 10 adverse effects.

Generally speaking, *Camellia sinensis* was the plant most frequently involved in adverse effects (55 citations): in 53 cases was consumed as green tea only, in 1 case as green, white and oolong teas, and in 1 case as green, black and oolong teas. Other frequently cited plants were *Paullinia cupana* (30) and *Panax ginseng* (24).

Plants contained in mono-ingredient PFS were ranked according the number of cases identified: *Glycine max* was the most involved (19 cases), followed by *Vaccinium macrocarpon* (12), *Camellia sinensis* (5), *Capsicum annuum*, *Cynara*

Table 5.8 Plants involved in at least ten cases of adverse effects collected by ANSES

Plant/ingredient		
Latin name	Common name	Number of cases
Camellia sinensis	Tea	55
Paullinia cupana	Guaranà	30
Panax ginseng	Ginseng	24
Vitis vinifera	Grape vine	24
Glycine max	Soybean	22
Cynara scolymus	Artichoke	19
Citrus aurantium	Bitter orange	18
Zingiber officinale	Ginger	17
Vaccinium macrocarpon	Cranberry	17
Ilex paraguayensis	Yerba mate	15
Melissa officinalis	Lemon balm	15
Rosmarinus officinalis	Rosemary	14
Taraxacum officinale	Common dandelion	14
Angelica archangelica	Garden angelica	12
Opuntia ficus-indica	Prickly pear cactus	12
Piper nigrum	Black pepper	12
Raphanus sativus	Radish	12
Vaccinium myrtillus	Common bilberry	12
Citrus sinensis	Sweet orange	11
Eschscholtzia californica	California poppy	11
Passiflora incarnata	Passionflower	11
Cola nitida	Kola nut	10
Curcuma longa	Turmeric	10
Dioscorea villosa	Wild yam	10
Foeniculum vulgare	Fennel	10
Harpagophytum procumbens	Devil's claw	10
Prunus cerasus	Sour cherry	10
Sambucus nigra	Elderberry	10

scolymus, *Harpagophytum procumbens*, *Melissa officinalis, Panax ginseng* and *Taraxacum officinale* (3 cases/each).

Considering the organ/systems involved, liver was the most frequent target (59 cases) (Table 5.9), followed by skin/mucosa (39), nervous system (38), gastrointestinal tract (33), cardiovascular system (29), hematopoietic apparatus (26). Among symptoms, allergic reactions were observed in 20 cases; asthenia, fever, pain and other symptoms were reported in 35 cases.

As regarding the symptomatology, 238 patients showed one main symptom, 29 showed symptoms localized in two, and 6 in three systems. Symptomatology was mainly considered of minor/moderate entity; however, some severe cases were collected. Four fatal cases were collected but their association with the intake of botanicals should be considered extremely doubtful:

Table 5.9 Target systems, signs and symptoms of adverse effects collected by ANSES

Signs/symptoms	Number of cases
Liver	59
Skin/mucosa	39
Nervous system (including psychiatric disorders)	38
Other symptoms	34
Gastrointestinal tract	33
Cardiovascular system	29
Hematopoietic apparatus	26
Allergic reactions	17
Neuromuscular function	12
Kidney	10
Metabolism	7
Respiratory	3
Death	1

1. The first case involved in a 72 years-old male patient, who consumed *Angelica archangelica*, *Camellia sinensis*, *Cynara scolymus*, *Panax ginseng*, *Raphanus sativus*, *Rosmarinus officinalis*, *Vaccinium myrtillus* (contained in three different products) for 15 days. He developed cholestatic hepatitis and jaundice before death. A further clinical investigation revealed a viral hepatitis, which was the probable reason of death;
2. The second fatal case, whose causality was evaluated as "possible", occurred in a 72 years old man who died after developing encephalopathy, jaundice and fulminant hepatitis. He was taking a supplement containing *Desmodium adscendens* from two days and some concomitant drugs, including sulpiride, aspirin and other medications to reduce blood pressure, such as acebutolol and perindopril;
3. A 39 years-old man died after developing a subacute fulminant hepatitis. He was taking a supplement containing *Vaccinium myrtillus*, *Cynara scolymus* and *Foeniculum vulgare* for 7 months; however, an autoimmune cirrhosis was finally diagnosed and was the probable reason of death;
4. A 73 years-old woman, who was taking *Ginkgo biloba*, *Pao pereira* and *Rawolfia vomitoria*, developed hepatic fibrosis with a fatal clinical outcome. Since the patient showed the presence of hepatic metastasis, the association of the adverse effect and the supplements intake was obviously extremely doubtful.

Moderate/severe hepatotoxic effects were associated more frequently with few botanicals: among them, *Camellia sinensis* (14 cases), followed by *Panax ginseng, Paullinia cupana, Piper nigrum* and *Thymus vulgaris* (5 cases/each). However, only

the effects associated to *Camellia sinensis* (green tea) had been described previously in the scientific literature (Federico et al. 2007; Gloro et al. 2005; Mazzanti et al. 2009). Hepatotoxicity was mainly due to green tea derivatives, while *Camellia sinensis* consumed as black or oolong tea caused cardiovascular effects of minor/moderate entity (tachycardia), probably due to caffeine. This difference in toxicity confirms the already reported hypothesis that catechins (mainly EGCG), present in higher amounts in green tea, are the main responsible for hepatotoxic effects (Di Lorenzo et al. 2015a). Other plants determined clinical problems previously reported: among them, *Paullinia cupana* (4) and *Citrus aurantium* (2) were responsible for insomnia and anxiety, probably mediated by their content in active compounds (Di Lorenzo et al. 2015a).

As for mono-ingredient PFS, *Glycine max* was reported in 19 adverse events, including a case of anaphylactic shock and other allergic reactions (with a causality considered possible); interestingly, 4 cases of hypercalcemia were recorded in newborns, whose mothers consumed supplements containing partially hydrogenated oil (72.32 mg/capsule) from *Glycine max* (as source of poly-unsaturated fats), vitamins (B1, B2, B3, B5, B6, B8, B9, D, E) and minerals (Cu, I, Mg, Zn, Mn, Fe). Considering that this effect has not been previously described, it has been supposed that it was mediated by vitamin D taken with the supplement. *Vaccinium macrocarpon* was involved in 12 cases, including a neutropenia (severe, causality: possible) and purpura (mild severity, causality: likely) and *Camellia sinensis* in 5 cases, where hepatotoxicity was the main problem, as reported above.

Interactions with drugs were observed in three cases:

1. a reduction of blood levels of valproic acid was observed in association with a food supplement containing *Taraxacum officinale*, red yeast rice and policosanol, but the enzymatic induction was considered doubtful;
2. a drastic reduction of pharmacological effect of levothyroxine was observed in a patient (53, F) operated for a thyroid cancer who consumed in association several botanicals (*Agropyron repens, beta vulgaris, Cichorium intybus, Citrus paradisi,* essential oil of *Lavandula hybrida*, *Orthosiphon arisatus, Panax ginseng, Raphanus sativus, Rosa canina, Sambucus nigra, Vitis vinifera, Zea mais*) for 11 months; the causal relationship was considered likely. It is possible that the critical reduction of pharmacological effect of conventional drugs was due to *Citrus paradisi*, which contains bergamottin, a compound, which can interfere with CYP450 as previously reported by He et al. (1998);
3. A serotonin syndrome in a 40 years-old woman, due to the association of venlafaxine (belonging to the class of Selective Serotonin Reuptake Inhibitors, SSRI) and a single dose of a PFS containing *Escholtzia californica*, *Hypericum perforatum*, *Magnolia* spp., *Valeriana officianlis*. The effect was attribute to hyperforin, which inhibits serotonin reuptake enhancing the drug effect (Borrelli and Izzo 2009). The causal relationship was considered probable, and the clinical outcome was positive.

5.6 Data on Adverse Effects Published in the Website of CFSAN Adverse Event Reporting System (CAERS) of FDA

The Center for Food Safety and Applied Nutrition's (CFSAN) Adverse Event Reporting System (CAERS) is a database containing information on adverse events caused by commercial products including foods, dietary supplements, and cosmetics. Data are freely available at: https://www.fda.gov/Food/ComplianceEnforcement/ucm494015.htm.

Cases can be reported by consumers, health care practitioners (physicians, nurses, pharmacists) and industries by filling a specific document on-line about: product involved, symptoms suffered and patient outcomes.

In this study, only cases related to botanicals were selected and analyzed but they could not be strictly classified for causality.

A total number of 2450 reports was found in the last 5 years (2010–2016); 413 of them (17%) involved mono-ingredient PFS, and 2037 (83%) were due to products containing more than one botanical. Table 5.10 lists the plants, contained in mono-ingredient products, causing the highest number of reported adverse effects. The distribution among the severity of symptoms classified according to PSS (Persson et al. 1998) is also shown.

Serenoa repens, Silybum marianum, Ginkgo biloba and *Cinnamomum verum* were ranked in the first four positions. The whole classification of symptoms is illustrated in Table 5.11. Generally speaking, the adverse effects showing severe symptoms were 349 (14.2%); of them 82.5% were associated with multi-ingredient PFS. Fatal outcomes occurred in 15 cases; most of them (13) were due to products, containing from 2 to 14 botanicals. Table 5.12 describes in details the 15 fatal cases, reporting the name of the supplement, its composition as reported in the label and the symptomatology described in the published reports.

Organs and systems involved in the adverse effect were numerous. Considering the mono-ingredient PFS, the most frequent target was the cardiovascular system, followed by gastrointestinal tract and nervous systems.

In more detail, hepatotoxicity and cardiovascular adverse effects were often associated with the intake of *Silybum marianum* (13 and 10 cases, respectively). The intake of saw palmetto (*Serenoa repens*) was responsible for cardiovascular (18) and gastrointestinal effects (16). These observations are in agreement with previously published papers (Ball and Kowdley 2005). Cardiovascular effects were also reported in 10 cases involving *Gingko biloba* and 14 due to *Cinnamomum verum*. Cases of cardiotoxicity were previously described for *Ginkgo biloba* (Di Lorenzo et al. 2015b), while *Cinnamomum verum* was normally associated with problems localized in the oral cavity (Siqueira et al. 2009). On the other hands, some cardiovascular events were reported by ANSES.

Data published by CAERS have the great advantage of the free accession, so that any reasearcher can download and use them, as in the case of this chapter. On the other hands, these data present some scientific limitations:

Table 5.10 Botanicals in mono-ingredient supplements, which were associated with the highest number of adverse effects, published in the CAERS website

Plant		Severity of symptoms				
Latin name	Common name	Minor	Moderate	Severe	Death	Total
Serenoa repens	Saw palmetto	22	17	18	0	57
Silybum marianum	Milk thistle	28	18	2	1	49
Ginkgo biloba	Ginkgo	23	17	4	0	44
Cinnamomum verum	Cinnamon	16	14	4	0	34
Vaccinium myrtillus	Common bilberry	8	9	3	1	21
Trigonella foenum-graecum	Fenugreek	7	4	6	0	17
Vitis vinifera	Grape	7	6	3	1	17
Actaea racemosa	Black cohosh	9	3	3	**0**	15
Glycyrrhiza glabra	Licorice	7	4	3	0	14
Vaccinium macrocarpon	Cranberry	9	5	0	0	14
Crataegus monogyna	Common Hawthorne	5	6	2	0	13
Allium sativum	Garlic	8	3	1	0	12
Salvia hispanica L.	Chia	5	4	2	0	11
Valeriana officinalis	Valerian	6	2	3	0	11
Hypericum perforatum L.	St. John's wort	7	1	2	0	10
Camellia sinensis	Green tea	**2**	**7**	**0**	0	9
Olea europea	Olive	5	2	0	0	7
Citrus paradisi	Grapefruit	2	3	1	0	6
Taraxacum officinale	Common dandelion	1	4	1	0	6
Carica papaya	Papaya	3	2	0	0	5
Rhamnus purshiana	Cascara buckthorn	2	1	1	0	4
Borago officinalis	Borage	2	1	0	0	3
Euterpe oleracea	Acai	2	1	0	0	3
Griffonia simplicifolia	Griffonia	2	1	0	0	3
Harpagophytum procumbens	Devil's claw	2	1	0	0	3
Malaleuca alternifolia	Narow-leaved paperbark	0	3	0	0	3
Vitex agnus-castus	Chastetree	1	0	2	0	3
Matricaria chamomilla	Chamomile	1	1	0	0	2
Melissa officinalis	Lemon balm	2	0	0	0	2
Phyllantus emblica	Emblic	0	2	0	0	2
Hippophae rhamnoides	Sea buckthorn	2	0	0	0	2

(continued)

Table 5.10 (continued)

Plant		Severity of symptoms				
Latin name	Common name	Minor	Moderate	Severe	Death	Total
Trifolium pratense	Red clover	2	0	0	0	2
Garcinia sp.	Garcinia	1	0	0	0	1
Lycium barbarum	Goji	1	0	0	0	1
Ocimum tenuiflorum	Holy basil	1	0	0	0	1
Morinda citrifolia	Noni	0	1	0	0	1
Panax ginseng	Ginseng	0	1	0	0	1
Piper nigrum	Black pepper	1	0	0	0	1
Rosa canina	Dog rose	1	0	0	0	1
Rubus idaeus	Raspberry	1	0	0	0	1
Glycine max	Soybean	1	0	0	0	1
Solanum lycopersicum	Tomato	1	0	0	0	1

Table 5.11 Severity of symptoms of adverse effects due to mono- and multi-ingredients food supplements, published in the CAERS website

	Severity of symptoms				
Product	Minor	Moderate	Severe	Death	Total
Mono-ingredient PFS	206	144	61	2	413
Multi-ingredient PFS	888	848	288	13	2037
Total	1094	992	349	15	2450

1. The adverse event are reported as such without any assessment by toxicologists;
2. In most cases, there is no detail supporting the causal relationship between the product intake and the adverse reaction;
3. Since cases can be reported by any citizen in US (health-care professionals but also consumers), the reliability of the data provided is often unassessable. Details on duration of intake, concomitant medical conditions or drugs intake are included in very few cases.

5.7 Conclusions

Table 5.13 compares all sources reviewed in this paper, ranking the botanicals in the first 13 positions for number of adverse effects. To make the association more reliable, only data on adverse effects due to mono-ingredient PFS were included in the table. Botanicals present in at least three lists are indicated in bold.

Results obtained by the retrospective Poison Centres' study (Lüde et al. 2016), the Consumer survey (Restani et al. 2016) and Pavia Poison Centre were comparable: in all studies *Valeriana officinalis* and *Camellia sinensis* were ranked in the first two positions. A different classification was found in the FDA website and with

Table 5.12 Name and composition of PFS involved in fatalities reported by CFSAN (2010–2016)

Product	Composition (as reported in the label)	Symptoms
Animal Stak	Vitamin B_6 (10.5 mg), magnesium 450 mg, zinc 30 mg, vitamin D_3; Pro testosterone complex (1 500 mg): *Tribulus terrestris* extract 1000 mg, *Eurycome longifolia* (root), LJ-100 (Eurypeptides, glycosaponins and polysaccharides), Fenugreek (seed), Stinging nettle root, Maca extract (*Lepidium meyenii*); Growth Hormone Support (1500 mg): arginine complex, *Mucuna pruriens* (standardized 15% L-DOPA) extract 300 mg, Immunolin, Humanofort; Alpha Glycerylphosphorylcholine; Anti-aromatase (mg 300): *Polygonum cuspidatum* root (resveratrol), Calcium-D-glucarate, diindolylmethane; Hormone Amplifying Blend (500 mg): L-carnitine fumarate, AgmaPure (agmatine sulphate), GlycoCarn (glycine propionyl L-carnitine HCl), quercetin, AstaPure (staxanthin), bioperine fruit; Restorative Support Complex (500 mg): Milk thistle extract (silymarin), Astragalus (root), Ashwaganda extract, Na-R-Alpha lipoic acid, Coenzime Q 10	Loss of consciousness, pulse absent, cardiac arrest, cardiomyopathy
Colonix advanced internal cleansing program colonix intestinal cleanser	Psyllium ask, Flax seed, Fennel seed, Papaya fruit, grapefruit pectin, Slippery Elma Bark, Marshmallow root, Rhubarb root, guar gum, Alfalfa leaf, peppermint leaf, *Uva ursi* leaf, stevia leaf, licorice root, *Aloe vera* gel 200:1 concentrate	Neoplasm malignant (association unassassable)
Smooth move tea	Organic senna leaf (1080 mg), organic liquorice root, organic bitter fennel fruit, organic sweet orange peel, organic cinnamon bark, organic coriander fruit, organic ginger rhizome, organic orange peel on gum arabic	Long QT syndrome
Nutrilite Double X vitamin/ mineral/Phytonutrient supplement (2 deaths)	Vitamin C, vitamin E, vitamin B_{12}, pantothenic acid, selenium, molybdenum, alpha lipoic acid, inositol, lycopene, lutein esters, citrus bioflavonoid dehydrate, dried kale, alfalfa concentrate, apple extract powder, asparagus powder, holy basil extract blend (*Ocimum tenuiflorum*), blueberry (*Vaccinium angustifolium*) powder, grape (*Vitis vinifera*) extract, oregano (*Origanum vulgare*) powder extract, prune (*Prunus domestica*) extract, rosemary (*Rosmarinus officinalis*) extract, cranberry (*Vaccinium macrocarpon*) extract, parsley (*Petroselinum crispum*) dehydrate, pomegranate (*Punica granatum*) extract, and sage (*Salvia officinalis*) powder extract	Two cases: in the first the circumstances were not specified; in the second one, Alzheimer disease was reported

(continued)

Table 5.12 (continued)

Product	Composition (as reported in the label)	Symptoms
Emergen-C immune defense	Multi-vitamin supplements with hibiscus extract, edelberry concentrate	Death, pancreatic carcinoma
Hydroxycut pro-clinical tablets	Amount per caplet: *Coffea canephora* (100 mg) fruit extract, *Malpighia glabra* (acerola) 25 mg, *Coffea arabica* 100 mg (equiv caffeine 90 mg), *Panax ginseng* root (12.5 mg), *Opuntia ficus-indica* leaf (12.5 mg), *Lycium barbarum* fruit (7.5 mg), *Punica granatum* fruit (7.5 mg), Choline bitartrate, ascorbic acid, nicotinamide, pyridoxine hydrochloride, chromium picolinate	Cardiac arrest, nausea, brain death, pulmonary oedema, pleural effusion, pupil fixed, diastolic dysfunction, toxicity to various agents, respiratory alkalosis, respiratory distress, hypophosphataemia, dizziness, pain, tachycardia, transaminases increased, renal failure acute, lactic acidosis, metabolic acidosis, myxoedema, fatigue, dysarthria, mental disorder, vomiting, hypothyroidism, alanine aminotransferase increased, adverse drug reaction, ammonia increased, brain injury, aspartate aminotransferase increased, oedema, blood glucose decreased, anoxia, depressed level of consciousness, mental impairment, brain oedema, hypokalaemia, blood ethanol increased, intracranial pressure increased, blood fibrinogen increased, acute hepatic failure, body temperature decreased, blood lactic acid increased, drug screen positive, pneumothorax, convulsion, leukocytosis, tachypnoea, blood bilirubin increased, hypertension, insomnia, blood thyroid stimulating hormone increased, dyspnoea, unresponsive to stimuli
Nature's bounty complete pms support complex rapid release liquid softgels	Multi vitamins complex + Cranberry concentrate (*Vaccinium macrocarpon* fruit) 500 mg, borage oil (*Borago officinalis*) seed oil, which contains gamma linolenic acid (270 mg) and linoleic acid (430 mg)	Convulsions

4life transfer factor plus tri-factor formula	IP-6 (inositol-hexaphosphate), beta-sitosterol and other phytosterols, *Cordyceps sinensis* mycelia extract, *Saccharomyces cervisiae* extract, *Agaricus blazeii* fruit extract, Aloe (*Aloe barbadensis*) leaf gel extract, Oat (*Avena sativa*), Olive (*Olea europaea*) leaf extract, maitake (*Grifola frondosa*) fruit extract, Shitake (*Lentinus edodes*) fruit extract	Choking sensation, dyspnoea
4 life transfer factor recall	Magnesium (110 mg), Lemon balm (*Melissa officinalis*) herb extract, soy (*Glycine max*) seed extract, Choline bitartrate, *Bacopa monnieri* aerial parts extract, N-acetyl-tyrosine, N-acetyl-L-cysteine, *Ginkgo biloba* leaf extract, *Huperzia serrata* herb extract, vinpocetine	–
Protandim (US) (2 deaths)	Calcium (77 mg), milk thistle (*Sylibum marianum*) seed, Bacopa extract (*Bacopa monnieri*) whole herb, Ashwagandha extract (*Withania somnifera*) root, green tea extract (*Camellia sinensis*) leaf, turmeric extract (*Curcuma longa*) rhizome	In one case, heart transplantation rejection was reported
Puritans Pride grape seed extract 100 mg rapid release capsules	Grape seed extract (*Vitis vinifera*) (100 mg) standardized (50% polyphenols, 50 mg), Citrus (*Citrus limon*) (peel) bioflavonoid complex 30 mg	Dizziness, nausea, symcope, anorectal disorder, blood pressure increased, blood glucose increased
Nature made Bilberry 30 mg	*Vaccinium myrtillus* (60 mg) anthocyanosides 15 mg	Miocardial infarction
Puritans Pride milk thistle 500 mg rapid release capsules	Milk Thistle (*Silybum marianum*) (seed) 250 mg from a 4:1 extract (equivalent to 1000 mg of whole herb)	Cerebrovascular accident

Table 5.13 Ranking of botanicals for frequency of adverse effects produced as reported by the different data sources included in this paper (only mono-ingredient PFS)

Scientific literature review		PlantLIBRA Poisons Centres survey		Pavia Poison Centre		Self-reported cases PlantLIBRA consumers survey		ANSES		CAERS (FDA)	
Plant	**%***	**Plant**	**%***	**Plant****	**%***	**Plant**	**%***	**Plant**	**%***	**Plant**	**%***
Glycine max	19.3	*Valeriana officinalis*	14.3	*Camellia sinensis*	5.2	*Valeriana officinalis*	9.2	*Glycine max*	20.7	*Serenoa repens*	13.8
Glycyrrhiza glabra	12.2	*Camellia sinensis*	6.2	*Valeriana officinalis*	4.8	*Camellia sinensis*	8.0	*Vaccinium macrocarpon*	13.0	*Silybum marianum*	11.8
Camellia sinensis	8.7	*Melissa officinalis*	4.3	*Paullinia cupana*	4.4	*Ginkgo biloba*	6.9	*Camellia sinensis*	5.4	*Ginkgo biloba*	10.6
Ginkgo biloba	8.5	*Mentha x piperita*	4.3	*Capsicum annuum*		*Paullinia cupana*	6.9	*Capsicum annuum*	3.3	*Cinnamomum verum*	8.2
Citrus aurantium	5.1	*Passiflora incarnata*	4.3	*Panax ginseng*	3.5	*Cynara scolymus*	5.7	*Cynara scolymus*	3.3	*Vaccinium myrtillus*	5.1
Cinnamomum verum	4.7	*Paullinia cupana*	4.3	*Cola acuminata*	3.1	*Echinacea spp*	5.7	*Harpagophytum procumbens*	3.3	*Trigonella foenum-graecum*	4.1
Cimicifuga racemosa	4.7	*Glycyrrhiza glabra*	3.7	*Citrus aurantium*		*Olea europaea*	5.7	*Melissa officinalis*	3.3	*Vitis vinifera*	4.1
Echinacea purpurea	4.1	*Ilex paraguariensis*	3.7	*Piper nigrum*	2.2	*Red rice*	5.7	*Panax ginseng*	3.3	*Actaea racemosa*	3.6
Vitex agnus castus	3.9	*Panax ginseng*	3.1	*Crataegus monogyna*	2.2	*Panax ginseng*	5.7	*Taraxacum officinale*	3.3	*Glycyrrhiza glabra*	3.4
Hypericum perforatum	3.9	*Citrus aurantium.*	2.5	*Melissa officinalis*	2.2	*Equisetum arvense*	4.6	*Commiphora muku*	2.2	*Vaccinium macrocarpon*	3.4
Panax ginseng	3.3	*Cynara scolymus*	2.5	*Zingiber officinalis*	1.7	*Allium sativum*	3.4	*Cucurbita pepo*	2.2	*Crataegus monogyna*	3.1
Valeriana officinalis	2.8	*Dioscorea villosa*	2.5	*Citrus paradisi*	1.7	*Foeniculum vulgare*	3.4	*Desmodium adscendens*	2.2	*Allium sativum*	2.9
Vitis vinifera	2.8	*Allium ursinum*	1.9	*Ribes nigrum*	1.7	*Glycine max*	3.4	*Rhodiola rosea* *Ribes nigrum* *Sambucus nigra*	2.2	*Salvia hispanica L.*	2.7
Total cases	**492**	**Total cases**	**161**	**Total cases**	**229**	**Total cases**	**87**	**Total cases**	**92**	**Total cases**	**414**

Gray= present in 5 out of 6 lists Yellow = present in 4 out of 6 lists Light blue= present in 3 out of 6 lists

Gray present in 5 out of 6 lists, *Yellow* present in 4 out of 6 lists, *Light blue* present in 3 out of 6 lists

ANSES Nutrivigilance data when botanicals contained in mono-ingredient products are considered. FDA's data show that *Serenoa repens* and *Silybum marianum* were the botanicals most frequently involved in adverse effects, while *Valeriana officinalis* and *Camellia sinensis* represented 2.7% and 1.7% of cases, respectively.

The ANSES Nutrivigilance data indicate that *Camellia sinensis* is ranking third, after *Glycine max* and *Vaccinium macrocarpon. Valeriana officinalis* is ranking sixth (not shown on Table 5.13) since this botanical is most often formulated with other plants in food supplements marketed in France.

Making a general assessment of data collected, *Camellia sinensis* and *Panax ginseng* are always included in the first 13 positions apart from the CSAN list; *Valeriana officinalis* is present in 4 out 6 lists; *Citrus aurantium*, *Cynara scolimus*, *Ginkgo biloba*, *Glicine max*, *Glycyrrhiza glabra*, *Melissa officinalis* and *Paulinnia cupana* are present in 3 out of 6 lists.

On the basis of the data analysed, some final considerations can be done:

1. No information on patient history, dose and treatment duration was provided in the majority of the spontaneous reports;
2. Although adverse events analysed showed mainly mild to moderate symptoms, some fatal cases were recorded;
3. Reports of adverse events due to botanicals are often unassessable, as a consequence of: (a) the lack of adequate information on causality, (b) the presence of several ingredients, which makes difficult to establish an association with the adverse effects; (c) the frequent not-specific symptomatology or the presence of multiple symptoms;

4. It should be also noticed that PFS often contain also other ingredients, not derived from plants (such as vitamins or minerals) that could have a role in adverse effects development;
5. Although the limit indicated above, it is evident that some botanicals are more at risk for adverse effects, as discussed above;
6. The consumers should be informed about the correct use of PFS, their possible adverse effects and interactions with conventional drugs.

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Part II
The Quality Control for the Safety of Consumers

Chapter 6
Food Supplements Containing Botanicals: The Concept of Quality

Brunella Carratù and Stefania Giammarioli

Abstract The aim of this chapter is to provide a brief overview of the issues related to manufacturing process and principles of quality system, with particular attention to quality control, keeping in mind the unique characteristics of botanicals used as ingredients in these products. Therefore the main steps of the industrial process of plant-based preparations will be described. In addiction the main interrelated aspects of an integrated quality system, designed to assure that products will be consistently fit for their intended use, will be explained. A crucial point are quality controls that must be carried out in each step of the production process on the raw materials, intermediate/finished products. Finally will be treated the activities included in the process of post marketing surveillance, both active and passive, and by way of example they will be described the systems of post-marketing surveillance adopted in some countries.

Keywords Botanicals • Quality system • Quality control • Post-marketing surveillance

6.1 Introduction

Widespread and growing use of food supplements containing botanicals has created public health challenges globally in terms of quality, safety and efficacy. The development of parameters for standardization and quality control of botanicals is a demanding task. The aim of this chapter is to provide a brief overview of the issues related to manufacturing process and principles of quality system, with particular attention to quality control, keeping in mind the unique characteristics of botanicals used as ingredients in these products.

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6.2 Manufacturing of Different Plant Food Supplements Categories

Food supplements containing botanicals (PFS) are composed of plant substances or association of several plants or plant-based preparations and in case of some nutrients. The plant substances used are whole plants, or parts of plants (roots, barks, leaves, seeds, etc.) either whole or fragmented, but also juices removed by pressure or incision of the living plant (oleoresins, gums, latex, etc.) which have not undergone any specific treatment. Preparations are obtained from herbal material by various processes (e.g. extraction, fractionation, distillation, concentration, drying) (see Fig. 6.1).

The final products are sold in many forms: as fresh or dried products; dry, fluid or semi-fluid extracts; capsules; powders; tea bags; and other forms.

In the following sections the main steps of the industrial process of plant-based preparations will be described (Kindel 2014; Gil-Chávez et al. 2013; Gupta et al. 2012; European Medicine Agency 2010; Sapkale et al. 2010).

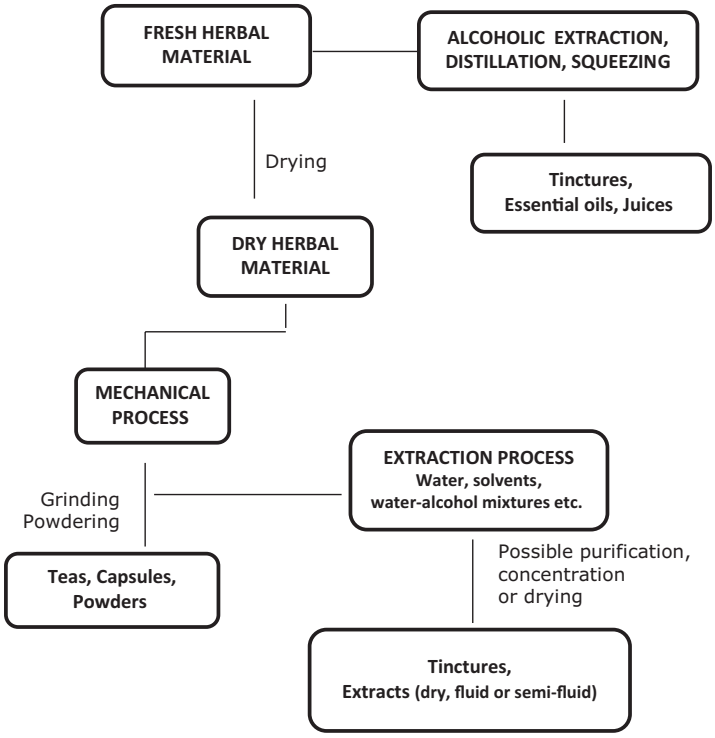

Fig. 6.1 Examples of plant-based preparations

6.2.1 Cleaning and Drying

After the plants are harvested, they must be cleaned. Cleaning may involve screening, washing, peeling, or stripping leaves from stems. Any unnecessary parts are removed prior to drying to avoid wasting time and energy. In some cases botanicals are used while fresh but generally they are dried first. The purpose of drying is to reduce the water content so that the plant can be stored. Most plants contain 60–80% moisture when harvested and must be dried to within 10–14% moisture before storage. Plants must be dried or processed as soon as possible after harvest because they begin to deteriorate immediately. Plants can be dried naturally or by a number of artificial methods. The type of plant or plant part being used will determine the appropriate drying technique.

6.2.2 Grinding

Grinding, or mincing, consists in mechanically breaking down either leaves, roots, seeds, or other parts of a plant into very small units ranging from larger course fragments to fine powder. Grinding is employed in the production of crude herbal products as well as in the initial phases of extracts. Some plant materials are packaged and sold at this point without any additional processing (teas, capsules, powders). Some proceed through an extraction process.

6.2.3 Extraction

The process of extraction is used in making juices, tinctures, dry, fluid or semi-fluid extracts. Extraction refers to separating by physical or chemical means the desired constituents from a plant. Solvent extraction (SE) is the most popular method of extraction. Table 6.1 lists some solvents suitable for extraction of particular classes of plant compounds (revised from Gupta et al. 2012).

The use of chloroform, dichloromethane, diethyl ether was reduced due to their toxicity and environmental impact. Sometimes mixtures of solvents are also used to get better extraction efficiency.

Hydro alcoholic solvent mixture (mixture of alcohol and water in varying proportions) is generally considered to give high extraction yields, which is owing to their expanded polarity range.

Extracts that has been separated from the plant material generally contain some unwanted substances such as tannins, pigments, microbial contaminants, etc. To separate impurities from the extract different methods such as decanting, filtration, sedimentation, centrifuging, heating, adsorption, precipitation and ion exchange are used.

Table 6.1 Plant compounds extracted by various solvents

Solvent	Compounds extracted
Chloroform	Terpenoids, flavonoids, alkaloids
Cyclohexane	Waxes, fats
Hexane	Waxes, fats
Dichloromethane	Terpenoids, alkaloids
Diethylether	Alkaloids
Ethylacetate	Alkaloids
Acetone	Flavonols, alkaloids
Ethanol	Tannins, polyphenols, flavonols, terpenoids, sterols, alkaloids, propolis
Methanol	Saponins, tannins, flavones, sugars, aminoacids, anthocyanins, terpenoids, quassinoids, lactones, polyphenols
Water	Sugars, aminoacids, saponins, tannins, lectins, terpenoids, anthocyanins, starches, polipeptides

SE has been improved by development of more modern extraction techniques such as microwave assisted extraction (MAE), ultrasound assisted extraction (UAE), supercritical fluid extraction (SFE), etc. in order to obtain better yields and promote the elimination of undesirable compounds from the extract.

Microwave-assisted extraction (MAE). When microwaves pass through a medium, their energy may be absorbed and converted into thermal energy. MAE works heating the moisture inside the cells that evaporates producing a high pressure on the cell wall. The pressure builds up inside the biomaterial modifying the physical properties of the biological tissues (cell wall and organelles) improving the porosity of the biological matrix. This allows better penetration of extracting solvent through the matrix improving yield of the desired compounds.

Ultrasound-assisted extraction (UAE) has been proposed as alternative to conventional SE, providing higher recovery of targeted compounds with lower solvent consumption. Its better extraction efficiency is related to the phenomenon called acoustic cavitation. When the ultrasound intensity is sufficient, the expansion cycle can create cavities or microbubbles in the liquid. The implosion of cavitation bubbles can hit the surface of the solid matrix and disintegrate the cells causing the release of the desired compounds.

Supercritical fluid extraction (SFE) is a method for extracting active ingredients using supercritical fluids, that are compounds (usually gases) at a temperature and pressure above their critical point, which have combined properties of gases and liquids. An advantage of supercritical extraction is that it can take place at low temperature thus preserving the quality of temperature-sensitive components. A wide range of compounds can be used as solvents in this technique. However, most separation systems use carbon dioxide due to its safety and low cost, in addition CO_2 ensure minimal alteration of the bioactive compounds. Polar molecules are poorly soluble in CO_2 and hence are not extractable, for this reason, the use of other solvent compounds (specially ethanol or methanol) is needed in order to enhance solubility and the selectivity of the process.

6.2.4 Steam Distillation

Steam distillation is another method for extracting active ingredients from plants. The water vapour carries small amounts of the vaporized compounds to the condensation flask, where the condensed liquid phase separates, allowing for easy collection. This process effectively allows for distillation at lower temperatures, reducing the deterioration of the desired products. Steam distillation is the most commonly used method for collecting essential oils.

6.2.5 Purification

Purification procedures may be applied to the extracts with the aim to reduce unwanted matter and/or to increase the content of active constituents (standardised extracts and quantified extracts). During the refining process the composition of the final preparation may vary to a greater or lesser extent but in general the refined extracts no longer have the total spectrum of constituents present in the original extract. The different purification steps lead from "total extracts" (natural multicomponent mixtures) via "refined extracts" (including mixtures of closely related constituents) finally to "isolated single constituents". The most common techniques used for the purification include: precipitation (salt, temperature, solvents, etc.), extraction (pure solvents, solvent mixtures, etc.), absorption (absorption chromatography, ion exchanger, etc.).

6.2.6 Concentration or Drying Process

After extraction of the plant and possible purification of extract, the resulting solutions can be concentrated into fluid or solid extracts. The result is separation of the extracted materials from the solvent that can be reused. Although there are still a number of liquid form extracts on the market (tinctures, fluid and semi-fluid extracts), the preferred industry method is to dry the extract to a solid form. The main reasons are: higher amount of active compounds, greater chemical stability and reduced cost. Tinctures, fluid and semi-fluid extracts are easily contaminated by bacteria and other micro-organisms. Liquid forms of extracts also promote chemical reactions, which may tend to break down the herbal compounds. A number of drying techniques are employed in the herbal processing industry, including freeze-drying and spray-drying. The result is a dried powdered extract that can then be put into capsules.

6.3 Principles of Quality System

Food supplements containing botanicals have to comply with all relevant aspects of legislation in their country of production in term of composition, manufacture and control. This means that manufacturers and distributors should apply an integrated system, usually named Quality Management, that provides assurance that products will be consistently fit for their intended use.

The definition of quality depends on both subjective and objective factors. The subjective factors include cultural, economic, psychological, religious and ethical aspects, creating a wide range of quality concepts. The objective factors include standardization of the organoleptic and physicochemical characteristics and food safety assurance (Da Cruz et al. 2006).

Quality Management is a wide-ranging concept covering all the arrangements made with the object of ensuring the quality of products, and include many interrelated aspects (see Fig. 6.2) , such as Quality Assurance, Quality Design, Quality Control, Quality Improvement and Manufacturing functions (Luning and Marcelis 2007).

Quality assurance: includes all activities and decisions to realize the quality. It deals with setting requirements on the quality system, evaluating its performance and organizing necessary changes.

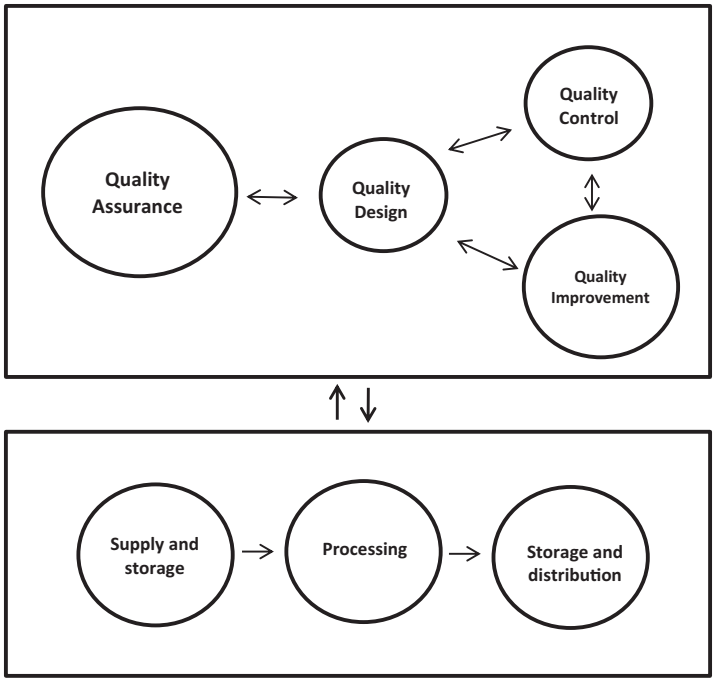

Fig. 6.2 Food quality management model

Quality design: starts with specifying consumer and/or customer demands and translating them into product and process specifications, i.e. formulation and selection of raw materials and choice of an appropriate manufacturing process and packaging materials.

Quality control: is a basic activity of quality assurance and its objective is to keep product properties, production processes between certain acceptable tolerances. Quality control must be an ongoing process to ensure that safety and quality of the product is maintained.

Quality improvement: involves a systematic approach to improving the system focusing on structural causes and solutions, in order to bring processes and resources at a higher level of quality.

Manufacturing functions: ensure that products are consistently produced and controlled according to quality standards and covers all aspects of production:

– supply and storage of incoming materials (i.e. raw and semi-processed materials, ingredients);
– transformation of incoming plant materials into processed products with desired physicochemical properties;
– packaging, storage and distribution of processed products applying appropriate conditions.

The following section will focus on critical aspects of Quality Control linked to the unique characteristics of the plant materials used in PFS, that make them different from other products as fortified foods or dietary supplements.

6.4 Quality Control

Appropriate quality controls must be carried out in each step of the production process (IADSA 2011) that can be summarized in:

- reception phase: visual inspection of raw materials/ingredients/supplies to verify any damage of the packaging and to check accompanying documentation (transport conditions and compliance with the order specifications);
- quarantine phase: release of raw materials/ingredients can either be based on certificates of analyses provided by the supplier, or sampled and tested in accordance with agreed specifications;
- production and packaging phase: defined and documented manufacturing procedures, including associated activities and precautions, are necessary to ensure the production of finished products which conform to their specifications and suitably protected against contamination or deterioration. Process conditions should be monitored and process controls carried out by suitable means including, as appropriate, sensory, instrumental and laboratory testing;
- approval phase of the intermediate/finished products: approval is based on checks by Quality Control for compliance with their specifications.

All lots of raw materials/ingredients, intermediate and finished products should be stored under the appropriate conditions (e.g. temperature and/ or relative humidity) and bear an identification mark that will provide a means of tracing products.

The laboratory tests represent a key element of Quality Control and require reliable and validated methods (see Chap. 4) to ensure the safety and quality of botanicals and botanical preparations intended for use in PFS.

The risk factors associated with the different stages of production require the drawing up of specifications and the performance of strict checks on raw materials and on intermediate/finished products.

6.4.1 Raw Materials

It is important that the batches of the botanical raw materials undergo appropriate testing before acceptance for further processing. Checks should include identification, absence of foreign material, and compliance with legal limits for contaminants and residues (Food Supplements Europe 2014; European Medicines Agency 2011; Sanzini et al. 2011; Franz et al. 2011; van Breemen et al. 2007; Council of Europe 2005).

6.4.1.1 Botanical Characterization and Identification

Botanical characterization and identification is imperative, particularly with botanicals harvested in the wild.

The scheme showed in Table 6.2 should be used for the characterization of a botanical. Great care should be exercised as there are many cases where botanicals have been renamed or reclassified. Attention must also be taken with the use of common names as these can vary from region to region and in some instances can be used for a different species. The characterization must be made on the basis of the Scientific (Latin) classification.

Table 6.2 Scheme for characterization of a botanical

Scientific (Latin) name	*Full systematic species name including Botanical family, genus, species, variety, subspecies, author's name, and chemotype if applicable*
Synonyms	*Botanical name(s) that may be or have been used interchangeably with the preferred scientific name*
Common names	*Vernacular name(s)*
Part used	*e.g. root, leaf, seed, etc.*
Geographical origin	*Continent, country, region*
Growth and harvesting conditions	*Wild or cultivated, cultivation practices, time of harvest in relation to both season and stage of the plant growth*

The characterization should be completed by proper identification of the botanical material by morphological description and chemical control, and eventually by DNA-based methods.

Morphological control include:

a. Macroscopic examination: consists of visual/olfactory observations of morphological and organoleptic characteristics of the plant (such as appearance, form, colour, fragrance and taste)
b. Microscopic examination: consists of plant organs and tissues analysis to identify the specific characteristics of the various genera and species

Chemical control: some phytochemical compounds are usually characteristic for a given plant, and their profile represents the fingerprint of the plant. The compounds considered may be the active substances but quite often characteristic substances only. The chemical fingerprint can be obtained by thin layer chromatography (TLC); high-performance liquid chromatography (HPLC); high performance thin layer chromatography (HPTLC) .

DNA-based identification: DNA fingerprinting makes it possible to identify the genus, species and variety of the raw material in an unambiguous and reproducible manner, even if the material has been processed (e.g. dried, grinded, pounded, lyophilized, squeezed, etc.). Most of DNA-based methods use polymerase chain reaction (PCR) to amplify the DNA region of interest.

In some cases other chemical and physical tests can support the identification. Examples of such tests are: total and insoluble ash, water soluble extractive, foreign organic substances, loss on drying, etc.

6.4.1.2 Foreign Matter

The foreign matter can be derived from various sources, this includes stones, soil, insects and insect parts, moulds, worms, rodent droppings, metal, textile fibres, pieces of glass, etc. Procedures for both visual and instrumental examinations for the presence of foreign matter should be in place and should be applied to all batches.

6.4.1.3 Contaminants and Residues

Botanicals can be susceptible to a wide range of contaminants and residues. Whilst many contaminants have environmental origin, the residues result from agricultural treatments during cultivation. Many of the contaminants and residues, that can potentially be found in botanicals, can be the subject of legal limits.

Following the main contaminants and residues are shown.

a. Microbiological contamination: is due to the presence of microbial pathogens relevant to human safety (e.g. Escherichia coli, Salmonella, Staphylococcus aureus, etc.) that can be a serious risk, particularly in situations where animal waste (faeces) is used as a fertiliser during cultivation or where surface water is used for irrigation. In addition, contamination may also occur during harvesting, post-harvesting, drying and subsequent processing stages;
b. Mycotoxins: are biochemical substances produced by the secondary metabolism of certain fungi or moulds colonizing the foodstuffs. Aflatoxins and ocratoxins A are the mycotoxins of major concern in botanicals;
c. Environmental contaminants: are organic and inorganic contaminants occurring in the environment and which can be found on botanical matter. The main ones are: toxic (heavy) metals (such as cadmium, lead, mercury, arsenic), dioxins, furans and dioxin-like polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs);
d. Residues: result from agricultural treatments during cultivation and storage and include pesticides (insecticides, fungicides and herbicides), ethylene oxide (not permitted in EU food law) and fumigants (e.g. phosphine or methyl bromide).

Finally no botanicals or botanical products, intended to be used in PFS, can be placed on the market in the EU if it is produced from a GMO or treated with ionizing radiation. Consequently it is necessary to carry out specific checks to verify the compliance with current legislation.

6.4.2 Intermediate/Finished Products

After botanical raw material has been identified and found to be free from hazardous contaminants and residues, it can be used in the production process.

The next step to ensure a safe, reliable and reproducible PFS is the standardization of intermediate/finished products together with purity control (absence of contaminants, residues and foreign matter) (Van Breemen 2015; Food Supplements Europe 2014; Sanzini et al. 2011; Speijers et al. 2010; van Breemen et al. 2007; Council of Europe 2005; Marcus and Grollman 2002; Fugh-Berman 2000).

6.4.2.1 Standardization

The standardization is carried out through the dosage of specific markers. A marker is a chemically defined characteristic constituent, or group of constituents, present in a specified botanical material. Markers can be used for control purposes, whether or not they have any physiological activity, as their function is to assist in

composition analysis and monitoring the batch-to-batch variation of the plant materials to ensure the uniformity of production.

Markers can be classified into two categories. The first is termed the "active marker(s)", which is a constituent or group of constituents that are generally accepted as contributing to a physiological effect. The second is known as "analytical marker(s)", which is a constituent or a group of constituents (in some case also toxic) that are known to be characteristic of the botanical material. The ideal marker is a constituent(s) for which there is an established and validated analytical method and for which the assay is not subject to interference from other constituents in the botanical source or from processing. Each batch of intermediate/final product should be analysed to confirm that specified marker substance levels are within the required range.

When multiple botanicals are used in a PFS, quality control can become very challenging. Furthermore, the quality control of supplements containing mixtures of botanicals is complicated due to the batch-to-batch variation in the chemical composition of each botanicals used in the product.

6.4.2.2 Contaminants, residues and foreign matter

All manufacturing operations must be performed in order to minimize the potential for growth of microorganisms, or for the degradation or contamination of intermediate/finished products. Chemical and microbial contaminants or foreign matter testing procedures shall be used where necessary to identify process failure.

For extracts special care should be given to the solvents used that must comply with the EU legislation. This legislation lists the permitted solvents in the production of foodstuffs and food ingredients, and for a number, conditions of use and maximum residue levels.

Another important issue is the possible contamination by pharmacological substances (see Table 6.3). Processing in pharmaceutical facilities might inadvertently introduce pharmaceutical compounds into PFS, but there is also the possibility of adulteration of supplements to improve their effectiveness through a deliberate

Table 6.3 Examples of pharmaceutical adulterants

Pharmacological class	Drugs
Analgesic-antiinflammatory	Aminophenazone, paracetamol, phenylbutazone, indomethacin
Antihistamines	Chlorphenamine
Corticosteroids	Betamethasone, prednisolone
Diuretics	Hydrochlorothiazide
Drugs for erectile dysfunction	Sildenafil
Stimulants	Caffeine
Tranquilizers	Diazepam

Table 6.4 Examples of misidentification/adulteration that have caused adverse reactions

Intended plant	Replaced plant	Effect
Illicium verum	*Illicium religiosum*	Convulsions
Gentiana lutea	*Pedophyllum emodi*	Gastro-intestinal and kidney symptoms
Panax ginseng	*Datura metel*	Anti-cholinergic symptoms
Stefania tetranda (guang fangji)	*Aristolochia fangji*	Nephrotoxicity

addition of pharmaceutical compounds frequently occurring in adverse events (also severe poisoning) or interactions.

6.4.3 Presence of Extraneous Plants

Controls should be carried out both on raw materials and intermediate/finished products to highlight the presence of extraneous plant material whose occurrence may be accidental or due to adulteration (Jordan et al. 2010; Smolinske 2005; Fugh-Berman 2000).

The first case is represented by:

- cross contamination with other plants that may occur in various stages (sowing, harvesting, transport, unloading and handling) from the field to the processing site;
- misidentification that occurs because many plants, especially those harvested in the wild, are similar in appearance during various growth phases;
- mislabelling, because of the similarity of common names for different species.

This sometimes can lead to the presence of plants containing toxic substances or responsible for allergic or idiosyncratic reactions.

The adulteration is due to the deliberate and intentional addiction of plants or their parts (not declared on the label) in order to improve the effectiveness of the product or for their lower cost or easier availability (see Table 6.4).

6.5 Post-Marketing Surveillance

Although PFSs are considered by the public as safe products, because they are made from natural ingredients, they are not risk free. There are a wide variety of risks associated with PFSs, as already mentioned above, which arise due to several reasons such as contaminations; unhygienic manufacturing; adulteration or

counterfeit; interactions with drugs, foods, other botanicals or dietary supplements; incorrect dosing and instructions, etc.

Governments are interested in monitoring the quality and safety of marketed products as the key to control potential consumption hazards. Post-marketing surveillance is a method designed to monitor the quality, safety, effectiveness and performance of PFSs.

The most crucial activity included in this process is that concerning safety, in particular collection of information or reports on adverse reactions, finding a causal relationship between adverse event and product.

This activity include (Health Canada 2008):

- identifying, as early as possible, potential safety issues;
- refining and adding information on suspected or known adverse reactions, and on a possible increase in their frequency;
- communicating new safety information to health professionals and public in order to improve the appropriate use of the supplements.

In order to be effective, the system should (Kingston 2012):

- be sufficiently "sensitive", such that potential threats or safety concerns would likely be included in the monitored events;
- be sufficiently specific to allow detection, differentiation and ultimately determination of real vs. perceived threats;
- identify intended and unintended patterns of use which may potentially contribute to "unintended effects";
- allow assessment of product performance by itself or in the presence of other products or substances;
- ensure that at-risk populations, such as children, pregnant women, elderly and people suffering with certain medical conditions, are considered when monitoring safety.

Post-marketing surveillance is an integrated set of activities requiring a careful structured methodological approach. There are mainly two types of post-marketing surveillance: passive surveillance, and active surveillance (Suman 2013; Murty 2007).

Passive Surveillance: include spontaneous reporting and is the most commonly used method for data collection and monitoring of adverse reactions. Physicians, pharmacists, and consumers voluntarily report any suspected reaction due to the use of PFS. The main advantage of spontaneous reporting is that its scope is national and able to cover diverse populations. The major disadvantages of this system include underreporting and poor quality of reports.

Active Surveillance: can be defined as regular periodic collection of case reports from health care providers or facilities. The active surveillance is a key to improve the quantity and the quality of adverse reaction reports and uses more rigorous data collection tools such as pilot studies in specific settings, observational cohort studies, targeted studies on important potential problems, etc. The disadvantages of this

system include the possibility of selection bias of reporters and patients, and requirement of more intense resources which makes it expensive.

Monitoring safety of PFSs is more challenging in comparison to drugs. The issues concern in particular causality assessment of adverse reaction, inadequate knowledge regarding characteristics of PFS, underreporting of adverse reactions.

In assessing the *causality of adverse reaction* it is necessary to take into account many factors. Most of the PFSs are mixtures of multiple herbs and ingredients that may cause adverse reactions; in these cases, it is hard to identify the causality to the product as a whole. Another important concern is the possible contamination due to for example to heavy metals and microbes, that pose difficulties in detecting whether adverse reaction was due to original product or to these contaminations. Other difficulties in assessing causality are adverse reactions produced from improper use of PFSs and/or drug-herb interactions.

The inadequate knowledge, regarding complex characteristics of PFSs, appropriate methods for safety, efficacy, harms/benefits assessment and unsuitability to specific population groups, pose the greatest challenge to monitoring the safety of these products. There are limited publications or data available that can provide reliable information regarding the risks and benefits of using PFSs. Recently, in response to this need, the European project (2010–2014) PlantLIBRA (Plant food supplements: Levels of Intake, Benefit and Risk Assessment) structured to develop, validate and disseminate data and methodologies for risk and benefit assessment and implement sustainable international cooperation was carried out.

The under-reporting of adverse reactions related to PFSs is an important issue in most countries. The main reason of underreporting is due to the fact that most of the consumers consider these products safe and there could not be any side effect with their use.

Today, many countries implement a post-marketing surveillance framework of the PFSs for the safety of their populations. Each country uses their own or similar systems for managing PFSs adverse reaction risks.

For example in **USA** (Felix et al. 2015; Frankos et al. 2010; Gardiner et al. 2008; Wallace et al. 2008; Kigston 2005) FDA regulates dietary supplements under a different set of regulations than those covering "conventional" foods and drug products i.e. the Dietary Supplement Health and Education Act of 1994 (DSHEA). According to this regulations, manufacturers and distributors are responsible for evaluating the safety and labelling of their products before marketing, to ensure that they meet all the requirements of DSHEA and FDA regulations. FDA is responsible for conducting post-marketing surveillance and taking action against any adulterated or mislabelled products.

For this purpose the FDA has created, through the Safety Reporting Portal (SRP), a suitable, secure, and efficient method for letting FDA know when industry or consumers find a problem with a dietary supplement. This method is an all-electronic version of the MedWatch, used by manufacturers, health care professionals, researchers, public health officials, and consumers to report problems (https://www.safetyreporting.hhs.gov/fpsr/FpsrRoutingPage.aspx Accessed February 3, 2017).

The data collected are entered into a computerized database called Adverse Event Reporting System which is further evaluated by multidisciplinary experts for causal assessment.

In **Canada** the post-marketing safety monitoring of all health products, including natural health products, is conducted by Health Canada (Scott 2012; Murty 2007). According to Health Canada, Natural Health Products (NHPs) are naturally occurring substances including chinese medicines, herbal remedies, ayurvedic medicines, homeopathic medicines, vitamins, minerals, probiotics and other products like amino acids and essential fatty acids.

Reports collection and assessment of suspected adverse reactions to health products marketed in Canada are performed through the Canada Vigilance Program and the submission of these reports occurs through the MedEffect Canada (http://www.hc-sc.gc.ca/dhp-mps/medeff/about-sujet-eng.php Accessed February 3, 2017). This system provides consumers, patients, and health professionals an easy access to report an adverse reaction or side effect; obtain new safety information on drugs and health products; learn and better understand the importance of reporting side effects. The information collected by the program can be accessed through the Canada Vigilance Online Database (http://www.hc-sc.gc.ca/dhp-mps/medeff/databasdon/index-eng.php. Accessed February 3, 2017).

In **Europe**, the European Medicines Agency (EMA) monitors only the safety of drugs and herbal medicinal products largely through passive surveillance. It collects information using EudraVigilance, an online system used for reporting and evaluating suspected cases of adverse reactions (https://eudravigilance.ema.europa.eu/Decommissioned/Decommissioned.html Accessed February 3, 2017).

Every EU state has its own specific surveillance system for PFS or uses for reporting the pharmacovigilance system.

For example in **Italy** botanicals are marketed mainly as food supplements and/or as medicinal products if nutritional/physiological effects, or therapeutic activities are claimed respectively.

The Italian Medicines Agency (AIFA) collects reports only for registered drugs and plant based medicinal products through the Italian Pharmacovigilance System, whereas the Italian National Institute of Health (ISS) manages a specific adverse reaction reporting system for natural health products (http://www.epicentro.iss.it/focus/erbe/sorv_reaz-avv.asp Accessed February 3, 2017).

The latter system (Menniti-Ippolito et al. 2008) collects in a database the spontaneous reports of suspected adverse reactions arisen after taking/administration of:

- food supplements
- galenic herbal preparations
- other natural preparations of non-vegetable origin (e.g. propolis, snail extracts, etc.)
- homeopathic remedies

Reports can be made by anyone who observes an adverse reaction (health care professionals, researchers, and consumers) by completing and faxing ISS a form

specially developed. Adverse events are then evaluated by a panel of experts consisting in two committees: a scientific and a coordinating committee.

Finally another source of data is represented, in many countries, by Poison Centres. A Poison Centre answers enquiries mainly about exposure to chemical agents, including pharmaceuticals, natural toxins, pesticides and industrial chemicals and also to particular products like those based on plants. However, as the purpose of these Centres is heavily focused on patient's treatment after the adverse event, and not on the collection of information regarding products or on establishing causal relationships, the resulting reports are very sensitive but not necessarily specific.

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Chapter 7
Protocols for Developing and Testing Methods Applied to the Quality Control of Botanicals

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Abstract Both the application of official/published methods and the development of a novel analytical protocol need specific guidelines for their validation. Methods developed for quality controls of botanicals could be classified as: (1) phytochemical assays, and DNA-based identification; (2) methods for the identification and quantification of specific compounds. The validation of the methods for phytochemical

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and DNA-based identification requires the following parameters: ruggedness, specificity and limit of detection (LOD). In relation to development and testing of methods for detection of specific compounds: beneficial and toxic substances, contaminants, residues and biomarkers, this chapter presents guidelines based on good analytical practices, to assist analyst in obtaining data of requested quality and to aid in the evaluation of the quality of obtained data. This chapter will be organised in the following three parts: (1) evaluation of the problem and characterization of analytical requirements; (2) development of methods; and (3) validation of methods.

The parameters that have to be evaluated for in-house validation will be different depending on the type of method (qualitative or quantitative, compounds in high concentration or traces).

Keywords Quality control • Botanical fingerprints • Contaminants • Adulterations

7.1 Introduction

Reliable analytical methods are required for compliance with national and international regulations in all areas of analysis. It is accordingly recognized that a laboratory must take appropriate measures to ensure that it is capable of providing data of the required quality. One of the analytical performance criteria is the use a "fully validated" method of analysis, which is now accepted or required in many sectors of analysis. Fully validated means that a method must have been assessed in a collaborative trial. There are, however, many situations where this is not feasible, and as result the need for laboratories to develop and use their own "in-house" methods is well recognized in analytical community. It has become recognized that such validation should be carried out on a more formal basis and a number of organizations have developed procedures and protocols, which meet such needs.

The authors of this chapter decided to develop the criteria of in-house validation; this approach could be considered adequate for the purpose since it is difficult of obtaining sufficient participants with the suitable expertise on botanicals to enable collaborative trials in every situation. Frequently, researchers in the field of botanicals have their expertise in specific analytical approaches and/or fields, so it is difficult to recruit an adequate number of laboratories for each analyte.

In view of different problem this chapter is divided in two distinct sections:

- development and testing of methods for plant/herb identification;
- development and testing of methods for detection of specific compounds: beneficial and toxic substances, contaminants, residues and biomarkers.

7.2 Development and Testing of Methods for Plant/Herb Identification

Methods for plant/identification can be divided into the following groups:

- Morphological
- Phytochemical
- DNA-based methods

7.2.1 Morphological Identification of Plant

Morphological characters can be evaluated at different levels on plant material:

- The macroscopic examination deals with characters visible with the naked eye. It is suitable for the description of whole plants and often also for whole plant parts;
- The micromorphology of a plant comprises characters that can be studied using a magnification glass or a stereomicroscope, affording magnifications up to ×40–50. Applicable to smaller plant parts, plant fragments, small flowers and plant trichomes;
- Morphology is studied using light microscopy. These techniques require usually a specific sample preparation as embedding, cutting, staining, etc. Typical magnifications are ×100–×400 (and may go up to ×1000). Additional techniques as the observation in the dark field or in the polarized light may enhance the visibility of certain structures. These techniques are well suited to study plant tissues, cell forms including plant trichomes and cell inclusions (e.g. Ca-oxalate crystals);
- Ultrastructure of plants and plant cells is accessible with scanning and transmission electron microscopy and an adequate sample preparation (e.g. ultra thin cutting, staining, vapour coating with gold). These techniques are elaborate and costly but are usually not needed for a correct plant identification;
- A set of technical terms (that can be defined in a glossary) is used to describe the characters at each level.

7.2.2 Phytochemical Identification of Plant

The phytochemical identification of plants aims to identify the plant/herbal using its chemical compositional profile of observed. It is performed by analytical methods such as or hyphenated techniques like GC-MS or UPLC-MS.

The development and testing of the methods for plant identification is based on three steps:

- Evaluation of the problem and characterization of analytical requirements;
- Development of the method;
- Validation.

The first two steps are common to the development and testing of methods for detection of specific compounds and they will be described at Sects. 7.3.1 and 7.3.2. The validation of the methods for plant identification requires the definition of the following parameters:

- Ruggedness;
- Specificity;
- Limit of detection (LOD).

The definition and methodology used to check each parameter will be explained in details in the specific sections. The definition of these parameters must follow the Guidelines of International Union of Pure and Applied Chemistry (IUPAC-Thompson et al. 2002), ISO (ISO 2007), AOAC International (AOAC 2002), EURACHEM (2016) and The International Council for Harmonisation (ICH 2005).

7.2.3 DNA-Based Identification of Plant

DNA-based identification of plants is based on the sequence of nucleotides on the DNA, which is unique for each individual. Some parts of the DNA are conserved within species but differ between species. Therefore the process in developing DNA-based methods is basically relying on the identification of suitable DNA-regions for species discrimination. To develop a reliable identification system, it is necessary to collect, besides a number of different samples from the target sample, also reference samples from closely related species.

Its main technology is the Polymerase Chain Reaction (PCR); for subsequent identification many different techniques can be applied, like direct sequencing or—more suitable for routine analysis—Amplification Refractory Mutation System (ARMS), High Resolution Melting (HRM) and Loop Mediated Isothermal (LAMP).

Sampling, DNA-isolation and detection are the three major steps that need to be validated, which can be done in a modular way.

The process is quite similar to that described in Section 7.2.2 and consists of the same three steps: (1) Evaluation of the problem and characterization of analytical requirements, (2) Development of the method, (3) Validation. In validation, the determination of ruggedness, specificity and LOD are required.

7.2.4 Guidelines for the Methods in Plant Identification

7.2.4.1 Morphological Approach

The morphological evaluation and identification of plant material has to follow a procedure that allows reproducibility, documentation and traceability of the obtained results (plant identity):

- Deposit a retainer sample or herbarium voucher specimen of the plant material assessed;
- For each specimen to be identified use a report sheet, where the main morphological characters of the assumed species are registered and mark which characters are present on the investigated specimen.
- Add an individual comment concerning the investigated specimen on the report sheet;
- For the foto documentation of macro- and micromorphology use an centimeter or millimeter scale;
- For the photo documentation of microscopic characters use a micrometer scale;
- Specify the sample preparation for microscopic investigation (embedding, cutting, staining);
- Specify the light microscopic technique (bright field, polarized light, etc.) and magnification used.

The complete document about the investigated specimen contains information, where retain samples or herbarium specimens are kept, the report with the observed characters, illustration with photos (macro- and microphotos) and a conclusion about the determined identity.

7.2.4.2 Phytochemical Approach

The development and testing of methods for plant identification cover different tasks:

- *Plant identification*: to identify a plant is necessary to distinguish the plant species from those of the same genus, as well as from other genera (especially of species often used as adulterants);
- *Method development*: it is advantageous to develop a standard method, which can be used for the identification of many plants species. The developed method has to be validated.
- *Matrix considered by the method*: the development of the methods should lead to approaches that can be used to analyse raw material as well as semi processed products and finished products (PFS). In the last two cases, it is necessary to divide the development of the methods in procedures for sample preparation and procedures for analysis.

7.2.4.3 DNA-Based Approach

As described above, also the DNA-based approach requires the development of a basic system that can be used for many plant species. In this case, the basic system consists in the concept of a "mastermix", a mixture of the components for PCR without primers and DNA to be analysed and a standard protocol for the PCR thermal profile. To the mastermix, only species specific primers and sample DNA have to be added.

Since PCR is a powerful method to detect extremely small quantities of a target, it is generally possible to use this technique also for processed materials. But—as in phytochemical identification—the methods could need some adaptations.

7.3 Development and Testing of Methods for Detection of Specific Compounds: Beneficial and Toxic Substances, Contaminants, Residues and Biomarkers

This section presents guidelines based on good analytical practices for the development and testing of methods for beneficial and toxic substances, contaminants, residues and biomarkers. The aim is to assist analysts in obtaining data of requested quality and to aid them in the relative evaluation.

7.3.1 Evaluation of the Problem and Characterization of Analytical Requirements

In setting up a method, at first it is indispensable to evaluate the analytical problem and to characterize analytical requirements following the stages listed below:

- Determine the applications of the method (plant species, compounds, matrix, etc.);
- Establish if the method is for qualitative or quantitative analysis;
- Choose the adequate analytical approach (chromatography, spectroscopy, electrochemical methods, others);
- Establish if the method will be developed ex-novo or if it will be an improvement of an existing method;
- Set up the performance criteria requested (specificity, precision, recovery, working range, etc.);
- Verify the existence of commercially available reference compound/material or the possibility to isolate the required substance from natural sources, or produce it synthetically.

7.3.2 Development of the Method

During the development of a method, it is necessary to follow some technical strategies as described below.

7.3.2.1 Elaborate a Method Protocol

The analytical protocol must include suitable procedures for:

- Sampling;
- Sample preparation, such as extraction and clean-up;
- Analytical determination:

  A. *Instrumental chromatographic methods.* Analytical determination with the selected technique, and optimization of derivatization conditions (if necessary) and setting up of suitable instrumental parameters. Among others: Gas chromatography (GC), High Performance Liquid Chromatography (HPLC), Gas Chromatography coupled with Mass Spectrometry (GC/MS), Liquid Chromatography coupled with Mass Spectrometry (LC/MS), Ultra Performance Liquid Chromatography (UPLC), etc.
  B. *Chromatographic methods without or with limited instrumental requirement.* Optimization of analytical conditions for separation and detection. Among others: Thin Layer Chromatography (TLC) and High Performance Thin Layer Chromatography (HPTLC).
  C. *Spectroscopic methods.* Optimization of analytical condition for chemical, immunochemical or enzymatic reaction (if necessary) and setting of suitable instrumental parameters. Among others: Spectroscopy and Photometry (UV/VIS), fluorimetry, Enzyme-Linked ImmunoSorbent Assay (ELISA), Atomic Absorption Spectroscopy (AAS), Nuclear Magnetic Resonance (NMR), etc.;
  D. *Electrochemical methods* (Electrophoresis, CE, etc.): optimization of analytical conditions for separation and detection and setting of suitable instrumental parameters.

- Elaboration of quali/quantitative results and discussion of data.

7.3.2.2 Preliminary Evaluation

To perform preliminary evaluation, some parameters have to be checked on the basis of the performance criteria established in the first steps: specificity, extraction and clean up efficacy, linearity, repeatability, etc.

This iterative process of development and evaluation continues until the method is deemed capable of meeting the requirements (Fig. 7.1); further development is unnecessary and it is possible to proceed with the whole validation.

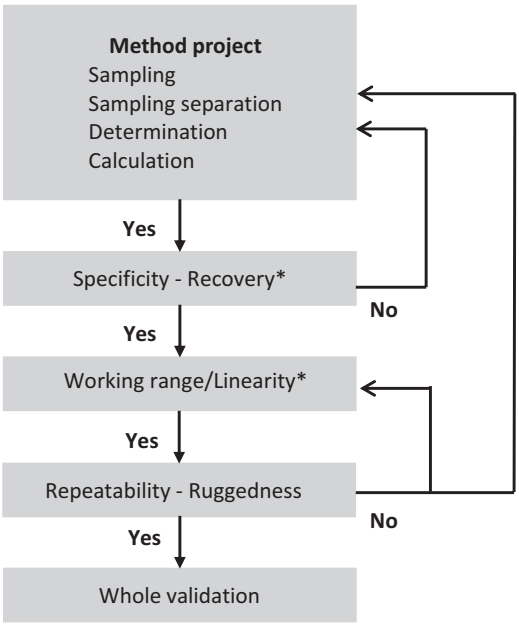

Fig. 7.1 Process of development and evaluation of an analytical method. * Refers to quantitative analysis

7.3.2.3 Validation

Validation of a method is the planned and documented procedure to establish its performance characteristics. The performance characteristics or the validation parameters of the method determine the suitability for its intended use. They define what the method can do under optimized conditions of sample preparation, analyte isolation, instrumental settings, and other experimental features.

The parameters that have to be evaluated for in-house validation will be different depending on the type of method (qualitative or quantitative), or analyte abundance (compounds in high concentration or traces). The schedule for validation is Illustrated in Table 7.1.

As indicated above, validation should be performed according to the Guidelines published by IUPAC, ISO, AOAC, ICH and EURACHEM. These associations have cooperated to produce agreed protocols or guidelines on the design, conduct and interpretation of method performance studies.

Definitions and methodology to check each parameter are listed below.

Specificity means the ability of a method to determine accurately and specifically the analyte of interest in the presence of other components in a sample matrix under the stated conditions of the test.

To check specificity there are two techniques:

– If a matrix blank is available, analyze an appropriate number of blank samples (at least five) and verify the absence of any interferences (signal, peaks, etc.);

Table 7.1 Protocol for the validation of an analytical method

Parameters	Method type		
	Qualitative	Quantitative (high concentration)[a]	Quantitative (traces)[b]
Specificity	+	+	+
Working range/linearity	–	+	+
Recovery	–	+	+
Trueness	–	+	+
Precision (repeatability, intermediate precision)	–	+	+
LOD (limit of determination)	–	–	+
LOQ (limit of quantification)	–	–	+
Ruggedness	+	+	+

[a]Bioactive compound
[b]Residue, contaminants

– If a matrix blank is unavailable, five times analyze samples fortified with the analyte at a range of concentrations (at least five) and compare the results with those obtained with pure analyte.

For instrumental chromatographic methods it is also possible to compare spectra of compounds in the matrix with that of pure reference compounds.

7.3.3 Working Range/Linearity

For any quantitative method, it is necessary to determine the working range, which is the range of analyte concentration over which the method may be applied. Within the working range there may exist a linear response range. Within the linear range signal response will have a linear relationship to analyte concentration or property value.

To check working and linear range:

1. Analyse one time reference materials or fortified sample at various concentrations for at least six concentrations. Plot measurement response (y-axis) against measured concentration (x-axis) and visually examine to identify approximate linear range and upper and lower boundaries of the working range.
2. Analyse reference materials or fortified sample at least six different concentrations within the linear range and repeat three times each. Plot measurement response (y-axis) against measured concentration (x-axis) and visually examine for outliers, which may not be reflected in the regression. Calculate appropriate regression coefficient. Calculate and plot residual values (difference between actual y value and the y value predicted by the regression line, for each x value). A close distribution about the regression line confirms linearity. Random/scattered distribution trends indicate non-linearity.

7.3.4 Recovery

Recovery means the percentage of the true concentration of a substance recovered during the analytical procedure.

To check the recovery analyze matrix blanks or unfortified samples (if matrix blank is unavailable) and samples fortified with the analyte of interest at a range of concentrations (at least at minimum, in the middle and at maximum of the working range), repeat the analysis six times.

7.3.5 Trueness

Trueness means the closeness of agreement between the average value obtained from a large set of test results and an accepted reference value.

Two basic techniques are available: checking against reference values for a characterized material or against another validated reference method.

To check trueness, the mean and standard deviation of a series, 10 replicate tests must be determined, and compared with accepted true value for the characterized material or with the results of the reference method.

Characterized materials for validation may be:

- *Reference material*: Material or substance one or more of whose property values are sufficiently homogeneous and well established to be used for the calibration of an apparatus, the assessment of a measurement method, or for assigning values to materials.
- *Certified Reference Material (CRM)*: Reference material, accompanied by a certificate, one or more of whose property values are certified by a procedure, which establishes its traceability to an accurate realisation of the unit in which the property values are expressed, and for which each certified value is accompanied by an uncertainty at a stated level of confidence.

In the absence of characterized materials or reference methods, trueness can be investigated by spiking and recovery (see recovery).

7.3.6 Precision

Precision means the closeness of agreement between independent test results obtained under stipulated conditions. Precision includes measures of reproducibility and repeatability. For in-house validation only repeatability is required.

Repeatability is expressed as standard deviation of test results obtained *with the same method on identical test items, in the same laboratory by the same operator using the same equipment.*

If possible intermediate precision that expresses within laboratories variation (different days, different analysts, different equipment, etc.) should be also determined.

To check precision it is necessary to repeat the tests at least six times.

7.3.7 Limit of Detection (LOD)

The limit of detection is the lowest concentration of analyte in a sample that can be detected, but not necessarily quantitated under the stated conditions of the test.

To determine LOD, analyse 10 independent sample blanks or sample blanks fortified at lowest acceptable concentration measured once each. Express LOD, as the analyte concentration corresponding to mean sample value ±3 s.

For instrumental chromatographic method it is also possible to refer to signal-to-noise-ratio (S/N) that should be ≥3.

7.3.8 Limit of Quantitation (LOQ)

The limit of quantitation' (LOQ) is the lowest concentration of analyte that can be determined with an acceptable level of repeatability precision and trueness.

To determine LOQ, analyse 10 independent sample blanks or sample blanks fortified at lowest acceptable concentration measured once each. Express LOQ, as the analyte concentration corresponding to mean sample value ±10 s.

For instrumental chromatographic method it is also possible to refer to signal-to-noise-ratio (S/N) that should be ≥10.

7.3.9 Ruggedness

The ruggedness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage.

To evaluate the ruggedness identify variables, which could have a significant effect on method performance. Set up experiments to monitor the effect on accuracy and precision of systematically changing the variables. Analyse each set of experimental conditions once. Determine the effect of each change of condition on the mean. Rank the variables in order of the greatest effect on method performance.

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Chapter 8
Classic/Recommended Methods and Development of new Methods to Monitor Phytochemical Composition of Plant Food Supplements and their Content in Active Molecules

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Abstract The object of this chapter is a short review of appropriate methods for the identification and authentication of plant materials and the quantification of bioactive compounds having physiological (or toxicological) relevance. The quality control of a botanical can be applied to raw material, a derivative (extract) or an ingredient of a commercial product (Plant Food Supplement or Traditional Medicine).

Using the most suitable assays, each batch of botanical should be identified using taxonomic classification, morphological examination and/or biochemical/chemical characterization. An example of the quality control, which can be performed on botanicals, is here presented taking into consideration *Camellia sinensis* (tea).

Keywords Botanicals • Quality Control • Phytochemical Profile • DNA Molecular Characterization • *Camellia sinensis*

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8.1 Introduction

Herbal materials, extracts and botanical preparations used in plant food supplements (PFS) must comply with EU food legislation regarding composition and safety. Since the consumers' expectations are primarily focused on beneficial effects, methods are necessary to test and quantify the biological activity of the complex mixture of phytochemicals present in the commercial products. The quality of raw material and botanical preparations is particularly relevant, when a company applies to EFSA for a functional claim to be added in the label or used in advertising.

Due to the fact that no compulsory guideline exists for PFS, groups of researchers or PFS producers have developed a number of analytical methods for quality control. They must be validated and tested on different raw materials, extracts and also on PFS from the market, since matrix can change dramatically the efficiency of an analytical technique. Moreover, considering the multi-component nature of a PFS, a proper and meaningful quality control must include the analysis of a broad spectrum of potential physiologically active phytochemicals, sometimes belonging to several chemical classes (Sanzini et al. 2011; Teken et al. 2004).

There are several standard and reference methods for the analysis of botanical derivatives; the most common ones are based on ISO Standards (Ameh et al. 2012), the current European Pharmacopoeia (2017) and the WHO Quality Control Methods (2011). Due to the request of analytical method to qualify and quantify botanical ingredients, the aim of this chapter is a short review of appropriate methods for:

- The identification and authentication of the plant material (phytochemical markers, molecular genetic methods);
- The identification and quantification of bioactive plant compounds of physiological (or toxicological) relevance.

The production of high quality food supplements containing botanicals begins with the identification of the correct species/varieties. Plants intended for use in food or supplements should be cultivated and harvested using suitable guidelines. The WHO Programme on Traditional Medicines (WHO 2014) reports guidelines for good agriculture and collection practices in the field of botanicals (WHO 2003). If wild plants are harvested or if the botanical (or its extracts) are purchased without any assurance of good agricultural practice, materials should be assayed with special attention.

For the complete identification of the botanical used in PFS, both as raw material and as derivatives (from extracts to final product), all data at disposal must be collected: (1) scientific name (plant family, genus, species, etc.); (2) common name in English and other languages, when necessary; (3) part(s) of the plant used; and (4) geographic origin, even though this information is not always at disposal (Schilter et al. 2003).

There are several methods validated for the authentication, standardization, and quality assurance; they are based on taxonomic identification, morphological and microscopic examination, fingerprint chromatography, DNA molecular characterization, and immunoassay of species-specific proteins.

An interesting description of the botanical characterization of a large number of plants is freely available at the website of the American Herbal Product Association (http://www.botanicalauthentication.org/index.php/Main_Page).

Macroscopic identification is usually performed on the intact whole plant during collection or harvesting. Unfortunately, this is not always possible and macroscopic examination in some cases is done on dry raw material. However, macroscopic examination cannot be enough to identify the species or distinguish sub-species differences in chemotype or ecotype (Sanzini et al. 2011; van Breemen et al. 2007; Yadav and Dixit 2008).

Microscopic examination allows the evaluation of whole, fragmented, or powdered plant material, but this approach requires professional microscopists trained in the analysis of botanical and reference standards, which are no always available (AHP 2011; Bisset and Wichtl 2000).

'Fingerprinting' is a useful tool based on chromatographic and/or spectroscopic profiling, obtained by high performance liquid chromatography (HPLC), Thin Layer Chromatography (TLC), High Performance Thin Layer Chromatography (HPTLC), gas chromatography (GC), or Fourier transform infrared (FTIR), near infrared (NIR), or nuclear magnetic resonance (NMR) spectrometry.

'Chemical fingerprinting' describes the compounds, which are characteristic or must be absent in a specific botanical; their analysis allows a whole characterization of the raw material or the final PFS, including the possible adulterations.

Due to accidental contamination or adulteration, a PFS might contain a mixture of the expected plant/s with other undesired/illicit ingredients. In these cases, the use of analytical methods aimed to detect specific compounds can be insufficient.

A DNA based identification can be obtained by using fresh or dried source material or higher processed preparations and has become a powerful tool for supplementing the identification process (Heubl 2010).

Several molecular techniques were implemented for the authentication of botanical products. Their explanation and general applicability including advantages and disadvantages are discussed in Chap. 9.

Camellia sinensis will be used in this chapter to describe an example of a possible quality control on a botanical preparation. Other examples regarding the quality control of botanical by DNA-based identification will be illustrated in Chap. 9, while further phytochemical characterizations will be described by the authors of Chap. 10.

8.2 Characterization of Botanical raw Material and Relative Derivatives: *Camellia sinensis*

Camellia sinensis, known as tea, is a common ingredient of food and food supplements; it is widely used to prepare the infusion, which is the second most consumed drink in the world after water. Teas are commercialized, as such or as derivatives, according to the processing applied to the leaves. The most important classification of tea is related to the manufacturing process:

1. **Black tea** is the product of enzymatic oxidation of *Camellia sinensis* fresh leaves. After the harvest leaves are spread in layers and maintained for up to 18 h at room temperature or under circulating warm air to reduce the moisture content to approximately 60% of the starting weight. Leaves are then crushed and macerated to promote enzymatic oxidation of the flavonols due to the presence of oxygen. During "fermentation", tea undergoes important compositional changes resulting in colour and flavour modification. At the end of this step, leaves are passed on trays through hot air driers to stop enzymatic activity and to decrease moisture to 3%.
2. In preparing **green tea**, the oxidizing enzymes are inactivated by steam-blasting the fresh leaves in perforated drums, followed by roasting in hot iron containers and rolling.
3. **Oolong tea** is obtained by rolling the leaves after a short "fermentation" process; during this period, chemical changes reach approximately one-half of those found in black tea.
4. **White tea** is considered the most delicate and freshest product available; leaves from the newest growth on the tea plant are handpicked and then rapidly and carefully dried, so the leaves are not allowed to oxidize.

Figure 8.1 shows the macroscopic aspects of the four main classes of commercial tea: white, green, oolong and black.

Tea contains several bioactive compounds and it is particularly rich in flavonoids, including catechins and their derivatives. The most abundant polyphenol is epigallocatechin 3-O-gallate (EGCG), which is the main responsible for the beneficial effects of tea (Chacko et al. 2010). Tea also contains caffeine, which is well-known for its effect on central nervous system (Nehlig et al. 1992) and theobromine and theophylline, which show diuretic and vasodilatorory activities (Martinez-Pinilla et al. 2015).

A description of the quality characterization of a PFS herbal ingredient, from the botanical aspects to the chemical composition, is here reported for *Camellia sinesis*, which is widely used in the international market.

8.2.1 Nomenclature

The botanical classification of *Camellia sinensis* (L.) Kuntze is illustrated in Table 8.1.

8.2.2 Morphology of Camellia sinensis

Figure 8.2 illustrates a plant of *Camellia sinensis*, cultivated in Japan and a page of a traditional herbarium, where the different parts of the plant are detailed. Tea is a small tree with a long life (25–90 years), mainly cultivated for its leaves. Leaves are simple and alternate, mostly oval and ovoid. Mature leaves are bright green, smooth and leathery. The young leaves are light green with white hairs in the underside

Fig. 8.1 Macroscopic differences between different tea samples: (**a**) white tea from Nepal; (**b**) green tea Gunpowder from China; (**c**) oolong Tea from China; (**d**) black tea from Sri Lanka

Table 8.1 Botanical classification of *Camellia sinensis* (tea)

Rank	Name
Kingdom	Plantae
Subkingdom	Tracheobionta
Superdivision	Spermatophyta
Division	Magnoliophyta
Class	Magnoliopsida
Subclass	Dilleniide
Order	Theales
Family	Theaceae
Genus	*Camellia* L.
Species	*Camellia sinensis* (L.) Kuntze

From: USDA (2017)

(used to prepare white tea). The classification of tea plants is mainly based on the leaf size (Pepó and Csajbók 2013; Piovan et al. 2014).

Fig. 8.2 Picture of the plant (*left*) and herbarium page (*right*) of *Camellia sinensis L.*

8.2.3 Organoleptic Characteristics

The organoleptic properties of tea are described by the American Herbal Products Association (AHPA 2013) as:

- *Aroma and odor*: characteristic;
- *Flavor/taste*: drying and astringent (due to tannins).

8.2.4 Microscopic Examination

Data on microscopic characteristics of *Camellia sinensis* are rare and often limited due to the lack of reference standard data. Papers published on this topic are very old, even though still cited in botanical studies (Clayton and Hassali 1909; Taylor 1889).

AHPA (2013) reports some sentences from the paper by Clayton and Hassali (1909):

1. *"Upper epidermal cells small and only slightly angular, in leaf of medium size; but larger, more angular, and with walls more distinctly visible, in the old and hard leaf. Hairs and stomata absent. Parenchymal cells similar to those of most other leaves, and not very distinctive.*
2. *Cells of the lower epidermis larger than those of the upper surface, and associated with stomata and hairs. Stomata, oval or sometimes nearly round, formed of two reniform cells (guard cells) encircling a very apparent aperture rather*

numerous, and confined to the under surface of the leaves. The epidermal cells are themselves curved in the neighborhood of the stomata. Hairs short, pointed, and undivided, confined to the under surface of the leaf: very numerous on young leaves, less abundant on old leaves. Wood fibre not characteristic."

8.2.5 DNA-Based Identification

Camellia sinensis L. Kuntze is one of the most important crops used for infusions and therefore an important commercial product. As a consequence, numerous efforts for unravelling the genetic background of this plant and for assessing the genetic diversity between cultivars, accessions, populations or provenances have been made. At least 72 publications concerning DNA based techniques in *Camellia sinensis* can be found in SCOPUS starting from 1994 (Matsumoto et al. 1994) onwards. Although *Camellia* is with 100–300 species a rather large genus and the classification of the varieties or subspecies within the species *Camellia sinensis* is still unresolved, only a few are addressing the identification of the species (Huang et al. 2014; Vijayan et al. 2009; Sharma et al. 2015).

In contrast to the low number of papers about species identification, a majority of DNA fingerprinting techniques like RAPD (Kaundun et al. 2000; Young-Goo et al. 2002) ISSR (Mondal 2002), AFLP (Wachira et al. 2001), CAPS (Kaundun and Matsumoto 2003b) and RFLP (Kaundun and Matsumoto 2003a) focused on the genetic diversity between varieties of *Camellia sinensis*.

These techniques provide useful information for plant breeding-programs, but the general applicability of these methods for identification of PFS in a standardized way is doubtful, although the authors were convinced of their suitability for routine analysis. Methods, which can be more easily transferred to other molecular labs like SNP markers (Fang et al. 2016; Zhang et al. 2014), or the use of highly variable DNA sequences (Katoh et al. 2003; Lee et al. 2016) might be more accurate, easier to use and appropriate for a differentiation of varieties of *Camellia sinensis*. For a secure identification on the species level, sequence analyses (Huang et al. 2014; Vijayan et al. 2009; Sharma et al. 2015; Lee et al. 2016) are more successful. DNA barcoding used by Stoeckle et al. (2011) proved to be secure but only on the level of the genus. Nevertheless, this approach can be used to detect possible adulterations with other plant genera. Dhiman and Singh (2003) found cashew husk (*Anacardium occidentale* L.) in *Camellia sinensis* samples. They developed species-specific PCR (polymerase chain reactions) primers for this particular adulterant, so that the identification process could be significantly improved.

This summary of DNA based methods used in *Camellia* demonstrates that there is no perfect stand-alone method. For an accurate approach, a combination of DNA methods—or even better—a combination of DNA methods with chemical fingerprinting would be the best solution.

8.3 The Identification and Quantification of Bioactive Plant Compounds of Physiological (or Toxicological) Relevance

Another approach useful for the identification of a specific botanical and/or its adultaration is the quantification of the molecules responsible for the physiological effects. In case of analytical problems, the identification of the botanical can be done by detecting and quantifying a molecule, which is devoid of any biological properties but is chosen as a specific marker.

Tea contains several bioactive compounds and it is particularly rich in catechins. Epigallocatechin gallate (EGCG) is the most abundant catechin, and its highest concentration is found in white and green tea. Tea catechins and polyphenols are effective scavengers of reactive oxygen species, and protect the consumers from the toxicological potential of Reactive Oxygen Species (ROS) (Higdon and Frei 2003). Tea contain caffeine, also known as theine, which is responsible for the stimulating activity and theanine, a non-protein amino acid involved in the specific tea taste (Vuong et al. 2011). Low amounts of other compounds are also present: teobromine, theophylline, amino acids, vitamins, minerals (among others fluoride).

The analytical approaches usually applied for the detection and quantification of catechins (the most representative tea molecules) are based on chromatographic techniques.

8.3.1 High Performance Thin Layer Chromatography Characterization

Thin layer chromatography or TLC is a fast chromatographic technique useful for qualitative or semi-quantitative analysis of herbal material and plant food supplements. Like all chromatographic techniques, it is based on the different distribution of the analytes in function of their affinity for the stationary and the mobile phase. The High Performance TLC (HPTLC) represents an improvement of the classical method; the stationary phase is generally prepared by using silica with a particle size of 5–6 μm, compared to 10–12 μm of those used in the original version. This leads to an increase of selectivity and efficiency in the chromatographic separation. The mobile phase is a solvent or a mixture of solvents specifically selected, having no affinity for the stationary phase and polarity suitable for the separation of analytes present in the sample. In the HPTLC technique, the reproducibility of the analysis is guaranteed by the automated deposition of samples and standards, which allows the loading of controlled and homogeneous quantities of the solutions. After the elution the plate is dried and exposed to lights at different wavelengths. The selection of wavelength and/or the use at derivatisation depends on the class of molecules considered.

HPTLC is a useful tool for the chemical characterization of *Camellia sinensis* derivatives and for a preliminary evaluation of the antioxidant activity of their active compounds.

8.3.1.1 Chemical Fingerprint of *Camellia sinensis* by HPTLC

Standard and sample preparation. Standard compounds used are caffeine, epicatechin (EC), epigallocatechin gallate (EGCG), epicatechin gallate (ECG), epigallocatechin (EGC), and catechin (C). They are solubilized and then diluted with methanol to reach final concentrations of 250 and 200 μg/mL.

Samples used in this assay are: one green teas, leaves (GT), one decaffeinated green tea, leaves (DGT), one green tea extract (GTE), two food supplements (PFS1 and PFS2), one black tea, leaves (BT), one decaffeinated black tea, leaves (DBT), and one black tea extract (BTE). Samples (0.1 g) were carefully ground and mixed, then added with 10 mL of a solution containing ethanol:water 80:20 (v/v). The resulting solution is sonicated for 10 min, filtered with a 0.45 μm filter and applied onto the plate.

Analytical protocol. The HPTLC equipment is from Camag (Muttenz, Switzerland) and consisted of an automatic applicator Linomat 5 and of a TLC Visualizer. The Vision Cat software Camag is used for data acquisition and processing. Plates of silica gel with fluorimetric indicator 60-F254 are from Merck (Darmstadt, Germany).

The analytical procedure is based on the semi-automatic application on the plate of 8 μL of hydro-alcoholic solution of sample or 4 μL of the standard methanolic solution at 250 μg/mL. The mobile phase contains toluene:acetone:0.1% formic acid 45:45:10 (v/v/v). The elution chamber is saturated with mobile phase for 20 min before each run.

At the end of the chromatographic run, the plate is dried, exposed at 254 nm. After heating at 110 °C and derivatization with Fast Blue Salt B solution (in water:methanol:dichloromethane 5:70:25, v/v/v), the plate is exposed at 366 nm and at visible light.

Chromatographic separation. Figure 8.3 shows the chromatographic separation of samples and standard solutions on the plate, which is exposed as such at 254 nm (a) and, after spraying with Fast-Blue Salt B reagent, at visible light (b). Purified standards are all well separated and the calculated *Ratio frontis* (*Rf*) are listed in Table 8.2.

Caffeine is well visible in all tea samples apart from decaffeinate green and black teas. In green tea samples, both with (GT) and without caffeine (DGT), the most abundant compound is EGCG, followed by ECG. Since Fast Blue Salt B reagent is specific for the detection of phenolic compounds, caffeine is visible only at 254 nm.

Tea derivatives, extracts and PFS, are well separated in the same chromatographic run; as expected, caffeine and catechin are more abundant in green tea extract. The different abundance of tea extract in PFS1 and PFS2 is easily remarkable and is in agreement with the different quantity of total catechins declared in the label: 75 mg/g for PFS1 vs. 300 mg/g of PFS2. The quantitative analysis was performed by HPLC, as reported in Sect. 8.3.1.3.

8.3.1.2 Semi-Quantitative Determination of the Antioxidant Activity of *Camellia sinensis* Derivatives

The HPTLC technique can be applied to the measure, in semi-quantitative way, of the antioxidant activity of tea active molecules.

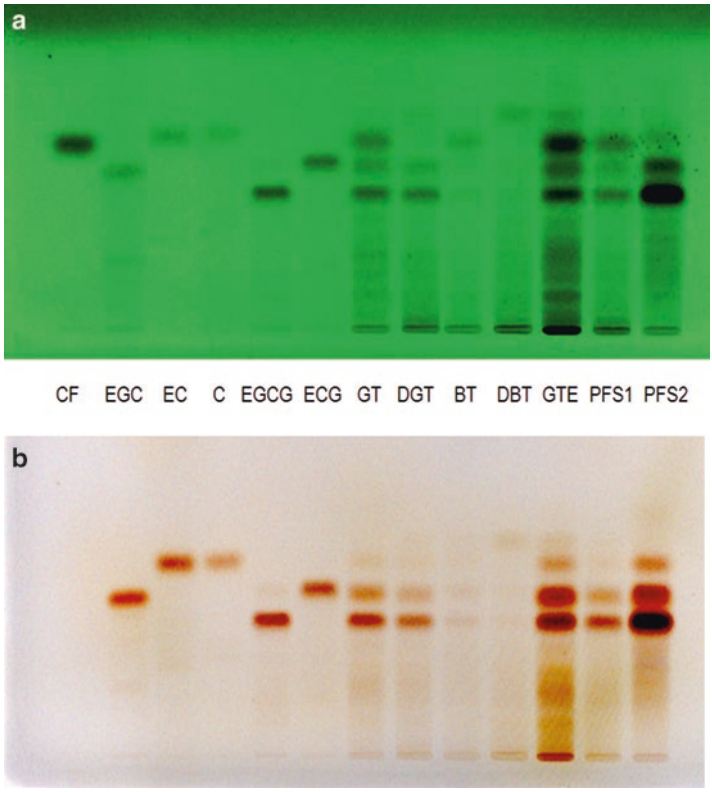

Fig. 8.3 HPTLC of of different tea samples. After chromatographic run, the plate is exposed as such to 254 nm (**a**) and, after spraying with Fast Blue Salt B reagent, at visible light (**b**). *CF* Caffeine, *EGC* Epigallocatechin, *EC* Epicatechin, *C* Catechin, *EGCG* Epigallocatechin-3-gallate, *ECG* Epicatechin-gallate, *GT* Green tea, *DGT* Decaffeinated green tea, *BT* Black tea, *DBT* Decaffeinated black tea, *GTE* Green tea extract, *PFS1* Food supplement 1, *PFS2* Food supplement 2

Table 8.2 *Rf* of standard catechins and caffeine for their identification by HPTLC in *Camellia sinensis* samples

Compound	Symbol	*Rf*
Caffeine	CF	0.44
Epigallocatechin	ECG	0.37
Epicatechin	EC	0.46
Catechin	C	0.47
Epigallocatechin-gallate	EGCG	0.32
Epicatechin-gallate	ECG	0.40

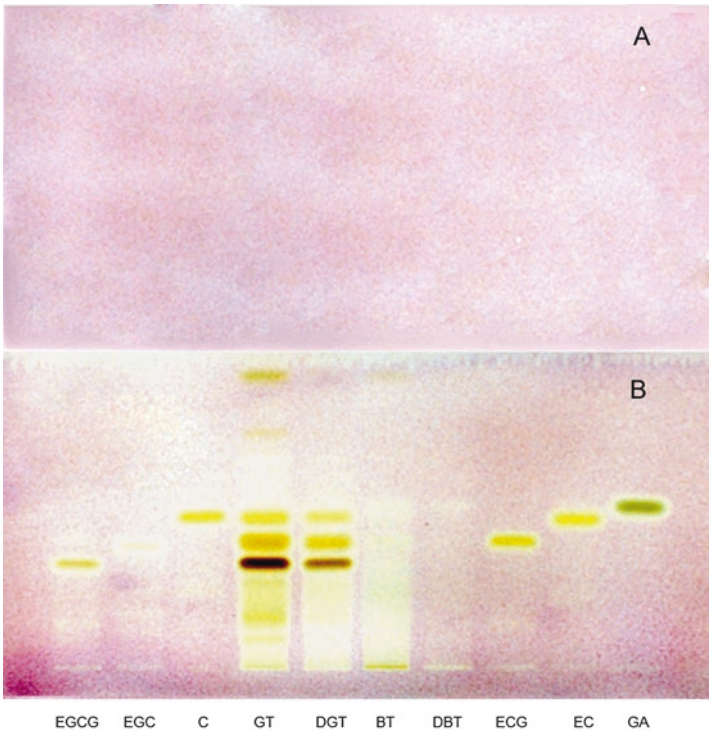

Fig. 8.4 HPTLC of tea samples for the semi-quantitative determination of antioxidant property. Plate is exposed at visible light and shown before (**a**) and 30 min after spraying with DPPH (**b**). *EGCG* Epigallocatechin-3-gallate, *EGC* Epigallocatechin, *C* Catechin, *GT* Green tea, *DGT* Decaffeinated green tea, *BT* Black tea, *DBT* Decaffeinated black tea, *ECG* Epicatechin gallate, *EC* Epicatechin, *GA* Gallic acid

Standard and sample preparation. Standard solutions are prepared as described above. Samples (0.5 g of tea leaves or 0.2 g of tea extracts/PFS) are added with 5 mL of methanol. Then, the solution is sonicated for 10 min and filtered with a 0.45 μm filter. Finally, standards and samples are applied onto the plate (4 μL).

Analytica.l protocol. The mobile phase has the same composition reported previously (toluene:acetone:0.1% formic acid 45:45:10, v/v/v). The elution chamber is saturated with mobile phase for 20 min before each run. At the end of the chromatographic run, the plate is dried for 15 min and sprayed with a DPPH methanolic solution (0.05%, v/v). Then, the plate is wrapped with an aluminum foil and placed in a dark room for 30 min. The plate is then exposed at visible light.

Semi-quantitative measure of antioxidant activity. Figure 8.4 illustrates the result obtained by spraying HPTLC plate with DPPH solution; plate was exposed at visible light at time 0 (A) and after 30 min from spraying (B). The decoloration from violet to yellow/brown is due the reaction of catechins (as standards or in tea samples) with DPPH radical and it is proportional to the antioxidant potency. Significant differences are remarkable among samples. EGCG and ECG show the

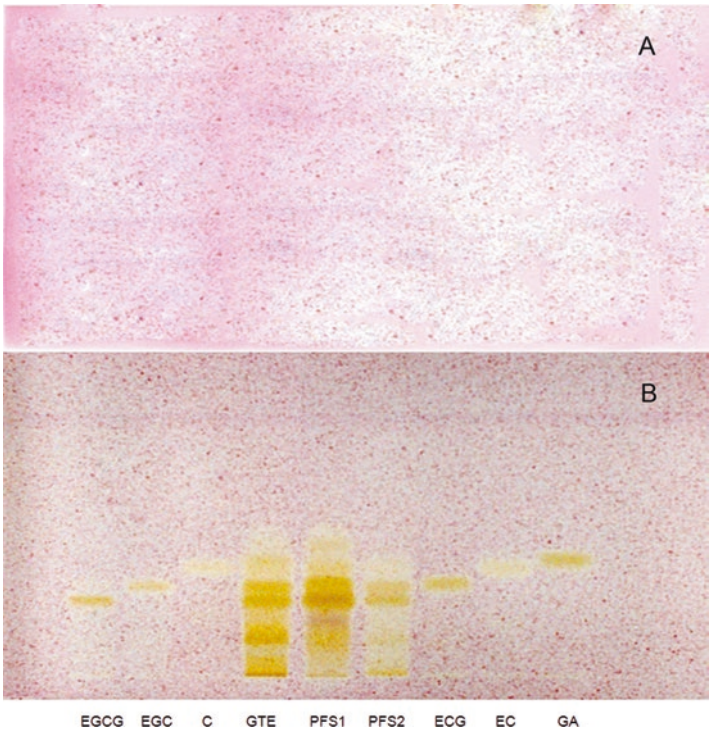

Fig. 8.5 HPTLC of tea derivatives (extract and PFS) for the semi-quantitative determination of antioxidant property. Plate is exposed at visible light and shown before (**a**) and 30 min after spraying with DPPH (**b**). *EGCG* Epigallocatechin-3-gallate, *EGC* Epigallocatechin, *C* Catechin, *GTE* Green tea extract, *FS1* Food supplement 1, *FS2* Food supplement 2, *ECG* Epicatechin-gallate, *EC* Epicatechin, *GA* Gallic acid

highest antioxidant activity, followed by EC. As expected, green tea (GT) shows the highest antioxidant activity, while black tea is almost free of chemical reactivity.

Figure 8.5 illustrates the antioxidant activity measured in tea extracts and in two food supplements. As reported previously for the catechin abundance, HPTLC allows a quick evaluation of the antioxidant properties of tea derivatives. Moreover, it is possible to identify the molecules responsible for that activity: EGCG, EGC and ECG. The lower antioxidant activity of PFS2 is confirmed by the lower amount of catechins both identified in HPTLC profile and reported in the label (see Sect. 8.3.1.1).

8.3.2 Quantification of Bioactive Compounds by High Performance Liquid Chromatography (HPLC)

The High Performance Liquid Chromatography (HPLC) is a liquid chromatography, in which separation is due to the partition between phases; the compounds are separated according to their different affinity for the stationary phase present in the chromatographic column and the mobile phase flowing through it.

During elution, the analytes establish a balance between the two phases on the basis of the polarity of the sample. The molecules having higher affinity for the stationary phase will move more slowly and will present a longer retention time.

The choice of the stationary and the mobile phase is carried out in function of the sample to be analyzed. Depending on the polarity of the stationary phase the HPLC is performed in normal or reverse phase:

1. In normal-phase, the stationary phase is polar and the mobile phase apolar. In this case, the typical stationary phase is silica;
2. In reverse phase, the mobile phase is more polar than stationary phases, which are hydrophobic and chemically derivatized silica.

The elution is monitored by using different equipments; the most used in the field of botanicals are: UV/Vis (including Diode Array) and fluorimetric detector, or mass spectrometer in its different options.

Bioactive molecules of *Camellis sinensis* can be identified and quantified by different HPLC methods; this chapter describes the method developed and validated during the PlantLIBRA project (Di Lorenzo et al. 2013).

Standard and sample preparation. Standards are solubilized in distilled water and then diluted with methanol to prepare the working solutions ranging from 0.09 to 100 µg/mL. Green and black tea leaves (1.5 g) are weighed and added with 200 mL hot water (80 °C). After 3 min, leaves are removed and the solution cooled at room temperature for 1 h. Then, the solution is filtered through a 0.45 µm filter and injected into the chromatographic system after suitable dilutions.

Samples of *Camellia sinensis* extract or PFS (1 g) are added with 100 mL water (HPLC grade), heated at 50 °C and stirred with magnetic bar (700 rpm) for 15 min. The solution is filtered with a 0.45 µm filter and injected into the chromatographic system after suitable dilution. Injected volume is 20 µL.

Analytical protocol. The HPLC is equipped with two pumps PU-1580 (Jasco, Tokyo, Japan) coupled with an autosampler AS-2059 plus Jasco (Tokyo, Japan) and a 975-UV detector (Jasco, Tokyo, Japan) set at 280 nm. The software ChromNAV (Jasco, Tokyo, Japan) is used for integration. The column used is a Kinetex 2.6 µ PFP, 100 A, 100 × 4.60 mm (Phenomenex, Torrance, CA, USA). The analysis is performed using a gradient elution at a flow rate of 1 mL/min; mobile phases is prepared by mixing two solutions: (A) 0.5% formic acid in water (v/v) and (B) 0.5% formic acid in acetonitrile (v/v). The gradient program is: 0–25 min, 96% A; 25–30 min, from 83 to 17% A; 30–35 min, 25% A; 35–40 min, from 25 to 96% A.

Chromatographic separation. Catechins and caffeine are the molecules involved in antioxidant activity and in main physiological properties of tea; their quantification can be useful for quality control at the place of production or as post-marketing surveillance. Catechins are often separated and quantified by HPLC-UV. Figures 8.6 and 8.7 show the chromatographic separations of: (1) standard catechin (EGC, EGCG, ECG) and caffeine; (2) green and black tea; (3) tea derivatives (extract and food supplements). Linear regression was used for quantification. Table 8.3 summarizes the catechins content (mg/g) of

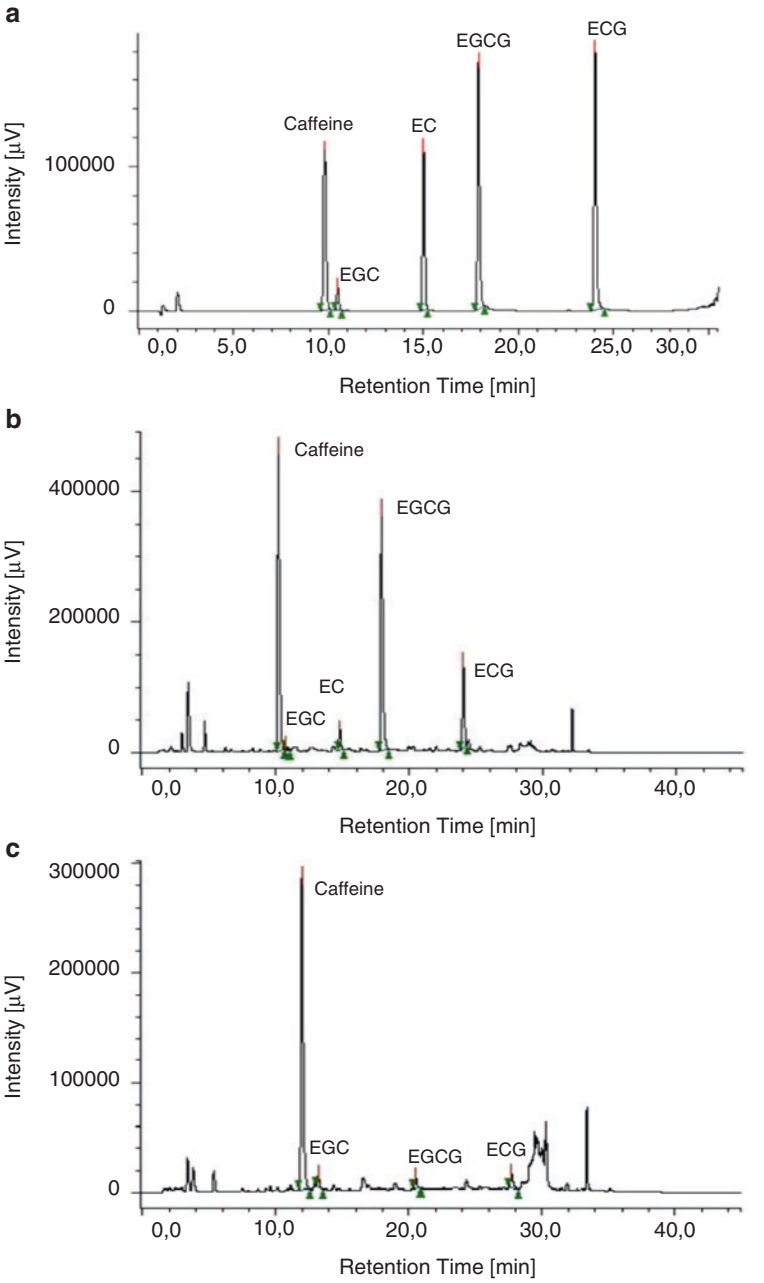

Fig. 8.6 HPLC/UV chromatograms of catechin and caffeine standards at 25 µg/mL (**a**); green tea (**b**); black tea (**c**); *EGC* Epigallocatechin, *EC* Epicatechin, *EGCG* Epigallocatechin-3-gallate, *ECG* Epicatechin-gallate

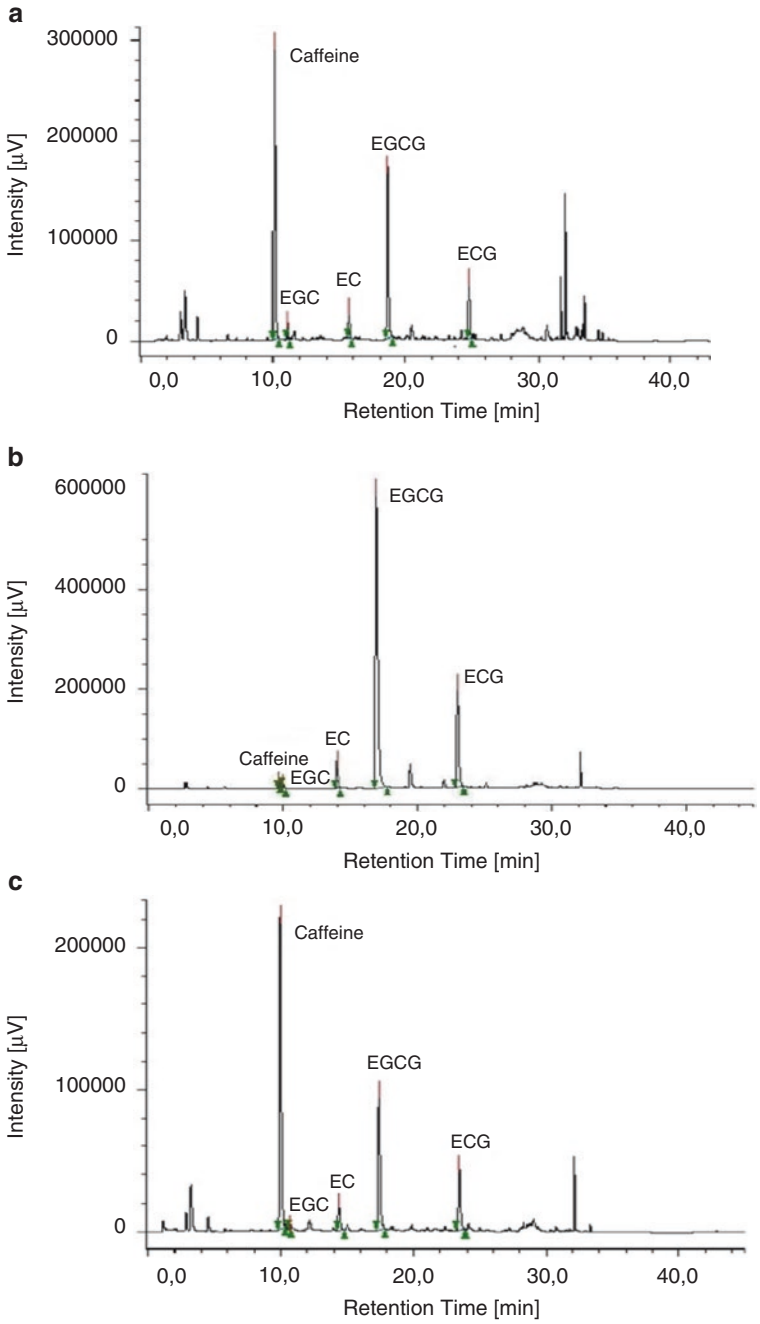

Fig. 8.7 HPLC/UV chromatograms of green tea extract (**a**); plant food supplement 1 (**b**); plant food supplement 2 (**c**). *EGC* Epigallocatechin, *EC* Epicatechin, *EGCG* Epigallocatechin-3-gallate, *ECG* Epicatechin-gallate

Table 8.3 Catechins content in tea samples (mg/g)

Sample	Total catechin content (mean ± SD)	EGCG (mean ± SD)	ECG (mean ± SD)	EC (mean ± SD)	EGC (mean ± SD)	Caffeine (mean ± SD)
GT	72.9 ± 4.4	47.1 ± 2.9	11.5 ± 0.9	9.1 ± 0.7	5.3 ± 0.2	20.2 ± 1.4
DGT	51.3 ± 2.8	34.0 ± 1.9	7.5 ± 0.7	5.0 ± 0.2	4.0 ± 0.4	ND
BT	4.8 ± 0.6	1.5 ± 0.3	1.5 ± 0.2	ND	1.8 ± 0.01	19.0 ± 0.5
DBT	9.0 ± 0.2	2.2 ± 0.3	1.8 ± 0.2	ND	5.0 ± 0.4	ND
GTE	172.5 ± 0.2	88.5 ± 5.5	27.2 ± 1.4	32.4 ± 2.0	24.4 ± 1.3	71.9 ± 5.1
PFS1	17.7 ± 0.8	12.2 ± 0.5	2.7 ± 0.1	2.9 ± 0.1	ND	10.8 ± 0.7
PFS2	288.3 ± 20.5	199.4 ± 19.1	47.6 ± 4.0	30.1 ± 2.9	11.3 ± 0.4	0.46 ± 0.03

GT green tea, *DGT* decaffeinated green tea, *BT* black tea, *DBT* decaffeinated black tea, *GTE* green tea extract, *PFS* plant food supplement, *ND* below LOD

the samples used in this chapter as examples. Each analysis was performed in triplicate and data are expressed as mean ± standard deviation (SD).

In green tea, caffeine and EGCG are the most abundant compounds in green tea, followed by ECG, EC and EGC (Fig. 8.6 and Table 8.2). In black tea, the most abundant active compound is caffeine; the catechins, apart from EC, have similar abundance and their total amount is 4.8 ± 0.6 mg/g, approximately 20 times less than their content in green tea (72.9 ± 4.4 mg/g). EGCG and caffeine are the most abundant compounds in green tea extract (GTE), followed by EC, ECG, and EGC (Fig. 8.7 and Table 8.2). The relative abundance of catechins shows some differences between samples; this is probably due to a different solubility of each catechin in the extraction medium. PFS1 shows a profile similar to that of extract, although with a higher content in EGCG and ECG. As expected, caffeine is not detectable in decaffeinated samples.

Generally speaking, both green tea and extracts/PFS show the highest catechins content (mainly EGCG and ECG), when compared with black tea derivatives. These differences could be explained by the "fermentation" process, which is responsible for the catechin oxidation (Subramanian et al. 1999). Other factors that may contribute to the changes in catechin concentration are the geographical origin, leaves quality and harvesting time. A high variability in terms of catechin concentration is observed in PFS, but results confirm concentrations indicated in the labels.

From the quantitative point of view (Table 8.3), the values of total catechins determined in PFS2 are in agreement with the quantity of total catechins and EGCG reported in the label: 300 mg/g and 209.16 mg/g, respectively. On the contrary, the total catechin concentration calculated in PFS1 is approximately 24% of the declared value (75 mg/g).

8.4 Conclusions

This chapter is an example of the quality control performed on a botanical, taking into consideration both morphological aspects and composition in active molecules. There are different analytical approaches: they can be used to confirm data obtained with other methods or to integrate information as in the case of antioxidant activity

measured directly on the HPTLC plate. The analytical approach used depend on the expected goal, and in some cases, a screening separation by TLC or HPTLC can be the best solution. In other cases, when quantitation is critical for confirming the identity or quality of a specific botanical preparation, more advanced and expensive methods are unavoidable.

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Chapter 9
How Far Advanced is the DNA-Based Identification of the BELFRIT-List?

Johannes Novak, Joana Ruzicka, and Corinna Schmiderer

Abstract The sector of botanicals is characterized by the huge number of species found in products on the market. Some governments issue positive and negative lists of plants in order to create legal certainty and to increase safety of those products. The most advanced positive list in Europe is the BELFRIT-List with 991 species in 594 genera, co-ordinate between Belgium, France and Italy. DNA-based methods for the identification of botanicals supplement nowadays the other identification methods based on morphology or phytochemistry. Molecular phylogenetic and population studies are helpful tools to develop a specific DNA-based method. In this contribution, the BELFRIT-List was reviewed for the availability of either a DNA-based identification method or molecular phylogenetic information. For 286 genera (48% of the genera), no helpful information could be found. Two hundred and thirty eight references with the intention to identify species by DNA demonstrate the already advanced field of this method. Such new methods are not developed systematically for all species of such lists, but more on a case-by-case approach for species difficult to identify. Therefore, the high number of papers already dealing with this method or bringing valuable information for their development should encourage combined efforts in standardizing validation.

Keywords Botanicals • Plant food supplements • DNA-based identification • DNA barcoding • BELFRIT list

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Botanicals are plants or plant parts used for their activity on human physiology, flavour or scent in medicines, plant food supplements, cosmetics, etc. Wolrdwide, mankind uses 72,000 botanicals alone for their medicinal activity (Schippmann 2006). This demonstrates the necessity of a correct species identification before processing and use. In order to harmonize the use of botanicals and avoid misuse of dangerous botanicals, some countries have established (or are in the process of establishing) positive lists of botanicals, safe to use or negative lists of botanicals that are potentially toxic. Europe, Belgium, France and Italy were the first to harmonize their national positive lists, finally agreeing on 991 species in 2013, called the BELFRIT-list (Cousyn et al. 2013) (Table 9.2). Their approach can be seen as first step towards a harmonization of botanicals for the whole EU.

With such a high number of botanicals in use, the first step to guarantee quality, efficacy and safety is a correct identification of the botanical and analysis for substitutions or admixtures. Species identification can be done on several levels with different methodological approaches: (1) macromorphology, (2) micromorphology, (3) composition of plant secondary compounds and (4) identification based on DNA sequence variation between taxa.

Methodologically, there are several ways to use taxa specific polymorphic DNA sequences to identify an unknown sample, like e.g. the DNA sequence obtained by sequencing, DNA fingerprinting or other molecular markers. To use DNA sequencing efficiently for many different species, a procedure was proposed called **'DNA barcoding'** (Hebert et al. 2003). For this method, always the same short DNA sequences variable between species are used and amplified by PCR using primers from conservative adjacent regions so that the same primers work in a wide range of species ('universal primers'). Sequences of reference materials (voucher specimens) are used in order to create a database with which the DNA sequence of an unknown sample can be compared and the taxon determined. In the meantime, a whole range of software and databases is available to identify plant species (Bhargava and Sharma 2013). The term 'DNA barcoding' is nowadays often extended to other DNA based methodologies for species identification (e.g. molecular markers).

A molecular marker is in its basic sense a particular segment of the DNA that represents differences on the genomic level (Agarwal et al. 2008). If these segments should be useful to distinguish species, the marker needs to be different between species but uniform between individuals of the same species. Insofar, DNA sequencing ('DNA barcoding') described above is also a molecular marker technique. Some popular marker techniques are briefly described in the glossary and some basic characteristics listed in Table 9.1.

In botanicals used in medicine DNA-based identification gained already some importance due to some species difficult to distinguish with conventional identification approaches (Techen et al. 2014; Heubl 2010; Coutinho Moraes et al. 2015; Parveen et al. 2016; Sarwat and Yamdagni 2016; Sun et al. 2016a). However, it should be kept in mind that DNA-based identification is not a simple straightforward approach since all raw materials—although denominated with a single species

Table 9.1 Characteristics of the most popular marker techniques ((Yip et al. 2007), modified; – … low, + … high, ++ … very high)

	RFLP	RAPD	AFLP	SSR	ISSR	SCAR	ARMS	HRM	DNA barcoding	Mini-Barcodes	DNA Meta-barcoding
Development costs	–	–	–	+	–	++	+	++	+	+	–
Running costs	+	–	+	–	–	–	–	–	+	+	++
Reproducibility	++	–	+	+	+	++	++	++	++	++	++
Prior sequence knowledge required?	No	No	No	Yes	No	Yes	Yes	Yes	Yes	Yes	Yes
Detection *in* mixtures	–	–	–	–	–	++	++	++	+	+	++
Detection *of* mixtures	–	–	–	–	–	–	–	++	+	+	++
Whole species composition of mixtures	–	–	–	–	–	–	–	–	–	–	++
Detection of fragmented DNA (processed intermediates / final products)	–	–	–	–	–	+	+	++	+	++	+
Technical requirement	–	–	–	–	–	–	–	+	+	+	++

name—are always mixtures. Therefore, careful interpretation of results as well as the correct choice of approaches and methods is a necessity. For more complex approaches (e.g. in quantifying amounts of adulteration) basics are still missing. Another important issue is the possible degradation of DNA in raw materials and especially in processed intermediates and final products.

Molecular phylogenetics, a branch of molecular systematics, explains the evolutionary relationship of organisms by differences in proteins or DNA sequence. The beginnings were in the 1960s and were sped up with the advent of DNA sequencing and PCR (Suárez-Díaz and Anaya-Muñoz 2008). Molecular phylogenetics created the basis for DNA barcoding and offers a wealth of valuable supportive information.

The intention of this paper is to check the species from the BELFRIT-list for the availability of information helpful for DNA-based identification by searching in the abstract and citation database SCOPUS. Beside papers on DNA based identification, also papers on phylogenetic analysis comprising the target species and a subset of molecular markers were regarded as helpful. The literature about molecular markers of BELFRIT-species is much richer than presented here, but the search was narrowed here on reproducible methods and methods suitable for routine samples. The search was conducted by combining (AND) the genus name with the logical disjunction (OR) of the keywords 'barcoding', 'taxonomy', 'molecular marker', 'authentication', 'phylogeny', 'phylogenetic*', 'chloroplast', 'nuclear', 'SCAR', 'ARMS', 'HRM', 'SSR', 'PCR'. From the hits, the papers meeting the criteria mentioned above were selected. Other molecular markers like RAPD may appear, but only when used in combination with one of the selected marker methods. Furthermore, only those papers with molecular markers were selected that include besides the target species also other species of the genus.

The BELFRIT-list is a quite comprehensive list with 991 species listed that belong to 594 genera. For 286 genera (48% of the listed genera) no valuable information could be retrieved. The number of papers with the intention to identify a species was with 238 quite high. However, this number is not spread equally but focussed on difficult and/or economically important species. Leading is here the genus *Panax*. For this genus 14 valuable papers were found of which 11 papers were dealing with identification (Table 9.2).

There is no coordinated systematic approach to develop methods for DNA-based identification of all species of such lists or a pharmacopoeia. They are developed on a case by case basis selecting those species which are of importance and/or are difficult to identify by other methods. Therefore, the number of already developed methods or information helpful to develop such methods is encouraging and should foster combined efforts to develop standardized validation criteria.

Table 9.2 The BELFRIT-List and DNA-based identification methods and helpful information for developing DNA-based identification methods

Botanical name	Family	References
Abelmoschus esculentus (L.) Moench; *A. moschatus* Medik.	Malvaceae	Sawadogo et al. (2009), Werner et al. (2016)
Abies alba Mill.; *A. balsamea* (L.) Mill.; *A. nordmanniana* subsp. *equi-trojani* (Asch. & Sint. ex Boiss.) Coode & Cullen; *A. sibirica* Ledeb.	Pinaceae	Hansen et al. (2005), Sánchez-Robles et al. (2012), Xiang et al. (2009, 2015)
Abroma augusta L. f	Malvaceae	–
Acacia catechu (L.f.) Willd.; *A. decurrens* Willd.; *A. nilotica* (L.) Delile; *A. senegal* (L.) Willd.; *Acacia seyal* Delile	Fabaceae	–
Acanthus mollis L.	Acanthaceae	–
Acer campestre L.; *A. negundo* L.; *A. saccharinum* L.	Sapindaceae	–
Achillea ageratum L.; *A. atrata* L.; *A. erba-rotta* All.; *A. maritima* (L.) Ehrend. & Y.P.Guo; *A. millefolium* L.; *A. nana* L.; *A. ptarmica* L.	Asteraceae	Newmaster et al. (2013)
Achyranthes bidentata Blume	Amaranthaceae	–
Acmella oleracea (L.) R.K.Jansen	Asteraceae	–
Acorus calamus L.	Acoraceae	Duvall et al. (1993), Goremykin et al. (2005), Joshi et al. (2012), Ryuk et al. (2014)
Actaea heracleifolia (Kom.) J.Compton; *A. racemosa* L.	Ranunculaceae	Ren et al. (2014), Newmaster et al. (2013)
Actinidia chinensis Planch.; *A. deliciosa* (A.Chev.) C.F.Liang & A.R.Ferguson	Actinidiaceae	(Li et al. (2002), Huang et al. (1998); Hirao et al. (2009), Fraser et al. (2001, 2005)
Adansonia digitata L.	Malvaceae	–
Adiantum capillus-veneris L.; *A. pedatum* L.	Pteridaceae	Hasebe et al. (1993), Wolf et al. (2003)
Adoxa moschatellina L.	Adoxaceae	Eriksson and Donoghue (1997)
Aegopodium podagraria L.	Apiaceae	Downie et al. (2000a, b)
Aesculus hippocastanum L.	Hippocastanaceae	Shi et al. (2013b)
Aframomum angustifolium (Sonn.) K.Schum.; *A. exscapum* (Sims) Hepper	Zingiberaceae	–
Agathosma betulina (P.J.Bergius) Pillans; *A. crenulata* (L.) Pillans; *A. serratifolia* (Curtis) Spreeth	Rutaceae	–
Agave americana L.; *A. sisalana* Perrine; *A. tequilana* F.A.C.Weber	Asparagaceae	–

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Table 9.2 (continued)

Botanical name	Family	References
Agrimonia eupatoria L.; *A. repens* L.	Rosaceae	–
Ajuga chamaepitys (L.) Schreb.; *A. iva* (L.) Schreb.; *A. reptans* L.	Lamiaceae	–
Albizia julibrissin Durazz.	Fabaceae	Sun and Chen (2013), Zhao et al. (2014b)
Alcea rosea L.	Malvaceae	–
Alchemilla vulgaris L.	Rosaceae	–
Aletris farinosa L.	Nartheciaceae	–
Alisma plantago-aquatica L.	Alismataceae	Ma et al. (2015)
Alliaria petiolata (M.Bieb.) Cavara & Grande	Brassicaceae	–
Allium ampeloprasum L.; *A. ascalonicum* L.; *A. cepa* L., *A. sativum* L., *A. schoenoprasum* L. *A. ursinum* L.	Amaryllidaceae	–
Alnus glutinosa (L.) Gaertn.; *A. incana* (L.) Moench	Betulaceae	Ren et al. (2010)
Aloe africana Mill.; *A. arborescens* Mill.; *A. ferox* Mill; *A. perryi* Baker; *A. plicatilis* (L.) Mill.; *A. vera* (L.) Burm. f.	Xanthorrhoeaceae	Ranghoo-Sanmukhiya et al. (2010), Gul et al. (2016), Daru et al. (2013)
Aloysia citriodora Palau	Verbenaceae	–
Alpinia galanga (L.) Willd.; *A. hainanensis* K.Schum.; *A. officinarum* Hance; *A. oxyphylla* Miq.	Zingiberaceae	Kress et al. (2005), Vaughn et al. (2014)
Althaea officinalis L.	Malvaceae	–
Amaranthus caudatus L.; *A. cruentus* L.	Amaranthaceae	Xu and Sun (2001), Ray and Roy (2009), Newmaster et al. (2013)
Ammi visnaga Lam.	Apiaceae	–
Amomum villosum var. *xanthioides* (Wall. ex Baker) T.L.Wu & S.J.Chen	Zingiberaceae	Huang et al. (2014b)
Amorphophallus konjac K.Koch	Araceae	Mekkerdchoo et al. (2011, 2013)
Amyris balsamifera L.	Rutaceae	–
Ananas comosus (L.) Merr.	Bromeliaceae	Duval et al. (2003), Faria et al. (2013)
Andrographis paniculata (Burm. f.) Nees	Acanthaceae	Osathanunkul et al. (2015), Arolla et al. (2015)

Anemarrhena asphodeloides Bunge	Asparagaceae	–
Anethum graveolens L.	Apiaceae	Jiménez-Mejías and Vargas (2015)
Angelica archangelica L.; *A. dahurica* (Hoffm.) Benth. & Hook.f. ex Franch. & Sav; *A. pubescens* Maxim.; *A. sinensis* (Oliv.) Diels; *A. sylvestris* L.	Apiaceae	Cui et al. (2007), Lee and Rasmussen (2000), Yan et al. (2016), Zhang et al. (2015a)
Angostura trifoliata (Willd.) T.S.Elias	Rutaceae	–
Annona muricata L.	Annonaceae	Larranaga and Hormaza (2015)
Anredera baselloides (Kunth) Baill.	Basellaceae	Eriksson (2007)
Antennaria dioica (L.) Gaertn.	Asteraceae	–
Anthemis tinctoria L.	Asteraceae	Oberprieler (2001), Presti et al. (2010)
Anthriscus cerefolium (L.) Hoffm.	Apiaceae	–
Anthyllis vulneraria L.	Fabaceae	–
Antirrhinum majus L.	Plantaginaceae	–
Aphanes arvensis L.	Rosaceae	Eriksson et al. (2003)
Apium graveolens L.	Apiaceae	Newmaster et al. (2013)
Arachis hypogaea L.	Fabaceae	Gimenes et al. (2007), Madesis et al. (2013), Tallury et al. (2005), Wang et al. (2011)
Aralia elata (Miq.) Seem.; *A. racemosa* L.	Araliaceae	Wen et al. (1998)
Arbutus unedo L.	Ericaceae	–
Arctium lappa L.; *A. minus* (Hill) Bernh.; *A. tomentosum* Mill.	Asteraceae	Newmaster et al. (2013), López-Vinyallonga et al. (2009, 2011)
Argania spinosa (L.) Skeels	Sapotaceae	–
Artemisia abrotanum L.; *A. absinthium* L.; *A. capillaris* Thunb.; *A. dracunculus* L.; *A. frigida* Willd.; *A. genipi* Weber ex Stechm.; *A. glacialis* L.; *A. judaica* L.; *A. pontica* L.; *A. umbelliformis* Lam.; *A. vallesiaca* All.; *A. verlotiorum* Lamotte; *A. vulgaris* L.	Asteraceae	Liu et al. (2012a, b), Doh and Oh (2012), Garcia et al. (2011), Lee et al. (2008), Mahmood et al. (2011)
Artocarpus altilis (Parkinson ex F.A.Zorn) Fosberg	Moraceae	Zerega et al. (2015)

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Table 9.2 (continued)

Botanical name	Family	References
Ascophyllum nodosum (L.) Le Jolis	Fucaceae	–
Asimina triloba (L.) Dunal	Annonaceae	–
Aspalathus linearis (Burm. f.) R.Dahlgren	Fabaceae	Edwards et al. (2008)
Asparagus cochinchinensis (Lour.) Merr.; *A. officinalis* L.; *A. racemosus* Willd.	Asparagaceae	Castro et al. (2013), Fukuda et al. (2012), Rai et al. (2012)
Astracantha adscendens (Boiss. & Hausskn.) Podlech; *A. certica* (Lam.) Podlech; *A. gummifera* (Labill.) Podlech; *A. microcephala* (Willd.) Podlech	Fabaceae	–
Astragalus membranaceus Moench	Fabaceae	Wojciechowski et al. (1999), Guo et al. (2010a), Long et al. (2013), Xiao et al. (2011), Zheng et al. (2014a), Bartha et al. (2013), Dong et al. (2003), Javanmardi et al. (2012), Kazemi et al. (2009), Liu et al. (2008), Qian et al. (2009), Safar et al. (2014)
Astrantia major L.	Apiaceae	–
Athamanta macedonica (L.) Spreng.	Apiaceae	–
Atractylodes lancea (Thunb.) DC.; *A. macrocephala* Koidz.; *A. ovata* (Thunb.) DC.	Asteraceae	Shao et al. (2015), Yu et al. 2014b, Huh and Bang (2006)
Avena fatua L.; *A. sativa* L.	Poaceae	Peng et al. (2010)
Baccharis trimera (Less.) DC.	Asteraceae	–
Bacopa monnieri (L.) Wettst.	Plantaginaceae	–
Bactris gasipaes Kunth	Arecaceae	Eiserhardt et al. (2011)
Balanites aegyptiaca (L.) Delile	Zygophyllaceae	–
Ballota nigra L.	Lamiaceae	–
Bambusa bambos (L.) Voss; *B. vulgaris* Schrad.	Poaceae	–
Barbarea verna (Mill.) Asch.; *B. vulgaris* R.Br.	Brassicaceae	Toneatto et al. (2012)
Bellis perennis L.	Asteraceae	–

Berberis aquifolium Pursh.; *B. aristata* DC.; *B. vulgaris* L.	Berberidaceae	Roy et al. (2010), Yu and Chung (2014)
Bertholletia excelsa Bonpl.	Lecythidaceae	–
Beta vulgaris L.	Amaranthaceae	Dohm et al. (2014), Richardson et al. (2016)
Betula alleghaniensis Britton; *B. lenta* L.; *B. pendula* Roth, *B. pubescens* Ehrh.	Betulaceae	Cräutlein et al. (2011)
Bixa orellana L.	Bixaceae	–
Blainvillea acmella (L.) Philipson	Asteraceae	–
Borago officinalis L.	Boraginaceae	–
Boronia megastigma Nees ex Bartlett	Rutaceae	Bayly et al. (2015)
Boswellia sacra Flueck.; *B. serrata* Roxb. ex Colebr.	Burseraceae	–
Brassica cretica Lam.; *B. napus* L.; *B. nigra* (L.) K.Koch; *B. oleracea* L.; *B. rapa* L.	Brassicaceae	Arias and Chris Pires (2012), El-Esawi (2016), Etoh et al. (2003), Ganopoulos et al. (2013b), Iñiguez-Luy et al. (2006), Kiefer et al. (2014), Kumar et al. (2011), Willis et al. (2014)
Bupleurum chinense DC.; *B. rotundifolium* L.	Apiaceae	Chao et al. (2014), Han et al. (2016a, b), Yu et al. 2014a
Bursera tomentosa (Jacq.) Triana & Planch.	Burseraceae	–
Caesalpinia bonduc (L.) Roxb.	Fabaceae	Gagnon et al. (2013)
Cakile maritima Scop.	Brassicaceae	Willis et al. (2014), Arias and Chris Pires (2012)
Calendula arvensis (Vaill.) L.; *C. officinalis* L.	Asteraceae	–
Calluna vulgaris (L.) Hull	Ericaceae	–
Camelina sativa (L.) Crantz	Brassicaceae	–
Camellia sinensis (L.) Kuntze	Theaceae	Huang et al. (2014a), Su et al. (2009), Vijayan et al. (2009)
Cananga odorata (Lam.) Hook.f. & Thomson	Annonaceae	Surveswaran et al. (2010)
Canarium acutifolium (DC.) Merr.	Burseraceae	–

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Table 9.2 (continued)

Botanical name	Family	References
Capparis spinosa L.	Capparaceae	Hall (2008)
Capsella bursa-pastoris (L.) Medik.	Brassicaceae	Khosravi et al. (2009)
Capsicum annuum L.	Solanaceae	Castañón-Nájera et al. (2014), Costa et al. (2016b), Jeong et al. (2010), Nicolaï et al. (2013), Raveendar et al. (2015), Shirasawa et al. (2013), Vallejo et al. (2012)
Carex arenaria L.	Cyperaceae	Starr et al. (2009), Clerc-Blain et al. (2010), Escudero et al. (2008), Ford et al. (2012), Jiménez-Mejías et al. (2012), Saarela et al. (2013), Shekhovtsov et al. (2012)
Carica papaya L.	Caricaceae	Kyndt and Gheysen (2007)
Carissa carandas L.	Apocynaceae	–
Carlina acaulis L.	Asteraceae	–
Carpinus betulus L.	Betulaceae	–
Carthamus lanatus L.; *C. tinctorius* L.	Asteraceae	Sehgal et al. (2009), Vilatersana et al. (2000)
Carum carvi L.	Apiaceae	Zakharova et al. (2012)
Cassia fistula L.	Fabaceae	Seethapathy et al. (2015), Mohanty et al. (2010), Purushothaman et al. (2014)
Castanea sativa Mill.	Fagaceae	Gismondi et al. (2015), Yousefzadeh et al. (2014)
Catalpa bignonioides Walter	Bignoniaceae	–
Ceanothus americanus L.	Rhamnaceae	–
Cecropia peltata L.	Urticaceae	–
Cedrus libani A.Rich.	Pinaceae	Fady et al. (2003), Gernandt et al. (2008)
Ceiba pentandra (L.) Gaertn.	Malvaceae	–
Centaurea behen L.; *C. calcitrapa* L; *C. centaurium* L.; *C. cyanus* L.; *C. jacea* L.; *C. montana* L.	Asteraceae	Boršić et al. (2011), Font et al. (2009), Garcia-Jacas et al. (2006), López-Alvarado et al. (2014)

Centaurium erythraea Rafn	Gentianaceae	–
Centella asiatica (L.) Urb.	Apiaceae	–
Centranthus ruber (L.) DC	Caprifoliaceae	Raymúndez et al. (2002)
Cerasus mahaleb (L.) Mill.	Rosaceae	Xu et al. (2015), Barac et al. (2014), Kato et al. (2014), Ohta et al. (2006), Shi et al. (2013a), Turkoglu et al. (2010)
Ceratonia siliqua L.	Leguminoseae	–
Cercis siliquastrum L.	Leguminoseae	–
Cetraria islandica (L.)	Parmeliaceae	Nelsen et al. (2011), Thell et al. (2009)
Chaenomeles speciosa Nakai	Rosaceae	Bartish et al. (2000)
Chamaecrista nomame (Sieber) H.Ohashi	Fabaceae	Seethapathy et al. (2015)
Chamaemelum nobile (L.) All.	Asteraceae	Oberprieler (2002)
Chelone glabra L.	Plantaginaceae	–
Chenopodium quinoa Willd.; *Chenopodium vulvaria* L.	Amaranthaceae	–
Chimaphila umbellata (L.) Nutt.	Ericaceae	–
Chiococca alba (L.) Hitchc.	Rubiaceae	–
Chionanthus virginicus L .	Oleaceae	Arias et al. (2011), Hong-Wa and Besnard (2013)
Chlorella vulgaris Beijerinck	Chlorellaceae	Alemzadeh et al. (2014), Baytut et al. (2014), Hadi et al. (2016), Heeg and Wolf (2015)
Chondrus crispus Stackhouse	Gigartinaceae	Janouškovec et al. (2013), Kim et al. (2014)
Chrysanthellum americanum (L.) Vatke	Asteraceae	–
Chrysanthellum indicum subsp. *afroamericanum* B.L.Turner	Asteraceae	–
Chrysophyllum cainito L.	Sapotaceae	Swenson and Anderberg (2005), Swenson et al. (2008)
Chrysopogon zizanioides (L.) Roberty	Poaceae	–
Cichorium endivia L.; *C. intybus* L.	Asteraceae	Gemeinholzer and Bachmann (2005)

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Table 9.2 (continued)

Botanical name	Family	References
Cinchona calisaya Wedd.; *C. lancifolia* Mutis; *C. micrantha* Ruiz & Pav.; *C. nitida* Ruiz & Pav.; *C. officinalis* L.; *C. pitayensis* (Wedd.) Wedd.; *C. pubescens* Vahl	Rubiaceae	Palhares et al. (2014)
Cinnamomum camphora (L.) J.Presl; *C. cassia* (Nees & T.Nees) J.Presl	Lauraceae	Newmaster and Ragupathy (2009), Sun and Chen (2013), Swetha et al. (2014), Yang et al. (2015)
Cistanche salsa (C.A. Mey.) G.Beck	Orobanchaceae	Han et al. (2010)
Cistus creticus L.; *C. incanus* L.; *C. monspeliensis* L.	Cistaceae	Marieschi et al. (2010)
Citrullus lanatus (Thunb.) Matsum. & Nakai	Cucurbitaceae	Chomicki and Renner (2015), Nimmakayala et al. (2010), Schaefer and Renner (2011)
Citrus aurantiifolia (Christm.) Swingle; *C. aurantium* L.; *C. limon* (L.) Osbeck; *C. maxima* (Burm.) Merr.; *C. medica* L.; *C. myrtifolia* Raf.; *C. nobilis* Lour.; *C. paradisi* Macfad.; *C. reticulata* Blanco; *C. sinensis* (L.) Osbeck;	Rutaceae	Araújo et al. (2003), Biswas et al. (2011), Curk et al. (2016), Distefano et al. (2013), Distefano et al. (2015b), Garcia-Lor et al. (2013), Hazarika et al. (2014), Herrero et al. (1996a, b, Hynniewta et al. (2014), Kumar et al. (2013), Kyndt et al. (2010), Li et al. (2010), Luro et al. (2001, 2008), Mahadani and Ghosh (2014), Morton (2009), Nicolosi et al. (2000), Penjor et al. (2010), Sun et al. (2015, 2016b)
Cladonia rangiferina (L.) Weber ex F.H.Wigg.	Cladoniaceae	Pino-Bodas et al. (2010, 2013), Steinová et al. (2013), Stenroos et al. (2015)
Clematis armandii Franch.; *C. chinensis* Osbeck	Ranunculaceae	Guo et al. (2010b), Zhang et al. (2015d)
Clinopodium nepeta subsp. *glandulosum* (Req.) Govaerts; *C. vulgare* L.	Lamiaceae	Bräuchler et al. (2010)
Clitoria ternatea L.	Fabaceae	–
Cnicus benedictus L.	Asteraceae	–
Cochlearia officinalis L.	Brassicaceae	–
Cocos nucifera L.	Arecaceae	Baker et al. (2011), Ronde et al. (1999)

Codonopsis pilosula (Franch.) Nannf.	Campanulaceae	He et al. (2014a), Wang et al. (2013c)
Coffea arabica L.; *C. canephora* Pierre ex Froehner	Rubiaceae	Amidou et al. (2007), Davis et al. (2007), Maurin et al. (2007)
Coix lacryma-jobi L.	Poaceae	–
Cola acuminata (P.Beauv.) Schott & Endl.	Malvaceae	–
Cola nitida (Vent.) Schott & Endl.	Malvaceae	–
Combretum micranthum G.Don	Combretaceae	Jordaan et al. (2011), Maurin et al. (2010)
Commiphora africana (A.Rich.) Endl.; *C. habessinica* (O.Berg) Engl.; *C. mukul* Engl.; *C. myrrha* (Nees) Engl.; *C. schimperi* (O.Bergman) Engl.	Burseraceae	Gostel et al. (2016)
Conyza canadensis (L.) Cronquist	Asteraceae	–
Copaifera langsdorffii Desf.	Fabaceae	–
Coptis japonica (Thunb.) Makino; *C. teeta* Wall.; *C. trifolia* (L.) Salisb.	Ranunculaceae	He et al. (2014b)
Corallina officinalis L.	Corallinaceae	Brodie et al. (2013), Hind et al. (2014)
Cordia myxa L.	Boraginaceae	–
Coriandrum sativum L.	Apiaceae	Terentieva et al. (2015)
Cornus florida L.; *C. mas* L.; *C. officinalis* Siebold & Zucc.; *C. sanguinea* L.	Cornaceae	Xiang et al. (2006), Hou et al. (2013b), Gismondi et al. (2012)
Corrigiola telephiifolia Pourr.	Molluginaceae	Kool et al. (2012)
Corylus avellana L.	Betulaceae	Whitcher and Wen (2001), Madesis et al. (2013), Bassil et al. (2013)
Corymbia citriodora (Hook.) K.D.Hill & L.A.S.Johnson	Myrtaceae	Shepherd et al. (2008)
Coscinium fenestratum (Goetgh.) Colebr.	Menispermaceae	–
Crambe maritima L.	Brassicaceae	Warwick and Gugel (2003)
Crataegus azarolus L.; *C. curvisepala* Lindm.; *C. laevigata* (Poir.) DC.; *C. monogyna* Jacq.; *C. pentagyna* Waldst. & Kit.	Rosaceae	Piedra-Malagón et al. (2016), Zarrei et al. (2015)
Crithmum maritimum L.	Apiaceae	–

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Table 9.2 (continued)

Botanical name	Family	References
Crocus sativus L.	Iridaceae	Babaei et al. (2014), Erol et al. (2014), Gismondi et al. (2013), Huang et al. (2015b), Jiang et al. (2014), Larsen et al. (2015), Petersen et al. (2008), Sucher and Carles (2008), Seberg and Petersen (2009), Zheng et al. (2015)
Crossostephium chinense (A.Gray ex L.) Makino	Compositeae	–
Cruciata laevipes Opiz	Rubiaceae	–
Cryptocarya agathophylla van der Werff	Lauraceae	–
Cucumis melo L., *C. sativus* L.	Cucurbitaceae	Renner et al. (2007), Zhuang et al. (2004)
Cucurbita maxima Duch.; *C. pepo*.L.	Cucurbitaceae	–
Cuminum cyminum L.	Apiaceae	Banasiak et al. (2016)
Cupressus sempervirens L.	Cupressaceae	–
Curcuma longa L.; *C. xanthorrhiza* Roxb.; *C. zedoaria* (Christm.) Roscoe	Zingiberaceae	Cao et al. (2001, 2010), Chen et al. (2015a), Deng et al. (2011, 2015), Dhanya and Sasikumar (2010), Dhanya et al. (2011), Parvathy et al. (2015), Sasaki et al. (2002), Sasikumar et al. (2004), Sucher and Carles (2008)
Cuscuta chinensis Lam.; *C. epithymum* Murray	Convolvulaceae	Abdin et al. (2012), Costea et al. (2011, 2015), García et al. (2014), Stefanović et al. (2007)
Cyamopsis tetragonoloba (L.) Taub.	Fabaceae	–
Cyathula officinalis K.C.Kuan	Amaranthaceae	Tian et al. (2010)
Cyclanthera pedata (L.) Schrad.	Cucurbitaceae	–
Cydonia oblonga Mill.	Rosaceae	–

Cymbopogon citratus (DC.) Stapf; *C. flexuosus* (Nees ex Steud.) W. Watson; *C. jwarancusa* subsp. *olivieri* (Boiss.) Soenarko; *C. martini* (Roxb.) W.Watson; *C. nardus* (L.) Rendle; *C. schoenanthus* (L.) Spreng.; *C. winterianus* Jowitt ex Bor	Poaceae	–
Cynara cardunculus L.; *C. scolymus* L.	Asteraceae	Curci et al. (2015)
Cyperus rotundus L.	Cyperaceae	Larridon et al. (2011a, b, (2013)
Cytinus hypocistis (L.) L.	Cytinaceae	–
Daemonorops draco (Willd.) Blume	Arecaceae	Wang et al. (2015b)
Dahlia pinnata Cav.	Asteraceae	–
Daucus carota L.	Apiaceae	Arbizu et al. (2014), Spooner et al. (2013), Banasiak et al. (2016)
Dendranthema grandiflorum (Ramat.) Kitam.	Asteraceae	–
Descurainia sophia (L.) Webb ex Prantl	Brassicaceae	
Dianthus caryophyllus L.	Caryophyllaceae	–
Dimocarpus longan Lour.	Sapindaceae	–
Dioscorea alata L.; *D. collettii* Hook. f. var. *hypoglauca* (Palib.) C.Pei & C. T.Ting; D.; *D. composita* Hemsl.; *D. oppositifolia* L.; *D. polystachya* Turcz.; *D. villosa* L.	Dioscoreaceae	Caddick et al. (2002), Girma et al. (2016), Sun et al. (2012)
Diospyros kaki Thunb.; *D. virginiana* L.	Ebenaceae	Yonemori et al. (2008),
Diplotaxis tenuifolia (L.) DC	Brassicaceae	Arias and Chris Pires (2012)
Dipsacus fullonum L.; *D. inermis* Wall.; *D. japonicus* Miq.	Caprifoliaceae	–
Dorstenia contrajerva L.	Moraceae	–
Dracocephalum moldavica L.	Lamiaceae	Horn et al. (2014)
Drimys winteri J.R.Forst. & G.Forst.	Winteraceae	–
Drosera anglica Huds.; *D. intermedia* Hayne; *D. peltate* Thunb.; *D. ramentacea* Burch ex DC; *D. rotundifolia* L.	Droseraceae	–
Dunaliella salina (Dunal) Teodoresco	Dunaliellaceae	Assunção et al. (2012), González et al. (1998, 2001)

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Table 9.2 (continued)

Botanical name	Family	References
Durvillea antartica (Chamisso) Hariot	Durvillaeaceae	–
Dysphania botrys (L.) Mosyakin & Clemants	Amaranthaceae	–
Echinacea angustifolia DC.; *E. pallida* (Nutt.) Nutt.; *E. purpurea* (L.) Moench	Echinacea	Adinolfi et al. (2007), Kapteyn et al. (2002)
Echium plantagineum L.	Boraginaceae	–
Eisenia bicyclis (Kjellman) Setchell	Lessoniaceae	–
Elaeis guineensis Jacq.	Arecaceae	Baker et al. (2011)
Elettaria cardamomum (L.) Maton.	Zingiberaceae	–
Eleutherococcus senticosus (Rupr. & Maxim.) Maxim	Araliaceae	Song et al. (2012), Zhao et al. (2015), Sun and Chen (2013)
Elymus repens (L.) Gould	Poaceae	Bieniek et al. (2015), Dizkirici et al. (2010), Fahleson et al. (2008), Kawahara (2009)
Embelia ribes Burm.f.	Primulaceae	Devaiah and Venkatasubramanian (2008)
Epilobium angustifolium L.; *E. parviflorum* Schreb.	Onagraceae	–
Equisetum arvense L.; *E. fluviatile* L.; *E. hyemale* L.; *E. telmateia* Ehrh.	Equisetaceae	Guillon (2007), Saslis-Lagoudakis et al. (2015)
Erica cinerea L.; *E. tetralix* L.	Ericaceae	–
Eriobotrya japonica (Thunb.) Lindl.	Rosaceae	Zhao et al. (2011)
Eriodictyon californicum (Hook. & Arn.) Torr.	Boraginaceae	–
Erodium cicutarium L 'Hérit.	Geraniaceae	–
Eruca vesicaria L. Cav.	Brassicaceae	–
Eryngium campestre L.	Apiaceae	Jawdat et al. (2010), Kadereit et al. (2008)
Eschscholtzia californica Cham.	Papaveraceae	–
Eucalyptus dives Schauer; *E. globulus* labill.; *E. odorata* Behr; *E. radiate* Sieber ex DC.; *E. smithii* R.T.Baker	Myrtaceae	Brondani et al. (2006), McKinnon et al. (2010), Richero et al. (2013), Tsoktouridis et al. (2014), van der Nest et al. (2000)

Eucheuma horridum J. Agardh; *E. spinosum* J. Agardh.	Solieriaceae	Tan et al. (2012, 2013)
Eucommia ulmoides Oliv.	Eucommiaceae	–
Eugenia uniflora L.	Myrtaceae	Nogueira et al. (2016)
Euphrasia rostkoviana Hayne; *E. stricta* D. Wolff ex J.F.Lehm.	Orobanchaceae	–
Euterpe oleracea Mart.	Arecaceae	–
Evernia prunastri (L.) Ach.	Parmeliaceae	–
Exostema caribaeum (Jacq.) Schult.	Rubiaceae	–
Fabiana imbricata Ruiz & Pav	Solanaceae	–
Fagopyrum esculentum Moench	Polygonaceae	Zhou et al. (2014a)
Fagus sylvatica L.	Fagaceae	Bruni et al. (2015), Gailing and Wuehlisch (2004), Seifert et al. (2012)
Fallopia japonica (Houtt.) Ronse Dec.	Polygonaceae	Gammon and Kesseli (2010)
Ferula assa-foetida L.	Apiaceae	Kurzyna-Młynik et al. (2008), Valiejo-Roman et al. (2006)
Ficus benghalensis L.; *F. carica* L.; *F. religiosa* L.	Moraceae	Li et al. (2012a), Phromthep (2012), Roy et al. (2010)
Filipendula ulmaria (L.) Maxim; *F. vulgaris* Moench.	Rosaceae	Hawkins et al. (2015)
Foeniculum vulgare Mill.	Apiaceae	–
Forsythia suspensa (Thunb.) Vahl	Oleaceae	Suh et al. (2011)
Fragaria x *ananassa* (Duchesne ex Weston) Duchesne ex Rozier; *F. vesca* L.; *F. viridis* Weston	Rosaceae	Njuguna and Bassil (2011), Eriksson et al. (2003)
Frangula alnus Mill.; *F. purshiana* Cooper	Rhamnaceae	–
Fraxinus excelsior L.; *F. ornus* L.	Oleaceae	Arca et al. (2012), Hinsinger et al. (2014), Jeandroz et al. (1997), Raquin et al. (2002), Wallander (2008)
Fucus serratus L.; *F. vesiculosus* L.	Fucaceae	Billard et al. (2005), Kucera and Saunders (2008), Pereyra et al. (2013)

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Table 9.2 (continued)

Botanical name	Family	References
Fumaria officinalis L.	Papaveraceae	–
Galeopsis segetum Neck.	Lamiaceae	–
Galium aparine L.; *G. mollugo* L.; *G. odoratum* (L.) Scop.; *G. verum* L.	Rubiaceae	–
Garcinia mangostana L.; *G. gummi-gutta* (L.) Roxb.	Clusiaceae	Yapwattanaphun et al. (2004)
Gardenia jasminoides J. Ellis	Rubiaceae	Suwannakud et al. (2014)
Gastrodia elata Blume	Orchidaceae	–
Gaultheria procumbens L.	Ericaceae	Bush et al. (2009), Ren et al. (2011)
Gelidium amansii J.V. Lamouroux; *G. sesquipedale* (Clemente) Thuret	Gelidiaceae	Boo et al. (2016), Iha et al. (2015)
Gentiana lutea L.	Gentianaceae	–
Geranium maculatum L., *G. pratense* L.; *G. robertianum* L.; *G. sanguineum* L.	Geraniaceae	–
Geum rivale L.; *G. urbanum* L.	Rosaceae	–
Ginkgo biloba L.	Ginkgoaceae	Little and Gulick (2014), Gong et al. (2008), Newmaster et al. (2013)
Glycine max (L.) Merr.	Fabaceae	Gupta et al. (2014), An et al. (2009), Chen and Nelson (2004), Madesis et al. (2012a), Sharma and Kobayashi (2014)
Glycyrrhiza glabra L.; *G. uralensis* Fisch. ex DC.	Fabaceae	Simmler et al. (2015)
Gossypium herbaceum L.; *G. hirsutum* L.	Malvaceae	Ahmad et al. (2007), Ashfaq et al. (2013), Feng et al. (2011), Mehboob-ur-Rahman et al. (2012), Tabbasam and Zafar (2014), Yu-xiang et al. (2013)
Griffonia simplicifolia (DC.) Baill.	Fabaceae	–
Grindelia camporum Greene; *G. hirsutula* Hook. & Arn.; *G. robusta* Nutt.; *G. squarrosa* (Pursh) Dun.	Asteraceae	Moore et al. (2012, 2014)
Guaiacum officinale L.; *G. sanctum* L.	Zygophyllaceae	–
Guazuma ulmifolia Lamk.	Malvaceae	–

Gynostemma pentaphyllum (Thunb.) Makino	Cucurbitaceae	Zhou et al. (2015)
Gypsophila paniculata L.	Caryophyllaceae	Calistri et al. (2016)
Haematococcus pluvialis Flotow	Haematococcaceae	Buchheim et al. (2013)
Haematoxylum campechianum L.	Fabaceae	–
Hamamelis virginiana L.	Hamamelidaceae	Palhares et al. (2015)
Haplopappus baylahuen Remy	Asteraceae	–
Harpagophytum procumbens (Burch.) DC.; *H. zeyheri* Decne.	Pedaliaceae	–
Hebanthe eriantha (Poir.) Pedersen	Amaranthaceae	–
Hedeoma pulegioides (L.) Pers.	Lamiaceae	–
Hedera helix L .	Araliaceae	Green et al. (2011)
Hedychium coronarium J.Koenig	Zingiberaceae	Wood et al. (2000), Vaughn et al. (2014), Cao et al. (2001)
Helianthus annuus L.	Asteraceae	Kumar et al. (2011)
Helichrysum arenarium (L.) Moench.; *H. italicum* (Roth.) G.Don.; *H. stoechas* (L.) Moench	Asteraceae	Galbany-Casals et al. (2012, 2014)
Heracleum sphondylium L.	Apiaceae	Logacheva et al. (2008)
Herniaria glabra L.; *H. hirsuta* L.	Caryophyllaceae	–
Hesperis matronalis L.	Brassicaceae	–
Hibiscus sabdariffa L.	Malvaceae	
Hieracium pilosella L.	Asteraceae	Tyler and Jönsson (2013)
Hierochloe odorata (L.) Beauv.	Poaceae	–
Himanthalia elongata (L.) S.F.Gray	Himanthaliaceae	–
Hippophae rhamnoides L.	Eleagnaceae	Hyvönen (1996), Liu et al. (2015), Ma et al. (2014b)
Hizikia fusiformis (Harvey) Okamura	Sargassaceae	Jia et al. (2014), Stiger et al. (2003)
Hordeum vulgare L.	Poaceae	Badr et al. (2000), Aalami et al. (2012), El-Rabey et al. (2002)

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Table 9.2 (continued)

Botanical name	Family	References
Houttuynia cordata Thunb.	Saururaceae	–
Humulus lupulus L.	Cannabaceae	–
Huperzia serrata (Thunb.) Trevis.	Lycopodiaceae	Ji et al. (2007), Shao et al. (2010)
Hydrastis canadensis L.	Ranunculaceae	–
Hygrophila auriculata (Schumach.) Heine	Acanthaceae	–
Hymenaea courbaril L.	Leguminoseae	–
Hypericum perforatum L.	Hyperaceae	Howard et al. (2009), Costa et al. (2016a), Nürk et al. (2013)
Hyssopus officinalis L.	Lamiaceae	Moon et al. (2010)
Ilex paraguariensis A.St.-Hil.	Aquifoliaceae	Gottlieb et al. (2005), Yi et al. (2014)
Illicium verum Hook. f.	Schisandraceae	Zhang et al. (2015b)
Impatiens balsamina L.	Balsaminaceae	Ruchisansakun et al. (2015)
Indigofera tinctoria L.	Fabaceae	Schrire (2013), Gupta (2012)
Inula britannica L.; *I. helenium* L.	Asteraceae	Ma et al. (2014a), Anderberg (1991)
Ipomoea batatas (L.) Poir	Convolvulaceae	Stefanović et al. (2003), Das (2011), Dhillon and Ishiki (1999)
Isatis tinctoria L.	Brassicaceae	Moazzeni et al. (2010), Sun and Pang (2013)
Jasminum grandiflorum L.; *J. officinale* L.	Oleaceae	–
Jateorhiza palmata (Lam.) Miers	Menispermaceae	–
Juglans cinerea L.; *J. regia* L.	Juglandaceae	Orel et al. (2003), Ross-Davis et al. (2008), Xiang et al. (2011), Zhao and Woeste (2011), Zhao et al. (2014a)
Jumellea fragrans (Thouars) Schltr.	Orchidaceae	Caetano Wyler and Naciri (2016)
Juniperus communis L.	Cupressaceae	–
Justicia adhatoda L.; *J. pectoralis* Jacq.	Acanthaceae	Kiel and McDade (2014)
Kaempferia galanga L.	Zingiberaceae	–

Kavalama urens (Roxb.) Raf.	Malvaceae	–
Kickxia spuria (L.) Dumort.	Plantaginaceae	Ghebrehiwet (2000)
Knautia arvensis (L.) Coult.	Caprifoliaceae	Rešetnik et al. (2014)
Krameria lappacea (Dombey) Burdet & B.B.Simpson	Krameriaceae	–
Lactuca indica L.; *L. sativa* L.	Asteraceae	Wang et al. (2013d)
Lagerstroemia speciosa (L.) Pers.	Lythraceae	–
Laminaria digitata (Hudson) J.V.Lam.; *L. hyperborean* (Gun.) Foslie; *L. japonica* J.E.Areschoug; *L. palmate* Bory de St.Vincent	Laminariaceae	Shi et al. (2005)
Lamium album L.	Lamiaceae	Krawczyk et al. (2014)
Larix decidua Mill.; *L. occidentalis* Nutt.	Pinaceae	Gros-Louis et al. (2005), Semerikov and Lascoux (2003), Semerikov et al. (2006), Wei and Wang (2004)
Lavandula x intermedia Emeric ex Loisel.; *L. angustifolia* Mill.; *L. latifolia* Medik; *L. stoechas* L.	Lamiaceae	–
Lawsonia inermis L.	Lythraceae	–
Ledum palustre L.	Ericaceae	Gao et al. (2002)
Lens culinaris Medik.	Fabaceae	–
Leonurus cardiaca L.; *L. japonicus* Houtt.	Lamiaceae	Bendiksby et al. (2011), Marciniuk et al. (2014)
Lepidium campestre (L.) R.Br.; *L. coronopus* (L.) Al-Shehbaz; *L. latifolium* L.; *L. meyenii* Walp.; *L. sativum* L.	Brassicaceae	Chen et al. (2015b)
Leptospermum petersonii subsp. *petersonii*.; *L. scoparium* J.R.Forst. & G.Forst.	Myrtaceae	–
Lespedeza capitata Michx.	Fabaceae	–
Leucanthemum vulgare (Vaill.) Lam.	Asteraceae	Konowalik et al. (2015)
Levisticum officinale W.D.J.Koch	Apiaceae	Zhang et al. (2015a), Yuan et al. (2015)
Ligusticum striatum DC.	Apiaceae	–

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Table 9.2 (continued)

Botanical name	Family	References
Lilium brownii F.E. Br. ex Miellez	Liliaceae	Zheng et al. (2014b)
Lindera aggregata (Sims) Kosterm.	Lauraceae	–
Linum usitatissimum L.	Linaceae	Kumar et al. (2012), Mcdill and Simpson (2011)
Liquidambar styraciflua L.	Altingiaceae	–
Litchi chinensis Sonn.	Sapindaceae	–
Lithothamnion calcareum (Pallas) Areschoug	Hapalidiaceae	Carro et al. (2014), Pardo et al. (2014)
Litsea cubeba (Lour.) Pers.	Lauraceae	–
Lobaria pulmonaria (L.) Hoffm.	Lobariaceae	Moncada et al. (2013)
Lonicera japonica Thunb.	Caprifoliaceae	Jiang et al. (2012), Hou et al. (2013a), Sun et al. (2011a, b), Caetano Wyler and Naciri (2016)
Lotus cornicolatus L.	Fabaceae	–
Luma chequen (Molina) A.Gray	Myrtaceae	–
Lycium barbarum L.; *L. chinense* Mill.	Solanaceae	Sze et al. (2008), Xin et al. (2015b), Sun and Chen (2013)
Lycopersicon esculentum Mill.	Solanaceae	Dodsworth et al. (2016), Tepe et al. (2016)
Lycopodium clavatum L.	Lycopodiaceae	–
Lycopus europaeus L.; *L. virginicus* Michx.	Lamiaceae	Moon et al. (2010)
Lysimachia vulgaris L.	Primulaceae	Zhang et al. (2012)
Lythrum salicaria L.	Lythraceae	–
Macadamia ternifolia F.Muell	Proteaceae	–
Macrocystis pyrifera (L.) C.Ag.	Laminariaceae	Macaya and Zuccarello (2010)
Magnolia champaca (L.) Baill. ex Pierre; *M. officinalis* Rehder & Wilson	Magnoliaceae	Li et al. (2012b), Sun and Chen (2013)
Malpighia glabra L.	Malpighiaceae	–
Malus domestica Borkh.; *M. pumila* Mill.; *M. sylvestris* L.	Rosaceae	Robinson et al. (2001), Yao et al. (2010)

Malva sylvestris L.	Malvaceae	–
Mammea americana L.	Calophyllaceae	–
Mangifera indica L.	Anacardiaceae	Hidayat et al. (2011), Yonemori et al. (2002)
Manihot esculenta Crantz	Euphorbiaceae	–
Manilkara zapota (L.) P.Royen	Sapotaceae	–
Maranta arundinacea L.	Marantaceae	–
Marchantia polymorpha L.	Marchantiaceae	–
Marrubium vulgare L.	Lamiaceae	–
Marsdenia cundurango Rchb. f.; *M. sylvestris* (Retz.) P.I.Forst.	Apocynaceae	Zhang et al. (2013c)
Mastocarpus stellatus (Stackhouse) Guiry	Phyllophoraceae	Robba et al. (2006), Le Gall and Saunders (2010)
Matricaria chamomilla L.	Asteraceae	Palhares et al. (2015), Newmaster et al. (2013)
Medicago sativa L.	Fabaceae	Steele et al. (2010)
Melaleuca alternifolia (Maiden & Betche) Cheel; *M. cajeputi* Powell; *M. leucadendra* (L.) L.; *M. linariifolia* Sm.; *M. quinquenervia* (Cav.) S.T.Blake; *M. viridiflora* Sol. ex Gaertn.	Myrtaceae	Ladiges et al. (1999)
Melilotus altissimus Thuill.; *M. officinalis* (L.) Lam.	Fabaceae	–
Melissa officinalis L.	Lamiaceae	–
Melittis melissophyllum L.	Lamiaceae	Scheen et al. (2008)
Mentha aquatica L.; *M. arvensis* L.; *M. spicata* L.; *Mentha* x *piperita* L.	Lamiaceae	Mattia et al. (2011), Wang et al. (2013a)
Mentzelia cordifolia Dombey ex Urb. & Gilg	Losaceae	Hufford et al. (2003)
Menyanthes trifoliata L.	Menyanthaceae	–
Mesembryanthemum crystallinum L.	Aizoaceae	–
Mespilus germanica L.	Rosaceae	–
Mikania amara (Vahl.) Willd.	Asteraceae	–
Mitchella repens L.	Rubiaceae	Huang et al. (2013)

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Table 9.2 (continued)

Botanical name	Family	References
Momordica balsamina L.; *M. charantia* L.	Cucurbitaceae	–
Monarda didyma L.; *M. punctata* L.	Lamiaceae	–
Morinda citrifolia L.; *M. officinalis* F.C.How	Rubiaceae	–
Moringa oleifera Lam.	Moringaceae	–
Morus alba L.; *M. nigra* L.	Moraceae	Nepal and Ferguson (2012), Zeng et al. (2015), Zhao et al. (2007)
Murraya koenigii (L.) Spreng.	Rutaceae	–
Musa x *paradisiaca* L. (pro sp.)	Musaceae	Bekele and Shigeta (2011), Christelov et al. (2011), Liu et al. (2010)
Myrciaria dubia (H.B.K.) McVaugh	Myrtaceae	–
Myrica cerifera L.; M. gale L.	Myricaceae	–
Myristica fragrans Houtt.	Myristicaceae	–
Myroxylon balsamum var. *balsamum* (L.) Harms	Fabaceae	–
Myrtus communis L.	Myrtaceae	–
Nardostachys jatamansi (D. Don) DC.	Caprifoliaceae	–
Nasturtium officinale R. Brown	Brassicaceae	–
Nelumbo nucifera Gaertn.	Nelumbonaceae	–
Nepeta cataria L.; *N. tenuifolia* Benth.	Lamiaceae	–
Nephelium lappaceum L.	Sapindaceae	–
Nigella sativa L.	Ranunculaceae	–
Ocimum basilicum L.; *O. gratissimum* L.; *O. tenuiflorum* L.	Lamiaceae	Christina, Annamalai (2014), Mahajan et al. (2015), Sarwat et al. (2016)
Oenothera biennis L.	Onagraceae	–
Olea europaea L.	Oleaceae	Ganopoulos et al. (2013b), Kumar et al. (2011)
Ononis spinosa L.	Fabaceae	Turini et al. (2010)

Onopordon acanthium L.	Asteraceae	–
Ophiopogon japonicus (Thunb.) Ker Gawl.	Asparagaceae	Li et al. (2011)
Opopanax chironius (L.) W.D.J.Koch	Apiaceae	–
Opuntia ficus-indica (L.) Mill.	Cactaceae	Lyra et al. (2013), Majure et al. (2012)
Orchis mascula L.	Orchidaceae	–
Origanum compactum Bentham; *O. dictamnus* L.; *O. majorana* L.; *O. vulgare* L.	Lamiaceae	Lukas et al. (2013), Theodoridis et al. (2012), Mattia et al. (2011)
Orthosiphon aristatus (Blume) Miq.	Lamiaceae	–
Oryza sativa L.	Poaceae	Cheng et al. (2003), Zeng et al. (2012)
Oxalis acetosella L.	Oxalidaceae	Newmaster et al. (2013)
Pachira aquatica Aubl.; *P. insignis* (SW.) Savigny	Malvaceae	–
Padus avium var. *avium*	Rosaceae	–
Paeonia lactiflora Pall.; *P. officinalis* L.; *P.* x *suffruticosa* Andrews	Paeoniaceae	Sun and Chen (2013)
Palmaria palmata (Linnaeus) Weber & Mohr	Palmariaceae	–
Panax ginseng C.A.Mey.; *P. notoginseng* (Burkill) F.H.Chen.; *P. pseudoginseng* Wall.; *P. quinquefolius* L.	Araliaceae	Chen et al. (2013a, b), Dong et al. (2014), Kim et al. (2015), Komatsu et al. (2005), Liu et al. (2016c), Song et al. (2015a, 2015b, 2015c), Wallace et al. (2012), Zhan et al. (2012), Zhu et al. (2003), Zuo et al. (2011), Song et al. (2012)
Panicum miliaceum L.	Poaceae	Zimmermann et al. (2013)
Panzerina lanata (L.) Bunge	Fabaceae	–
Papaver rhoeas L.	Papaveraceae	Zhang et al. (2015c), Carolan et al. (2006)
Parietaria officinalis L.	Urticaceae	–
Parmelia saxatilis (L.) Ach.	Parmeliaceae	Divakar et al. (2016)
Parthenium hysterophorus L.	Asteraceae	Kumar et al. (2009)
Parthenocissus tricuspidata (Siebold & Zucc.) Planch.	Vitaceae	Ingrouille et al. (2002), Wen et al. (2007)

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Table 9.2 (continued)

Botanical name	Family	References
Passiflora incarnata L.	Passifloraceae	Giudicelli and Mäder (2015), Muschner et al. (2003), Palhares et al. (2015)
Pastinaca sativa L.	Apiaceae	Logacheva et al. (2008)
Paullinia cupana Kunth	Sapindaceae	–
Pedalium murex L.	Pedaliaceae	–
Pelargonium graveolens L'Herit; *P. radens* H.E.Moore; *P. sidoides* DC:	Geraniaceae	–
Perilla frutescens (L.) Britton	Lamiaceae	Ito et al. (1998)
Persea americana Mill.	Lauraceae	–
Persicaria bistorta (L.) Samp.; *P. maculosa* Gray	Polygonaceae	–
Petiveria alliacea L.	Phytolaccaceae	–
Petroselinum crispum (Mill.) Nyman ex A.W.Hill	Apiaceae	Lohwasser et al. (2010)
Peucedanum ostruthium (L.) W.Koch	Apiaceae	Schmiderer et al. (2015)
Peumus boldus Molina	Monimiaceae	Palhares et al. (2015)
Phaseolus vulgaris L.	Fabaceae	Madesis et al. (2012b), Nicolè et al. (2011); Gupta et al. (2014), Kumar et al. (2014)
Phellodendron amurense Rupr.	Rutaceae	Zhang et al. (2016), Sun and Chen (2013)
Phillyrea latifolia L.	Oleaceae	–
Phlebodium aureum (L.) J.Sm.	Polypodiaceae	–
Phoenix dactylifera L.	Arecaceae	Ballardini et al. (2013), Naeem et al. (2014)
Photinia melanocarpa (Michx.) K.R.Robertson & J.B.Phipps	Rosaceae	–
Phyla scaberrima (Juss. ex Pers.) Moldenke	Verbenaceae	–
Phyllanthus amarus Schumach. & Thonn.; *P. emblica* L.; *P. niruri* L.	Phyllanthaceae	Srirama et al. (2014), Buddhachat et al. (2015)
Phymatolithon calcareum (Pallas) W.H.Adey & D.L.McKibbin	Hapalidiaceae	Hernández-Kantún et al. (2014), Peña et al. (2014), Pardo et al. (2014), Carro et al. (2014)
Physalis alkekengi L.; *P. peruviana* L.	Solanaceae	Feng et al. (2016)

Picea abies (L.) Karst.	Pinaceae	Bouillé et al. (2011), Ran et al. (2010)
Picramnia antidesma Sw.	Picramniaceae	–
Pimenta dioica (L.) Merr.; *P. racemosa* (Mill.) J.W.Moore	Myrtaceae	–
Pimpinella anisum L.; *P. major* (L.) Huds.; *P. saxifraga* L.	Apiaceae	Wang et al. (2014b), Valiejo-Roman et al. (2002)
Pinus koraiensis Siebold & Zucc.; *P. massoniana* Lamb.; *P. mugo* Turra; *P. pinaster* Ait.; *P. pinea* L.; *P. sylvestris* L.	Pinaceae	Ganopoulos et al. (2013a), Gernandt et al. (2005), Krupkin et al. (1996)
Piper aduncum L.; *P. longum* L.; *P. nigrum* L.	Piperaceae	Dhanya et al. (2009); Parvathy et al. (2014)
Pistacia lentiscus L.; *P. terebinthus* L.; *P. vera* L.	Anacardiaceae	Parfitt, Badenes (1998); Syouf et al. (2012); Yi et al. (2008)
Pisum sativum L.	Fabaceae	Schaefer et al. (2012)
Plantago afra L.; *P. arenaria* Waldst. & Kit.; *P. lanceolata* L.; *P. major* L.; *P. media* L.; *P. orbignyana* Steinh. ex Decne.; *P. ovata* Forssk.	Plantaginaceae	Ishikawa et al. (2009), Meyers and Liston (2008), Newmaster et al. (2013)
Platanus orientalis L.	Platanaceae	Grimm and Denk (2010)
Platycodon grandiflorus (Jacquin) A.DC.	Campanulaceae	–
Plectranthus barbatus Andrews	Lamiaceae	–
Pogostemon cablin (Blanco) Benth.	Lamiaceae	He et al. (2014c), Yao et al. (2016)
Polygala senega L.; *P. sibirica* L.; *P. tenuifolia* Willd.; *P. vulgaris* L.	Polygalaceae	–
Polygonatum odoratum (Mill.) Druce	Lilliaceae	–
Polygonum aviculare L.	Asparagaceae	–
Populus alba L.; *P. balsamifera* L.; *P. nigra* L.; *P. tremula* L.; *P. tremuloides* Michx.	Salicaceae	Feng et al. (2013), Liu et al. (2016b), Schroeder and Fladung (2015), Schroeder et al. (2012), Wang et al. (2015a)
Porphyra umbilicalis Kätzing	Bangiaceae	Broom et al. (2010), Kucera and Saunders (2012)
Portulaca oleracea L.	Portulacaceae	Newmaster et al. (2013)
Potentilla anserina L.; *P. argentea* L.; *P. erecta* (L.) Raeusch.; *P. reptans* L.	Rosaceae	Paule et al. (2011), Saarela et al. (2013)

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Table 9.2 (continued)

Botanical name	Family	References
Prangos pabularia Lindl.	Apiaceae	Valiejo-Roman et al. (2006)
Primula elatior Hill; *P. veris* L.	Primulaceae	–
Prunella vulgaris L.	Lamiaceae	–
Prunus africana (Hook. f.) Kalkman; *P. amygdalus* Batsch.; *P. armeniaca* L.; *P. avium* (L.) L.; *P. cerasus* L.; *P. domestica* L.; *P. dulcis* (Mill.) D.A.Webb; *P. persica* (L.) Stokes; *P. serotine* Erh.; *P. spinosa* L.	Rosaceae	Distefano et al. (2015a), Quan and Zhou (2011), Sarhan et al. (2015)
Psidium guajava L.; *P. guineense* Sw.	Myrtaceae	Tuler et al. (2015)
Pterocarpus erinaceus Poir.; *P. indicus* Willd.; *P. marsupium* Roxb.; *P. officinalis* Jacq.; *P. santalinus* L. f.	Fabaceae	Saslis-Lagoudakis et al. (2008)
Pueraria montana var. *lobata* (Willd.) Sanjappa & Pradeep; *P. tuberosa* (Willd.) DC.	Fabaceae	Egan and Pan (2015), Egan et al. (2016), Zeng et al. (2003)
Pulicaria dysenterica (L.) Gaertn.	Asteraceae	Englund et al. (2009)
Pulmonaria officinalis L.	Boraginaceae	–
Punica granatum L.	Lythraceae	–
Pyrola rotundifolia L.	Ericaceae	–
Pyrus communis L.	Rosaceae	Faria et al. (2013)
Quercus alba L.; *Q. coccifera* L.; *Q. ilex* L.; *Q. infectoria* G.Olivier; *Q. petraea* (Matt.) Liebl.; *Q. pubescens* Willd.; *Q. robur* L.; *Q. serrata* subsp. *serrata*; *Q. suber* L.	Fagaceae	Bruni et al. (2012), Ferri et al. (2009), Hubert et al. (2014), Piredda et al. (2011), Rellstab et al. (2016), Schroeder et al. (2016), Simeone et al. (2016), Simeone et al. (2013), Yang et al. (2016), Hawkins et al. (2015), Bruni et al. (2015)
Quillaja saponaria Molina	Quillajaceae	–
Raphanus raphanistrum subsp. *sativus* (L.) Domin; *R. sativus* L.	Brassicaceae	Pipan et al. (2013), Prieto et al. (2005), Qi and Zhang (2012), Thalmann et al. (2001), Wang et al. (2007), Warwick and Black (1991, 1997), Yamagishi and Terachi (2003), Yamane et al. (2005), Yang et al. (1998)

Raphia farinifera (Gaertn.) Hyl.	Arecaceae	–
Rehmannia glutinosa (Gaertn.) DC.	Plantaginaceae	Xia et al. (2016), Huang et al. (2016)
Reynoutria multiflora (Thunb.) Moldenke	Polygonaceae	–
Rhamnus alpina L.; *R. cathartica* L.	Rhamnaceae	Culley and Stewart (2010)
Rheum australe D.Don; *R.* x *hybridum* Murray; *R. officinale* Baill.; *R. palmatum* L.; *R. rhabarbarum* L.; *R. rhaponticum* L.	Polygonaceae	Kersten et al. (2008), Wang et al. (2012), Yang et al. (2004), Zhang et al. (2013b), Zhou et al. (2014b)
Rhodiola crenulata (Hook. f. & Thomson) Ohba	Crassulaceae	Deng et al. (2007), Xin et al. (2015a)
Ribes nigrum L.; *R. rubrum* L.; *R. uva-crispa* L.	Grossulariaceae	Lanham and Brennan (1999), Palmieri et al. (2013), Stanys et al. (2004)
Robinia pseudoacacia L.	Fabaceae	Chen and Filippis (1996), Liesebach and Schneck (2012), Wang et al. (2015c)
Roccella phycopsis Ach.	Rocellaceae	–
Rosa canina L.; *R.* x *centifolia* L. (pro sp.); *R. gallica* L.; *R. moschata* Mill.; *R. rubiginosa* L.; *R.* x *damascene* Mill.	Rosaceae	Bashir et al. (2014), Gardes et al. (2005), Iwata et al. (2000), Riaz et al. (2012), Ritz and Wissemann (2011), Takeuchi et al. (2000), Wu et al. (2000, 2001)
Rosmarinus officinalis L.	Lamiaceae	Ince et al. (2009), Bruni et al. (2015), Mattia et al. (2011)
Rubia cordifolia L.	Rubiaceae	–
Rubus caesius L.; *R. chingii* var. *suavissimus* (S. Lee) L.T.Lu; *R. fruticosus* L.; *R. idaeus* L.; *R. rosa* L.H.Bailey	Rosaceae	Alice et al. (2001), Bushakra et al. (2015), Castillo et al. (2010), Dossett et al. (2015), Girichev et al. (2015), Imanishi et al. (2008), Lewers et al. (2005), Potter et al. (2007), Rousseau-Gueutin et al. (2011), Stafne et al. (2005), Zorrilla-Fontanesi et al. (2011), Hawkins et al. (2015)

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Table 9.2 (continued)

Botanical name	Family	References
Rumex acetosa L.; *R. acetosella* L.; *R. alpinus* L.; *R. conglomeratus* Murray; *R. crispus* L.; *R. longifolius* DC.; *R. obtusifolius* L.; *R. patientia* L.; *R. sanguineus* L.	Polygonaceae	–
Ruscus aculeatus L.; *R. hypoglossum* L.	Asparagaceae	–
Sabatia angularis (L.) Pursh	Gentiananceae	–
Saccharina latissima (L.) C.E.Lane, C.Mayes, L.D.Druehl & G.W.Saunders	Laminariaceae	McDevit and Saunders (2010)
Saccharum officinarum L.	Poaceae	Asano et al. (2004), Chandra et al. (2013), Evans and Joshi (2016), Melotto-Passarin et al. (2011), Nair et al. (2005), Takahashi et al. (2005), Welker et al. (2015)
Salix alba L.; *S. caprea* L.; *S. fragilis* L.; *S. pentandra* L.; *S. purpurea* L.	Salicaceae	Hamza-Babiker et al. (2009), King et al. (2010), Liesebach et al. (2010), Lin et al. (2009), Oberprieler et al. (2013), Sulima and Przyborowski (2013), Triest et al. (2009)
Salvia miltiorrhiza Bunge; *S. officinalis* L.; *S. pratensis* L.; *S. sclarea* L.	Lamiaceae	Karaca et al. (2013), Niu et al. (2013), Olarte et al. (2013), Przyborowski et al. (2013), RadosavljeviĆ et al. (2012), Walker et al. (2004), Xu et al. (2009), Wang et al. (2013b)
Sambucus nigra L.	Adoxaceae	Marieschi et al. (2016)
Sanguisorba minor Scop.; *S. officinalis* L.	Rosaceae	–
Sanicula elata Buch.-Ham. ex D.Don	Apiaceae	–
Santalum album L.	Santalaceae	Dev et al. (2014)
Santolina chamaecyparissus L.	Asteraceae	–
Saponaria officinalis L.	Caryophyllaceae	–
Saposhnikovia divaricata (Turcz.) Schischk	Apiaceae	–
Sarcopoterium spinosum (L.) Spach	Rosaceae	–
Sargassum fusiforme (Harvey) Setchell	Sargassaceae	Liu et al. (2016a)

Sarracenia purpurea L.	Sarraceniaceae	Bayer et al. (1996), Stephens et al. (2015)
Satureja hortensis L.; *S. montana* L.; *S. thymbra* L.	Lamiaceae	Bräuchler et al. (2010)
Saussurea costus (Falc.) Lipsch.	Asteraceae	–
Schisandra chinensis (Turcz.) Baill.	Schisandraceae	Kim et al. (2012), Li et al. (2014)
Scorzonera hispanica L.	Asteraceae	–
Scrophularia ningpoensis Helmsl.	Scrophulariaceae	Chen et al. (2011)
Scutellaria baicalensis Georgi; *S. lateriflora* L.	Lamiaceae	Liu et al. (2016b), Xia et al. (2014)
Secale cereale L.	Poaceae	Bustos and Jouve (2002), Shang et al. (2006), Skuza et al. (2015)
Sedum acre L.; *S. album* L.; *S. roseum* (L.) Scop.	Crassulaceae	–
Selenicereus grandiflorus (L.) Britton & Rose	Cactaceae	–
Sempervivum tectorum L.	Crassulaceae	–
Senna alexandrina Mill.; *S. italica* Mill.; *S. obtusifolia* (L.) H.S.Irwin & Barneby; *S. occidentalis* (L.) Link; *S. tora* (L.) Roxb.	Fabaceae	Ghareeb et al. (1999), Monkheang et al. (2011), Seethapathy et al. (2015)
Sequoiadendron giganteum (Lindl.) J.Buchholz	Taxodiaceae	–
Serenoa repens (W.Bartram) Small	Arecaceae	–
Sesamum indicum L.	Pedaliaceae	Bedigian (2015), Brzezinski (2007), Ehlert et al. (2009), Ehlert et al. (2007), Hupfer et al. (2009), Köppel et al. (2009), Mustorp et al. (2008), Schöringhumer and Cichna-Markl (2007), Schöringhumer et al. (2009), Spaniolas et al. (2008, 2010), Uncu et al. (2015), Yamada et al. (1993), Madesis et al. (2013)
Seseli tortuosum L.	Apiaceae	–
Sideritis syriaca L.	Lamiaceae	Kalivas et al. (2014)
Sigesbeckia orientalis L.	Asteraceae	–
Silaum silaus (L.) Shinz & Thell.	Apiaceae	–

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Table 9.2 (continued)

Botanical name	Family	References
Silybum marianum (L.) Gaertn.	Asteraceae	–
Simmondsia chinensis (Link) C.K. Schneid.	Simmondsiaceae	–
Sisymbrium officinale (L) Scop.	Brassicaceae	–
Smilax aristolochiifolia Mill.; *S. aspera* L.; *S. china* L.; *S. cordifolia* Humb. & Bonpl. ex Willd.; *S. glabra* Roxb.; *S. officinalis* Kunth; *S. purhampuy* Ruiz; *R. regelii* Killip & C.V.Morton	Smilacaceae	Wang et al. (2014a), Qi et al. (2013); Kool et al. (2012)
Solanum melongena L.; *S. tuberosum* L.	Solanaceae	Ahmadvand et al. (2014), Reddy et al. (2015), Sakata and Lester (1997)
Solidago virgaurea L.	Asteraceae	–
Sorbus aucuparia L.	Rosaceae	Chester et al. (2007), Dłuzewska et al. (2013)
Sorghum bicolor (L.) Moench	Poaceae	Zhang et al. (2013a), Tavoletti et al. (2009), Ng'Uni et al. (2010), Liu et al. (2014)
Spatholobus suberectus Dunn.	Fabaceae	–
Spergularia rubra (L.) J.Presl & C.Presl.	Caryophyllaceae	–
Spinacia oleracea L.	Amaranthaceae	–
Spirulina major Kätzing ex Gomont; *S. maxima* (Setchell & Gardner) Geitler; *S. platensis* (Gomont) Geitler	Pseudanabaenaceae	–
Stachys officinalis (L.) Trevis.; *S. recta* L.; *S. sylvatica* L.	Lamiaceae	–
Stellaria media (L.) Vill.	Caryophyllaceae	Zhao et al. (2009)
Stemmacantha carthamoides (Willd.) Dittrich	Asteraceae	–
Styphnolobium japonicum (L.) Schott	Fabaceae	–
Symplocos racemosa Roxb.	Symplocaceae	–
Syringa vulgaris L.	Oleaceae	–
Syzygium aromaticum (L.) Merr. & L.M.Perry; *S. cumini* (L.) Skeels; *S. jambos* (L.) Alston; *S. malaccense* (L.) Merr. & L.M.Perry	Myrtaceae	Nogueira et al. (2016), Rai et al. (2013)
Tagetes erecta L.; *T. minuta* L.	Asteraceae	–
Tamarindus indica L.	Fabaceae	–

Tamarix gallica L.	Tamaricaceae	Terzoli et al. (2014)
Tanacetum balsamita L.; *T. parthenium* (L.) Sch. Bip.; *T. vulgare* L.	Asteraceae	–
Taraxacum officinale Web.	Asteraceae	Wallinger et al. (2012), Hawkins et al. (2015)
Terminalia bellerica (Gaertn.) Roxb.; *T. chebula* Retz.	Combretaceae	–
Thalictrum flavum L.	Ranunculaceae	–
Theobroma cacao L.	Malvaceae	Haase and Fischer (2007), Kane et al. (2012)
Thlaspi arvense L.	Brassicaceae	–
Thymus satureioides Coss.; *T. serpyllum* L.; *T. vulgaris* L.; *T. zygis* L.	Lamiaceae	–
Tilia americana L.; *T. cordata* Mill.; *T. platyphyllos* Scop.; *T. tomentosa* Moench; *T.* x *europaea* L.	Malvaceae	–
Tinospora sinensis (Lour.) Merr.	Menispermaceae	–
Trachyspermum ammi (L.) Sprague	Apiaceae	–
Tragopogon porrifolius L.	Asteraceae	–
Tribulus terrestris L.	Zygophyllaceae	Balasubramani et al. (2010)
Trichilia catigua A.Juss.	Meliaceae	–
Trichosanthes kirilowii Maxim.	Cucurbitaceae	Cui et al. (2006, 2007)
Tridax procumbens L.	Asteraceae	–
Trifolium arvense L.; *T. campestre* Schreb.; *T. pratense* L.; *T. repens* L.	Fabaceae	LeRoy et al. (2002)
Trigonella caerulea (L.) Ser.; *T. foenum-graecum* L.	Fabaceae	Dangi et al. (2016), Steele et al. (2010)
Trillium erectum L.	Melanthiaceae	Kazempour Osaloo et al. (1999)
Triticum aestivum L.; *T. aestivum* L.; *T. dicoccon* (Schrank.) Schäbl.; *T. durum* Desf.; *T. spelta* L.; *T. turgidum* L.	Poaceae	Kikkawa et al. (2015)
Tropaeolum majus L.; *T. minus* L.	Tropaeolaceae	–
Tsuga canadensis (L.) Carrière	Pinaceae	Havill et al. (2008), Wang et al. (1997)
Turnera diffusa Wild. ex Schult.	Passifloraceae	–

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Table 9.2 (continued)

Botanical name	Family	References
Ulmus glabra Huds.; *U. pumila* L.; *U. rubra* Muhl.	Ulmaceae	Collada et al. (2004), Cox et al. (2014), Goodall-Copestake et al. (2005), Whiteley et al. (2003), Brunet et al. (2013), Zalapa et al. (2008, 2009)
Ulva lactuca L.	Ulvaceae	Loughnane et al. (2008), O'Kelly et al. (2010), Shimada et al. (2003)
Uncaria gambir (Hunter) Roxb.; *U. rhynchophylla* (Miq.) Miq. ex Havil.; *U. tomentosa* (Willd. ex Schult.) DC.	Rubiaceae	–
Undaria pinnatifida (Harvey) Suringar	Alariaceae	Muraoka and Saitoh (2005)
Urtica dioica L.; *U. urens* L.	Urticaceae	–
Usnea barbata (L.) Weber ex F.H.Wigg.; *U. longissimi* Ach.; *U. plicata* Wiggers	Parmeliaceae	–
Vaccinium myrtilloides Michx.; *V. corymbosum* L.; *V. macrocarpon* Aiton; *V. myrtillus* L.; *V. oxycoccos* L.; *V. uliginosum* L.; *V. vitis-idaea* L.	Ericaceae	An et al. (2015), Bassil (2012), Bassil et al. (2010), Bell et al. (2008), Bian et al. (2014), Boches et al. (2005), Jaakola et al. (2010), Polashock and Vorsa (2002), Schlautman et al. (2015), Suda and Lysák (2001)
Valeriana jatamansi Jones; *V. officinalis* L.	Valerianaceae	Diao et al. (2010), Ruzicka et al. (2016), Slanc et al. (2006), Takeuchi et al. (2001), Yang et al. (2011)
Valerianella locusta (L.) Laterr.	Caprifoliaceae	–
Vanilla planifolia Jacks. ex Andrews	Orchidaceae	–
Verbascum densiflorum Bertol.; *V. phlomoides* L.; *V. thapsus* L.	Scrophulariaceae	Srivastava and Saggoo (2014)
Verbena officinalis L.	Verbenaceae	Ruzicka et al. (2009)
Veronica anagallis-aquatica L.; *V. beccabunga* L.; *V. chamaedrys* L.; *V. officinalis* L.	Plantaginaceae	–
Viburnum lantana L.; *V. opulus* L.	Adoxaceae	Clement et al. (2014), Winkworth and Donoghue (2004, 2005)

Vicia faba L.	Fabaceae	Shiran et al. (2014), Madesis et al. (2012b), Tavoletti et al. (2009)
Vigna angularis (Willd.) Ohwi & H.Ohashi	Fabaceae	Goel et al. (2002)
Viola calcarata L.; *V. odorata* L.; *V. palustris* L.; *V. tricolor* L.	Violaceae	Yockteng et al. (2003)
Viscum album L.	Santalaceae	Kwanda et al. (2013)
Vitex agnus-castus L.; *V. trifolia* L.	Lamiaceae	–
Vitis vinifera L.	Vitaceae	–
Withania somnifera (L.) Dunal	Solanaceae	Negi et al. (2000)
Xeranthemum annuum L.	Asteraceae	–
Yucca filamentosa L.; *Y. schidigera* Roezl ex Ortgies	Asaparagaceae	–
Zea mays L.	Poaceae	Buckler IV and Holtsford (1996), Fang et al. (2012), Gaut (1996), McIntosh et al. (2005), Hilton and Gaut (1998), White and Doebley (1998), Orton et al. (2016), Wallinger et al. (2012)
Zingiber officinale Rosc.	Zingiberaceae	Chavan et al. (2008), Ghosh et al. (2011), Theerakulpisut et al. (2012)
Ziziphus jujuba Mill.	Rhamnaceae	Huang et al. (2015a)

Glossary

AFLP (amplified fragment length polymorphism) combines RFLP and PCR-based technology to overcome the major disadvantage of RAPD (reproducibility). Adaptors (short DNA primer recognition sites) are ligated to DNA digested with restriction endonucleases and selectively amplified with a limited primer set.

ARMS (amplification refractory mutation system, synonyme: allele-specific PCR (AS-PCR)) is a PCR based on the fact that a mismatch at the 3′ primer end may lead to failure in PCR. If the primer is developed in a way that the specific DNA difference between two species is located at the 3′ end, a PCR product is formed only for one of the species. For a DNA identification system, it is advantageous to develop also a second assay for the complementary allele.

DNA Barcoding is a PCR technique combined with classical Sanger sequencing that uses conserved primers which should ideally result in amplification products with all species and which are spanning a region of enough DNA sequence variability to distinguish species.

DNA Metabarcoding is a technique combining the classical taxonomic DNA sequencing approach using conservative primers (see above) with next-generation sequencing technologies. With this technique, it became possible to study the composition of communities in one analysis.

DNA Mini-Barcoding corresponds in its principles to normal DNA barcoding. In this case, to overcome DNA degradation for processed samples, only a short fragment of the barcode region is amplified. Primers used in this approach are normally not so 'universal' and prior sequence knowledge is required for their development.

Indels (a combination of the terms insertions and deletions) describe length variations of DNA. Additionally inserted nucleotides are called insertion, the lack of nucleotides is called a deletion.

ISSR (intersimple sequence repeat) uses primers containing simple sequence repeats (SSR) amplifying closely neighbouring SSRs (from genomic 'SSR hotspots').

HRM (high resolution melting analysis) is a melting curve analysis of an amplicon produced in a prior real-time PCR with a DNA-intercalating fluorescence dye. After PCR, temperature is slowly increased. The decreasing fluorescence (due to DNA melting) is measured and results in sequence specific temperature profiles.

RAPD (random amplified polymorphic DNA) uses short primers (ten bases long) that bind randomly on the genome. Where two binding sites are close enough on the different DNA strands (within about 1–2 kb), the DNA polymerase is able to amplify this region. This results in typical banding patterns of the samples due to rearrangements or indels in the primer binding sites or within the amplicon.

RFLP (restriction fragment length polymorphism, not PCR based) uses restriction endonucleases to cut (digest) the DNA into short pieces. Differences between two samples are detected after separation of the DNA, usually by chemically

labeled DNA probes. As DNA is not pre-amplified, high amounts of DNA are required for a whole genome analysis.

SCAR (sequence characterized amplified regions) is a technique to convert polymorphisms from RAPD, ISSR, AFLP or other techniques into reproducible simple PCR reactions by (cloning and) sequencing the polymorphism and developing standard PCR primers differentiating the polymorphic site.

SNPs (single nucleotide polymorphism) are variations in single nucleotides. They are the most frequently occurring DNA polymorphisms. With the advances in sequencing technologies and the abundance of available sequence information SNPs became the most important markers.

SSR (simple sequences repeats, synonyms: short tandem repeats, microsatellites) are monotonous repetitions of very short DNA motifs that are very frequently interspersed in DNA. The reason for the variation in repeat number is strand slippage during DNA replication. Primers flanking those highly variable regions are normally used in a conventional PCR for the evaluation.

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Chapter 10
Profiling of Botanical Extracts for Authentication, Detection of Adulteration and Quality Control Using UPLC-QTOF-MS

Paul A. Steenkamp, Lucia H. Steenkamp, and Dalu T. Mancama

Abstract Herbs and plant extracts are very popular and are being used by all cultures for healthcare and as food supplements. The correct identification of the required plant material is vital for formulation of final formulas for use by mankind. The use of instruments such as UPLC-QTOF-MS can be advantageous in the identification and authentication of plant extracts and formulated products to ensure the safe use of these products. Furthermore, it is also imperative that the extracts are not contaminated with other chemicals or pesticides as this can be detrimental to humans during the consumption of these products. Organic extracts of six example plants were made as part of the PlantLIBRA collaboration. The development of UPLC-QTOF-MS profiling methods showed that separation of the major compounds found in the extracts was possible and allowed for high resolution mass spectral evaluation of the compounds detected. By using reference standards and published literature, the presence of active or marker compounds could be confirmed in the different plant extracts. The high mass accuracy of the TOF data also allowed for the tentative identification of extra compounds as well as the ability to differentiate between different formulations. Establishing the correct chemical profile of a plant before use as a food supplement or herbal formulation, would ensure the use of the correct plant species, but also detect any new compounds or contaminants such as pesticides or toxins which may be identified using UPLC-QTOF-MS technologies.

Keywords *Cinnamomum verum* • *Valeriana officinalis* • *Boswellia serrata* • *Passiflora incarnata* • *Peumus boldus* • *Harpagophytum procumbens* • Food supplements • UPLC-QTOF-MS

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10.1 Introduction

Historically the authentication of plant material was done by a skilled botanist, who specialised in the identification and characterisation of plants and collected plant specimens. The development of DNA fingerprinting added another level of certainty to the identification process, but did not highlight the chemical components of a specific botanical sample. The chemical complexity of plant material can be studied with a variety of instrumental techniques. The most prominent techniques include spectrometry and spectroscopy technologies. Both these techniques can be applied to solid plant material but usually require the preparation of a representative extract of the plant material to allow for the more general application of these analytical techniques. All of these techniques will aid in the authentication of plant material but suffer the same fundamental flaw—the inability to separate the chemical compounds prior to detection, characterization and identification.

The development of chromatography in all its forms allowed for the separation of compounds in solution for isolation, purification and characterization applications. Gas chromatography and liquid chromatography allowed for the separation of volatile and non-volatile compounds respectively, but still required a suitable detector for visualization. The rapid development of hyphenated/hybrid instrumentation offered the best of both techniques—chromatographic separation techniques combined with a choice of detectors. The use of liquid chromatography coupled to mass spectrometry has been successfully applied in chemical profiling (Kamatou et al. 2005; Nyiligira et al. 2008; Van Heerden et al. 2003), as well as forensic investigations (Steenkamp et al. 2002, 2004, 2006; Stoev et al. 2010). The recent developments in high performance liquid chromatography resulted in the development of ultra-high performance liquid chromatography (UPLC/UHPLC). Mass spectrometry developed in parallel take advantage of the superior resolution, speed and sensitivity offered by UPLC/UHPLC technologies. A recent thesis submitted by Almalki (2015) highlighted the importance of combining good chromatography with a time-of-flight (TOF) mass spectrometer to profile and characterise a traditional Chinese medicine YANG XIN® formulation.

Chemical profiling of raw materials used in the formulation of food supplements and herbal remedies has recently become a necessity to ensure quality and to authenticate raw materials prior to formulation.

Six plants were used in the study performed by CSIR-Biosciences. Different parts of the plants were obtained through the PlantLIBRA collaboration. All the plants are used as traditional herbal medicines or ingredients of food supplements. Identification or authentication of the correct plant and plant parts are therefore crucial to avoid exposure of consumers to incorrect formulations, which may result in severe side-effects or may even be fatal. Furthermore, detection of contamination of the plant material, with for instance pesticides, will also be possible once the chemical profile of the plant extracts has been elucidated and will be detected as new compounds. Using the advantage of the separation power of UPLC as well accurate mass determination, these new compounds can be identified and formulators

alerted of potential risks. The six plants used in the study (and described below) were: *Harpagophytum procumbens, Peumus boldus, Passiflora incarnata, Boswellia serrata, Valeriana officinalis* and *Cinnamomum verum.*

10.1.1 Harpagophytum procumbens

This plant is also referred to as Devil's Claw, grapple plant or wood spider. The plant is native to Southern Africa and more specifically in the eastern and south eastern parts of Namibia, Southern Botswana and the Kalahari region of the Northern Cape (Raimondo and Donaldson 2002).

The potential uses come from the secondary tuberous roots, which are harvested, sliced and dried before use (Wegener 2000). The dried secondary tubers of the plant contain iridoid glycosides, particularly harpagoside (*trans-cinnamoyl harpagide*) including small amounts of *trans-cinnamoyl harpagide*, procumbide and plant sterols, which are thought to have anti-inflammatory effects (Wichtl 2004). The plant extracts also have analgesic, sedative and diuretic properties as recognised by the British Herbal Pharmacopoeia and the European Pharmacopoeia recognises the herb as a component of a number of dietary supplements and over the counter preparations for use as an anti-rheumatic.

The plant has found applications such as being used as an analgesic, treating complications relating to pregnancy and using the plant in the form of an ointment to treat skin disorders by the Khoisan people in the Kalahari Desert (Wegener 2000). For gastrointestinal disturbances such as dyspepsia, the bitter-tasting part of the plant is used (Mills and Bone 2000). In the form of an infusion it can be used to treat blood disorders and as an anti-inflammatory and analgesic it can be used to treat post-partum pain, as well as pain associated with dysmenorrhoea, rheumatism and general musculoskeletal pains (Tyler 1993; Gagnier et al. 2004; Tippler et al. 1996; Loew et al. 2001). As an ointment it can be applied topically for sprains, sores, ulcers and boils (Mills and Bone 2000).

10.1.2 Peumus boldus

Peumus boldus is the only species in the genus Peumus and the tree is endemic to the central region of Chile (Heusser 1971). In South America the dried leaves of the plant have been used since the nineteenth century against diseases of the liver and gallstones (Lanhers et al. 1991). In different pharmacognostical articles and pharmacopoeias the therapeutic uses of the plant are listed as cholagogue as it causes the discharge of bile from the system, and choleretic as it causes increased bile production from the liver. It is also used for digestive disturbances, and its effects as a diuretic, hepatic stimulant, stomachic, anti-dyspeptic, sedative and antihelmintic (Speisky and Cassels 1994; Lanhers et al. 1991; Genest and Hughes 1968; Magistretti 1980). The leaves of the

plant have also been reported to be used for the treatment of headache, toothache, rheumatism and urinary tract inflammation (Speisky and Cassels 1994). Boldine seems to be an antioxidant and may protect the liver from toxins (Speisky and Cassels 1994; Jimenez and Speisky 2000). According to an article by O'Brien et al. (2006), research in the 1990s led to the discovery that boldine is one of the most potent natural antioxidants. According to Quezada et al. (2004) the antioxidant activity of boldo leaf extracts came mainly from the flavonoid fraction (44.1%) followed by the alkaloid fraction (15.6%), with catechin and boldine being the main contributors of the antioxidant activity of these two fractions (60.9% and 35.6% respectively) of the total activity. Besides the positive antioxidant properties contained in the plant, there is a huge concern about the suitability of the boldo leaves in traditional herbal medicinal products due to the presence of ascaridole, which is highly toxic. Furthermore, abortifacient and teratogenic effects have been observed in rats when fed high doses of a dry ethanolic extract and boldine. Boldo oil will contain ascaridole and should therefore not be used internally or externally. Because ascaridole has a very low solubility in water, aqueous extracts may be acceptable in products such as herbal teas. An ethanolic extract of boldo leaves will however contain potentially toxic levels of the ascaridole (HMPC 2009).

10.1.3 Passiflora incarnata

Passiflora incarnata is also referred to as maypop, true passionflower and wild apricot and is part of the Passifloraceae family. The plant is traditionally used in the fresh or dried form to treat nervous anxiety and insomnia in the form of a traditional European remedy (Handler 1962) or as a homeopathic formulation (Rawat 1987). In a report from 1787, Materia Medica Americana, it was highlighted that the plant can be used to treat epilepsy in the aged (Dhawan et al. 2004). Reports by Bartram (1995) and CSIR (1966a, b) indicated that the plant has properties which can be used in the treatment of spasmodic disorders and insomnia in the young and aged.

The FDA has listed *Passiflora incarnata* as a "safe herbal sedative" (HerbClip 1996). Due to the fact that this species of plant contains some cyanogenic compounds, Dhawan et al. (2004) stated that toxicity cannot be ruled out. Furthermore, *Passiflora incarnata* should be taken with caution along with other central nervous system depressants or stimulants (Felter and Lloyd 1983).

10.1.4 Boswellia serrata

Boswellia serrata is also known as Indian frankincense tree, Indian olibanum tree or salai in Hindi. The main product from the Boswellia tree is an aromatic resin called frankincense or olibanum. The bark of the tree is peeled and the sap collected to obtain the gum-like oleoresin in the form of hardened yellowish lumps [Steadyhealth.com].

A number of chronic inflammatory diseases such as rheumatoid arthritis, bronchial asthma, osteoarthritis, ulcerative colitis and Crohn's disease have been treated with the extracts of the plant and have reported positive results (Ammon 2006). A number of pharmacokinetic studies have been done on WokVel®, an extract from *Boswellia* (Sontakke et al. 2007; Kimmatkar et al. 2003; Sharma et al. 2004). Adbel-Tawab et al. (2011) indicated that the *Boswellia serrata* extract may promise to be an alternative to NSAIDs and should be tested further in formal pharmacological studies and clinical trials.

The extract of *Boswellia serrata* has been used in the treatment of experimental primary and secondary brain tumors to test the anti-neoplastic activity in vitro (Pang et al. 2009; Liu and Duan 2009) and in limited clinical research (Flavin 2007). Likewise Yadav et al. (2011) performed studies on the effect of Boswellia extract on human colorectal cancer, establishing that it inhibits growth of the cancer and also that it reduced metastasis. Researchers such as Lalithakumari et al. (2006) and Sengupta et al. (2008) reported that no adverse or toxic effects were observed with 5-Loxin which is a *Boswellia serrata* extract enriched with 3-O-acetyl-11-keto-beta-boswellic acid (AKBA) during double-blind studies on the anti-inflammatory properties of the gum resin.

10.1.5 Valeriana officinalis

The genus, Valeriana, contains over 250 species and a large number of subspecies, but it is specifically *Valeriana officinalis* L. which is used as an herbal treatment in Europe and other parts of the world (Circosta et al. 2007). The plant contains more than 150 chemical compounds (Jiang et al. 2007) from different compound classes.

The underground parts of the plant, namely the roots and rhizomes, contain two main groups of compounds, (1) the sesquiterpenes which include valerenic acid and its derivatives, valeranone, valeranal and kessyl esters and (2) the valepotriates which include valtrate, didrovaltrate, acevaltrate and isovaleroxyhydroxyvaltrate. Other compounds in the plant are flavonoids, triterpenes, lignans and alkaloids (Goppel and Franz 2004).

The roots of the Valerian plant are used in herbal medicine and food supplements. Valerenic acid and its derivatives (Stoll and Seebeck 1957) and the valepotriates (Thies 1966) are considered to be the most important compounds responsible for the sedative effect of the plant (Von Eikstedt and Rahman 1969; Hendriks et al. (1981, 1985). The roots of the plant are used as a natural or alternative treatment in medical conditions where benzodiazepines are normally used. The medical conditions therefore which find advantages from Valerian are nervous tension, excitability, stress, intestinal colic and cramps (National Institutes of Health 2007; Hadley and Petry 2003; Schmitz and Jäckel 1998).

According to Bos et al. (2002), the valepotriates have been investigated for their pharmacological action, but they are unstable and are often not present in commercial preparations. Based on the work by Bisset (1994) and Houghton (1999), the extracts

made from water alone or with alcohol tend to be sleep inducing and a sedative, while the extracts made with dilute alcohol have high levels of valepotriates which has a tranquilizing effect and reduces anxiety without causing drowsiness.

As reported by at least three articles, the valepotriates act as prodrugs and they are converted to homobaldrinal which has spasmolytic activity (Wagner et al. 1980; Schneider and Willems 1982; Veith et al. 1986). The two valepotriates, valtrate and didrovaltrate, were the first of the valerian compounds shown to have antispasmodic effects (Wagner and Jurcic 1979). Their effect is probably due to their influence on the influx of Ca^{2+} or their binding to the muscle.

Baldrinal and homobaldrinal are breakdown products from valtrate and isovaltrate respectively (Veith et al. 1986). These two breakdown products have been found to be mutagenic as well as having direct genotoxic effects (Von der Hude et al. 1986).

10.1.6 Cinnamomum verum (zeylanicum)

Cinnamon refers to a spice found in the inner bark of approximately a dozen species of trees from the genus Cinnamomum in the family Lauraceae. The species *Cinnamomum verum* (also classified as *Cinnamomum zeylanicum*) is the only species cultivated in Sri Lanka, which is the island to which this species is native [Wikipedia/Cinnamon].

Cinnamon has been reported to be remarkable in the treatment of Type II diabetes and insulin resistance (Khan et al. 2003; Verspohl et al. 2005; Couturier et al. 2010). Couturier et al. (2010) found that cinnamon alters body composition in association with improved insulin sensitivity in rats fed on a high fat/high fructose diet. Taher et al. (2006) reported that it is the compound cinnamtannin B1 isolated from *Cinnamomum zeylanicum,* which causes the therapeutic effect on Type II diabetes with the exception of postmenopausal patients studied on *Cinnamomum cassia* (Vanschoonbeek et al. 2006). Other traditional uses of cinnamon include the treatment of toothache, fighting bad breath, aiding in digestion and stave off the common cold (Hart-Davis 2007). The compounds—cinnamaldehyde, cinnamyl acetate, eugenol and anethole which are present in cinnamon leaf oil have been found to very effective in killing mosquito larvae (ScienceDaily 2004). Ranjbar et al. (2007) reported that the bark of *Cinnamomum zeylanicum* contains significant antioxidant potential and could therefore benefit humans by protecting them from oxidative stress which could lead to illness.

10.2 Materials and Methods

All chemicals including reference standards for UPLC-MS work were of ultra-pure LC-MS grade and purchased from Fluka (Steinheim, Germany) while ultra-pure solvents were purchase from Honeywell (Burdick & Jackson, Muskegon, USA).

Ultra-pure water was generated from a Millipore Elix 5 RO system and Millipore Advantage Milli-Q system (Millipore SAS, Molsheim, France). Plant material was sourced from the ECD unit of the CSIR (Dr. Marthinus Horak) locally while the PlantLIBRA plant material was supplied by Dr. Franz Chlodwig (PlantLIBRA).

10.2.1 Instrumentation and Analysis

For all the UPLC analysis the following instrument set-up was used: a Waters UPLC coupled in tandem to a Waters photodiode array (PDA) detector and a SYNAPT G1 HDMS mass spectrometer was used to generate accurate mass data. Two analytical procedures were used to analyse the samples. Up to four column chemistries were evaluated, namely the Waters CSH C18, HSS T3 HSS C18 UPLC columns, CSH Phenyl-hexyl and BEH C8. All the columns were 150 mm × 2.1 mm × 1.7/8 μm in dimension and column temperature controlled at 60 °C. The specific conditions used for each of the six plants are described separately below.

The SYNAPT G1 mass spectrometer was used in V-optics and operated in electrospray mode to enable detection of alkaloids and flavonoids. Leucine enkephalin solution (50 pg/mL in (1:1) water:acetonitrile) was used as reference calibrant to obtain typical mass accuracies between 3 and 5 mDa. The mass spectrometer was operated in positive and negative modes with a capillary voltage of 2.0 kV, the sampling cone at 30 V and the extraction cone at 4 V. The scan time was 0.1 s covering the 100–1000 Da mass range. The source temperature was 120 °C and the desolvation temperature was set at 400 °C. Nitrogen gas was used as the nebulisation gas at a flow rate of 800 L/h. The software used to control the hyphenated system and to perform all data manipulation was MassLynx 4.1 (SCN 704).

Waters MarkerLynx XS (SCN 678) software was used to evaluate similarities and/or differences between plant extracts and to visualise the similarities/differences in the chemical profile of plant material obtained from different sources.

10.2.2 Extraction and Chromatographic Conditions

10.2.2.1 *Harpagophytum procumbens*

In preliminary experimental work done on *Harpagophytum procumbens*, it was found that a 1:1 (v/v) mixture of methanol:acetonitrile (MeOH:ACN) produced a useful extract from dried plant material. This method was used by extracting 1 g of plant material with 10 mL of the solvent mixture in an ultrasonic bath. The samples were extracted four times and each extraction was sampled to determine the completeness of the extraction process. The supernatants were combined and used as test sample to develop the chromatographic procedure.

For the analysis, the HSS C18 column produced the best peak shape and was used to develop the chromatographic separation method. Chromatographic separation of the pooled extraction samples was done utilising a Waters HSS C18 column (150 mm × 2.1 mm, 1.8 μm) and the column temperature controlled at 60 °C. A binary solvent mixture was used consisting of water (Eluent A) containing 10 mM formic acid (natural pH of 2.3) and acetonitrile (Eluent B). The initial conditions were 95% A at a flow rate of 0.4 mL/min and were maintained for 1 min, followed by a multi-level gradient as follows: 90% A at 3.5 min, 80% A at 4 min, 75% A at 16 min, 40% A at 18 min and finally 10% A at 24 min. The conditions were kept constant for 3 min and then changed to the initial conditions. The runtime was 30 min and the injection volume was 1 μL. The PDA detector was scanned between 200 and 500 nm (1.2 nm resolution) while collecting 20 spectra per second.

10.2.2.2 *Peumus boldus*

A MeOH extract was prepared by extracting 1 g of plant material with 10 mL of MeOH in an ultrasonic bath. The sample was extracted four (4) times and each extraction was sampled to determine the completeness of the extraction process. The supernatants were combined and used as test sample to develop the chromatographic procedure.

Optimisation of the chromatographic separation was done utilising a Waters HSS C18 column (150 mm × 2.1 mm, 1.8 μm) and the column temperature controlled at 60 °C. A binary solvent mixture was used consisting of water (Eluent A) containing 10 mM formic acid (natural pH of 2.3) and acetonitrile (Eluent B). The initial conditions were 95% A at a flow rate of 0.5 mL/min and were maintained for 0.1 min, followed by a multi-level gradient as follows: 93% A at 1.5 min, 89% A2 at 4 min, 87% A at 12 min, and finally 5% A at 26 min. The conditions were kept constant for 2 min and then changed to the initial conditions. The runtime was 30 min and the injection volume was 1 μL. The PDA detector was scanned between 200 and 500 nm (1.2 nm resolution) while collecting 20 spectra per second.

10.2.2.3 *Passiflora incarnata*

The chromatographic separation of a 60% methanolic extract of the *Passiflora incarnata* leaf sample supplied, was evaluated in three UPLC column chemistries, namely HSS T3 C18, HSS C18 and CSH Phenyl-Hexyl. Optimisation of the chromatographic separation was done utilising a Waters CSH Phenyl-Hexyl column (150 mm × 2.1 mm, 1.7 μm) and the column temperature controlled at 60 °C. A binary solvent mixture was used consisting of water (Eluent A) containing 10 mM formic acid (natural pH of 2.3) and methanol (Eluent B). The initial conditions were 82% A at a flow rate of 0.4 mL/min and were maintained for 1 min, followed by gradient to 87% A at 15 min, finishing at 5% A at 26 min. The conditions were kept constant for 2 min and then changed to the initial conditions. The runtime was

30 min and the injection volume was 1 μL. The PDA detector was scanned between 200 and 500 nm (1.2 nm resolution) while collecting 20 spectra per second.

10.2.2.4 *Boswellia serrata*

An 80% MeOH extract was prepared by extracting 5 g of PlantLIBRA *Boswellia serrata* resin material with 20 mL of 80% (v/v) MeOH in an ultrasonic bath. The sample was extracted four times to ensure complete extraction of the sample. The supernatants were combined and used as test sample to develop the chromatographic procedure. Upon standing and subsequent evaporation, the supernatant became turbid and a precipitate formed. A portion of the precipitate was dissolved in methanol and analysed with the optimised method.

The chromatographic separation and peak shape of an 80% (v/v) methanolic extract of the *Boswellia serrata* resin sample supplied was evaluated in four UPLC column chemistries, namely CSH C18, T3 C18, HSS C18 and BEH C8. Optimisation of the chromatographic separation was done utilising a Waters BEH C8 column (150 mm × 2.1 mm, 1.7 μm) and the column temperature controlled at 60 °C. A binary solvent mixture was used consisting of water (Eluent A) containing 10 mM formic acid (natural pH of 2.3) and acetonitrile (Eluent B). The initial conditions were 50% A at a flow rate of 0.4 mL/min and were maintained for 1 min, followed by an initial gradient to 33% A at 19 min, followed by a second gradient to 5% A at 26 min. The conditions were kept constant for 1 min and then changed to the initial conditions. The runtime was 30 min and the injection volume was 1 μL. The PDA detector was scanned between 200 and 500 nm (1.2 nm resolution) while collecting 20 spectra per second.

10.2.2.5 *Valeriana officinalis*

Various extraction solvents were evaluated namely 40, 60, 80 and 100% (v/v) methanol. Plant material extracted with 80% (v/v) methanol produced the most complex and diverse selection of compounds. The chromatographic separation and peak shape of the 80% methanolic extract of the Valerian root sample supplied (PL Reference) was evaluated using three UPLC column chemistries, namely HSS T3 C18, HSS C18 and BEH C8. Optimisation of the chromatographic separation was done utilising a Waters BEH C8 column (150 mm × 2.1 mm, 1.7 μm) and the column temperature controlled at 60 °C. A binary solvent mixture was used consisting of water (Eluent A) containing 10 mM formic acid (natural pH of 2.3) and methanol (Eluent B). The initial conditions were 90% A at a flow rate of 0.4 mL/min and were maintained for 0.1 min, followed by a gradient to 20% A at 34.5 min and finishing at 5% A at 35 min. The conditions were kept constant for 1 min and then changed to the initial conditions. The runtime was 40 min and the injection volume was 1 μL. The PDA detector was scanned between 200 and 500 nm (1.2 nm resolution) while collecting 20 spectra per second.

10.2.2.6 *Cinnamomum verum*

Various extraction solvents were evaluated namely 60, 80 and 100% (v/v) methanol. Plant material extracted with 60% (v/v) methanol produced the most complex and diverse selection of compounds. The chromatographic separation and peak shape of the 60% methanolic extract of a commercial cinnamon bark sample purchased (COM1) was evaluated using three UPLC column chemistries, namely HSS T3 C18, HSS C18 and CSH Hexyl-Phenyl. Optimisation of the chromatographic separation was done utilising a Waters CSH Hexyl-Phenyl column (150 mm × 2.1 mm, 1.7 μm) and the column temperature controlled at 60 °C. A binary solvent mixture was used consisting of water (Eluent A) containing 10 mM formic acid (natural pH of 2.3) and acetonitrile (Eluent B). The initial conditions were 95% A at a flow rate of 0.4 mL/min and were maintained for 1 min, followed by multiple gradients to 85% A at 4 min, 80% A at 8 min and 5% A at 35 min. The conditions were kept constant for 1 minute and then changed to the initial conditions. The runtime was 30 min and the injection volume was 5 μL. The PDA detector was scanned between 200 and 500 nm (1.2 nm resolution) while collecting 20 spectra per second.

10.3 Results and Discussion

10.3.1 Harpagophytum procumbens

A number of methods with different alcohol to water ratios were attempted in preliminary extractions, but it was found that a mixture of 1:1 methanol:acetonitrile yielded an extract containing most of the major compounds identified by other studies using HPLC or HPTLC either linked with DAD or MS (Günther and Schmidt 2005; Abdelouahab and Heard 2008; Karioti et al. 2011; Seger et al. 2005; Babili et al. 2012).

An evaluation of the raw data obtained from the analysis of the CSIR ECD sample revealed that a large selection of the compounds listed in the Dictionary of Natural Products (DNP Ver.18.2) could be identified from the accurate mass data as presented in Fig. 10.1. To confirm the tentative identifications done from the mass spectral data, six reference standards were also analysed with the optimized method. Based on this data and the accurate mass data obtained from the TOF analysis, the identity of the six compounds could be positively confirmed. Figure 10.2 is a typical XIC mass chromatogram obtained from the analysis of the methanol extract using the optimized method. The compounds that were positively identified were labelled. Table 10.1 is a summary of the compounds detected and identified.

The UPLC method separated the major peaks and these could then be identified using reference standards. The presence of five compounds was confirmed namely harpagide, verbascoside, isoverbascoside, 6-acetylacteoside and harpagoside. The high mass accuracy of the TOF data also allowed for the tentative identification of three compounds, namely dihydrichinatrienone, totaratrienediol and a possible isomer of 6-acetylacteoside.

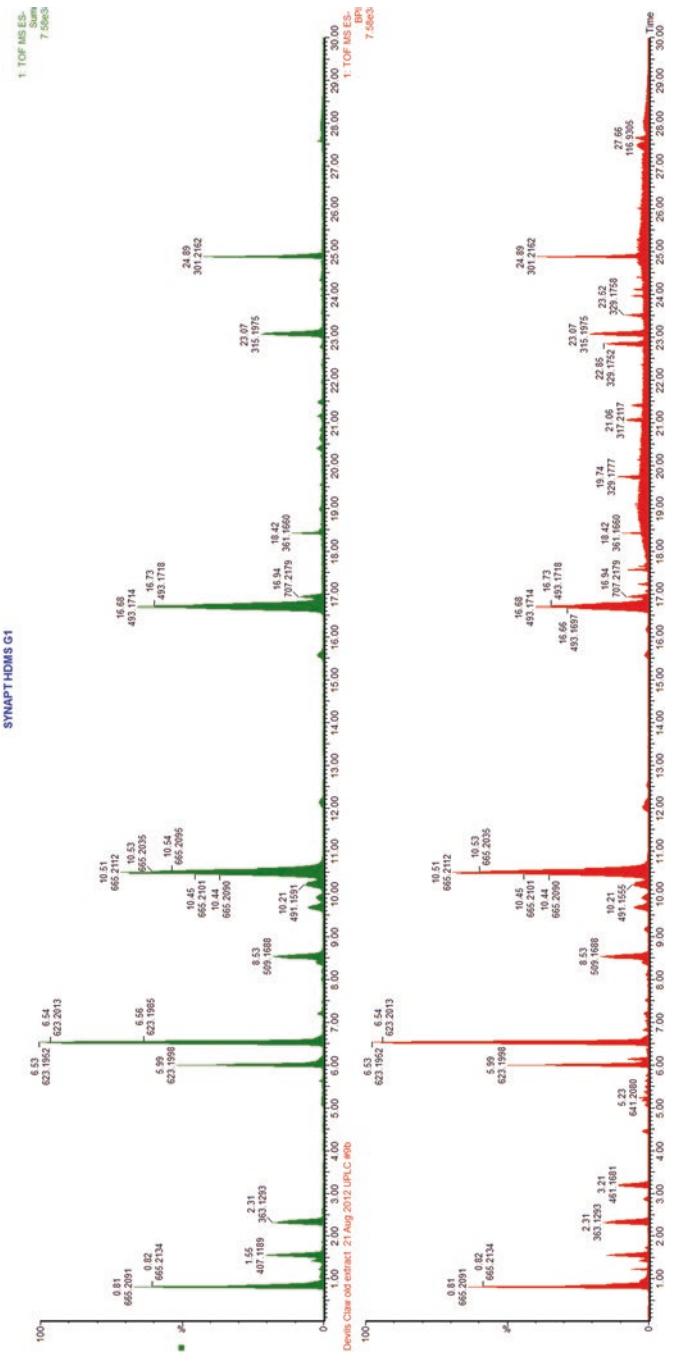

Fig. 10.1 Comparison of the BPI and XIC chromatograms of the MeOH/ACN CSIR ECD *Harpagophytum* sample extract

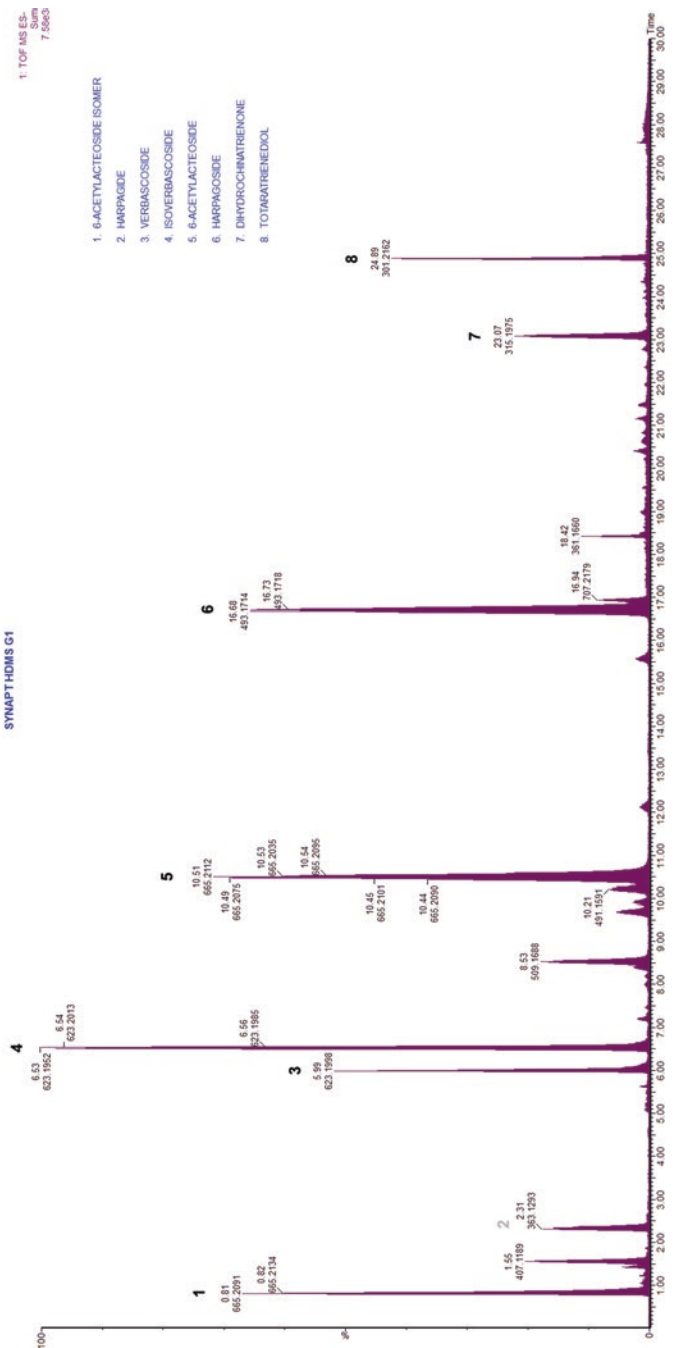

Fig. 10.2 Annotated XIC mass chromatograms of the MeOH/ACN CSIR ECD *Harpagophytum* sample extract

Table 10.1 Compounds detected and confirmed in the CSIR ECD sample

Compound	Formula	Accurate mass (Da)	Confirmed by standard	DBE score	Detected mass (Da)	RT (min)	Mass accuracy (mDa)
6-Acetylacteoside isomer	$C_{31}H_{38}O_{16}$	666.2160	No	13	665.2130	0.81	4.8
Harpagide	$C_{15}H_{24}O_{10}$	364.1370	Yes	4	363.1291	2.32	−0.4
Verbascoside	$C_{29}H_{36}O_{15}$	624.2054	Yes	12	623.1983	6.00	0.7
Harprocumbide B	$C_{24}H_{28}O_{12}$	508.1581	No	11	507.1503	6.13	0.0
Isoverbascoside	$C_{29}H_{36}O_{15}$	624.2054	Yes	12	623.1993	6.52	1.7
8-p-Coumaroylharpagoside	$C_{24}H_{30}O_{12}$	510.1737	Yes	10	509.1677	8.53	1.8
Pagoside	$C_{24}H_{28}O_{11}$	492.1632	No	11	491.1580	10.20	2.7
6-Acetylacteoside	$C_{31}H_{38}O_{16}$	666.2160	Yes	13	665.2089	10.51	0.7
Harprocumbide A	$C_{30}H_{40}O_{16}$	656.2316	No	11	655.2238	10.92	0.0
Harpagoside	$C_{24}H_{30}O_{11}$	494.1788	Yes	10	493.1710	16.72	0.0
2,6-Diacetylacteoside	$C_{33}H_{40}O_{17}$	708.2266	No	14	707.2186	16.94	−0.1
Ajugol, 6-Epimer, 8-cinnamoyl (*E*-)	$C_{24}H_{30}O_{10}$	478.1839	No	11	475.1630	17.56	1.1
Procumbide or Procumboside	$C_{15}H_{22}O_{10}$	362.1213	No	5	361.1662	18.42	1.1
6,12,13-Trihydroxy-5,8,11,13-chinatetraen-7-one	$C_{20}H_{26}O_4$	330.1831	No	8	329.1760	22.85	0.7
12,13-Dihydroxy-8,11,13-chinatrien-7-one	$C_{20}H_{28}O_3$	316.2038	No	7	315.1970	23.07	1.0
8,11,13-Totarariene-12,13-diol	$C_{20}H_{30}O_2$	302.2246	No	6	301.2175	24.88	0.7

10.3.2 Peumus boldus

From the LC-MS spectra of the different solvent extracts of *Peumus boldus* the chemical complexity of extracts derived from *Peumus boldus* leaves was highlighted. The water extract displayed more polar compounds while the methanol extract contained more of the apolar compounds. The 60% methanol extract represented the best of both extraction solvents and was used in all further development work.

An analysis of the mass spectrometric data of Fig. 10.3 (60% MeOH extract) revealed that at least 14 compounds could be tentatively identified—five flavonoids and nine alkaloids (Table 10.2). The identity of five compounds could be more positively confirmed by a process that analyses collisionally activated dissociated (CAD) mass spectra, through the use of a systematic bond-disconnection approach. With this approach, a second data channel is generated during the mass spectrometric analysis of the samples by running higher collision energies to invoke fragmentation of the molecules. By evaluating the fragmentation mass spectra obtained, the feasibility of the proposed structures is evaluated to produce the fragmentation mass spectra observed. The drawback of this approach is the inability to differentiate between isomers. If the compounds under investigation have the same basic structure with the only difference being the substitution pattern, this approach fails to deliver unambiguous results.

Without using certified reference standards for the expected compounds, it is very difficult to positively identify each of the compounds detected in the methanolic extract. The process of identification without reference standards is also complicated by the fact that electrospray ionisation (ESI) readily forms adducts resulting in more complex mass spectrometric data. By selectively extracting data according to the mass to charge ratio (*m/z*), some compounds can be easily detected and confirmed. Initially four *m/z* values were used to try and identify compounds. By selecting an *m/z* of 328.15 Dalton (Da), one major and five minor compounds are identified that contain a mass ion of 328.15 Da. Selecting an *m/z* of 317.06 Da resulted in the display of seven major and seven minor compounds. These complex situations do occur frequently in crude plant extracts but is not the general norm. The selection of *m/z* 291.08 and *m/z* 609.18 Da resulted in the display of one major and two minor compounds (*m/z* 291.08) and one major and three minor compounds (*m/z* 609.18) respectively. The compound with an *m/z* of 291.0861 was positively identified as catechin while the compound with an *m/z* of 609.1863 was identified as isorhamnetin 3-dirhamnoside.

A selection of the expected masses was made and used to extract compound-specific data from the analytical data obtained from the analysis of the crude 60% MeOH extract. The process of selecting only the masses of interest decreased the complexity of the BPI chromatogram and generated a customised XIC chromatogram for *Peumus boldus* extracts that is specific to those compounds typically found in the plant material.

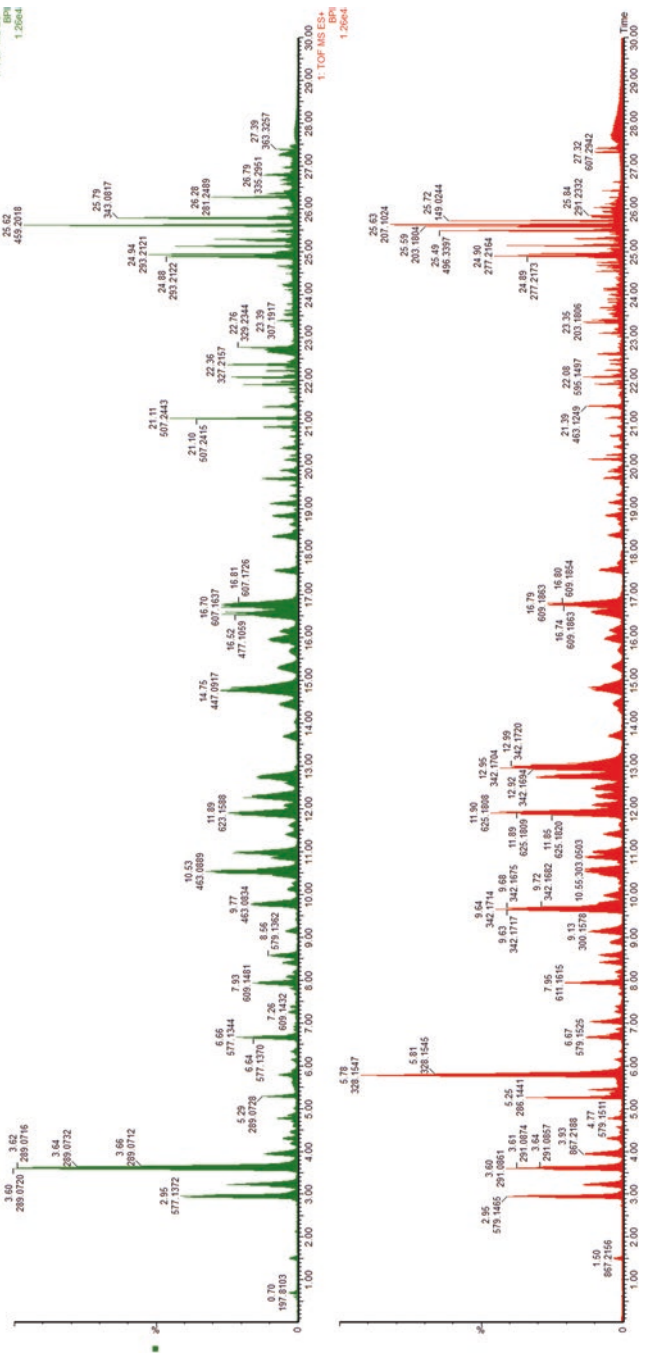

Fig. 10.3 ESIPos and ESINeg chromatographic separation of the 60% MeOH *Peumus boldus* extract

Table 10.2 Compounds detected in the 60% MeOH extract of *Peumus boldus*

Compound	Empirical formula	Accurate mass (Da)	Detected (Mode)	Retention time (min)	iFit error (mDa)	MassFrag confirmed
Catechin	$C_{15}H_{14}O_6$	290.2681	+/−	3.61	0.00 1.9	Yes
Boldine	$C_{19}H_{21}NO_4$	327. 1471	+	(5.78 and 6.65) (8.85 and12.45)	0.00 −0.3	No
Norisocorydine	$C_{19}H_{21}NO_4$	327.1471	+	(5.78 and 6.65) (8.85 and12.45)	N/A	N/A
Reticuline	$C_{19}H_{23}NO_4$	329.3902	+	6.99	0.00 0.4	Yes
Isoboldine	$C_{19}H_{21}NO_4$	327.1471	+	(5.78 and 6.65) (8.85 and12.45)	N/A	N/A
N-methyllaurotetanine	$C_{20}H_{23}NO_4$	341.4009	+	12.95	0.00 0.2	Yes
Isorhamnetin	$C_{16}H_{12}O_7$	316.0582	+	Multiple	N/A	N/A
Isocorydine	$C_{20}H_{23}NO_4$	341.1627	+	9.64	0.00 −0.8	No
Laurotetanine	$C_{19}H_{21}NO_4$	327.1471	+	(5.78 and 6.65) (8.85 and12.45)	N/A	N/A
Sinoacutine	$C_{19}H_{21}NO_4$	327.1471	+	(5.78 and 6.65) (8.85 and12.45)	N/A	N/A
Peumoside	$C_{27}H_{30}O_{15}$	594.1585	+/−	12.73	0.00 1.0	Yes
Isorhamnetin 3,7-diglycoside	$C_{27}H_{30}O_{15}$	594.1585	+/−	10.52	0.00 −0.01	Yes
Kaempferol-3-glucoside-7-rhamnoside	$C_{27}H_{30}O_{15}$	594.1585	+/−	10.91	0.00 0.00	Yes
Isorhamnetin 3-dirhamnoside	$C_{28}H_{32}O_{15}$	608.1741	+/−	16.72	0.00 0.8	Yes

It must be noted that some compounds could also be detected in the ESINeg ionisation mode (Table 10.2) and greatly assisted in the positive identification of these compounds. The iFit (normalised) values obtained during the elemental analysis is an indication of how well the predicted and actual isotope pattern of a compound correlate. The closer the value is to zero, the more likely the predicted elemental composition and related monoisotopic mass is the actual mass of the

compound detected. This however, does not supply any final structure relating to the mass spectral data and structural calculations such as rings and double bond equivalence (DBE).

By using the selected masses to generate a specific XIC chromatogram (Fig. 10.4) and by applying the knowledge gained from the literature and plant data bases, 11 compounds could be tentatively identified. At least 20 other related compounds were also detected but could not be identified.

An evaluation of the BPI chromatograms of the crude extract revealed that at least 130 compounds could be visually detected with ease while the ESIPos XIC chromatogram displayed approximately 31 compounds (results not shown).

10.3.3 Passiflora incarnata

Results obtained (results not shown) with different solvent extractions clearly highlight the chemical complexity of extracts derived from *Passiflora incarnata* leaves. The water extract displayed more polar compounds while the methanol extract contained more of the apolar compounds. The 60% methanol extract represented the best of both extraction solvents and was used in all further development work.

Figure 10.6 represents the chemical diversity observed with the 60% methanol extract. According to literature *Passiflora incarnata* contains alkaloids and flavonoids that should be detectable by LC-MS. An analysis of the mass spectrometric data (Fig. 10.5) (60% methanol extract) revealed that at least 17 compounds could be tentatively identified—however no alkaloids could be detected.

A selection of the expected masses was made and used to extract compound-specific data from the analytical data obtained from the analysis of the crude 60% MeOH extract. The process of selecting only the masses of interest decreased the complexity of the BPI chromatogram and generated a customised XIC chromatogram for *Passiflora incarnata* extracts that is specific to those compounds typically found in the plant material. Utilising the mass spectral data and MassFragment software, an attempt was made to annotate the XIC chromatograms (results not shown) with the possible identities of some of the detected compounds. The annotation is only tentative for those compounds of which the fragmentation mass spectra could be related to the proposed structure found in ChemSpider or the Dictionary of Natural Products. It must be noted that some compounds could be detected in both the ionisation modes (Table 10.3) and greatly assisted in the positive identification of these compounds.

Various Passiflora samples were received as part of this study and the samples were extracted as reported earlier. The extracts were analysed using the same analytical conditions and method as used for the initial Passiflora sample submitted (*Passiflora incarnata* leaves). The results indicated that although the various Passiflora leaf samples were in general similar, they did differ in their chemical profiles. The results are given in Table 10.4.

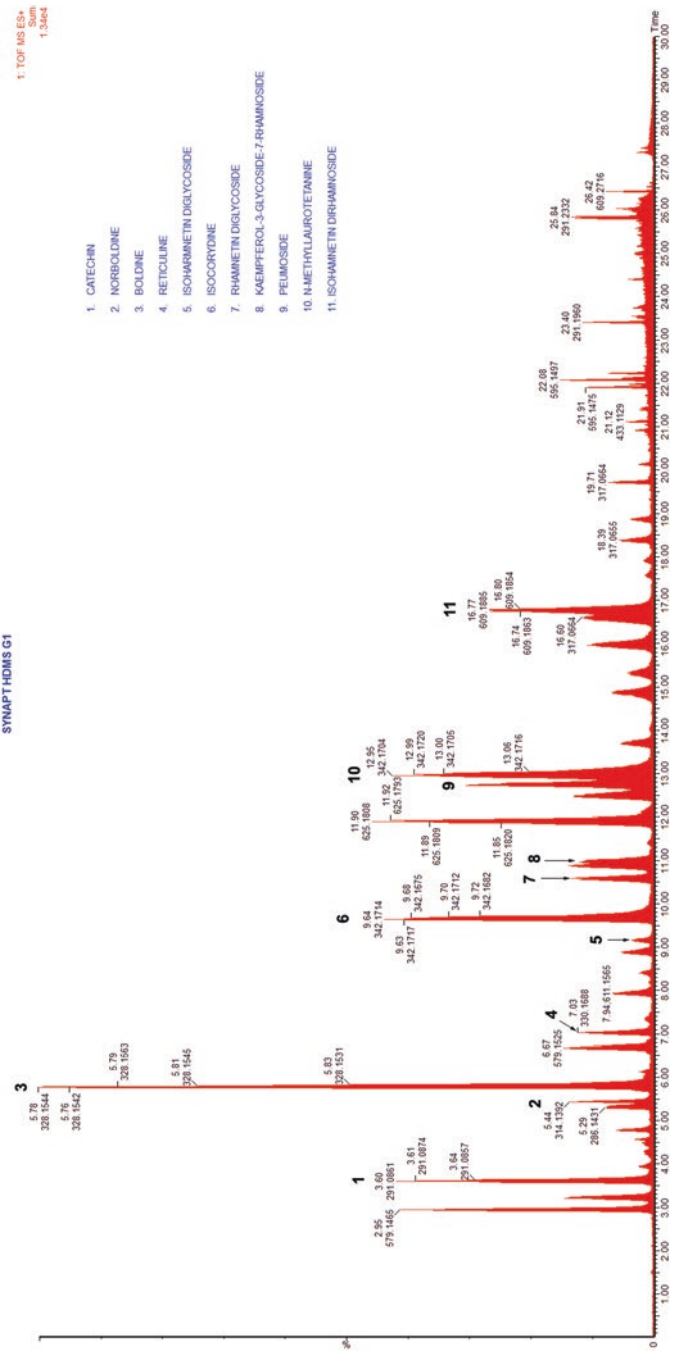

Fig. 10.4 Annotated XIC mass chromatogram of the 60% MeOH *Peumus* extract

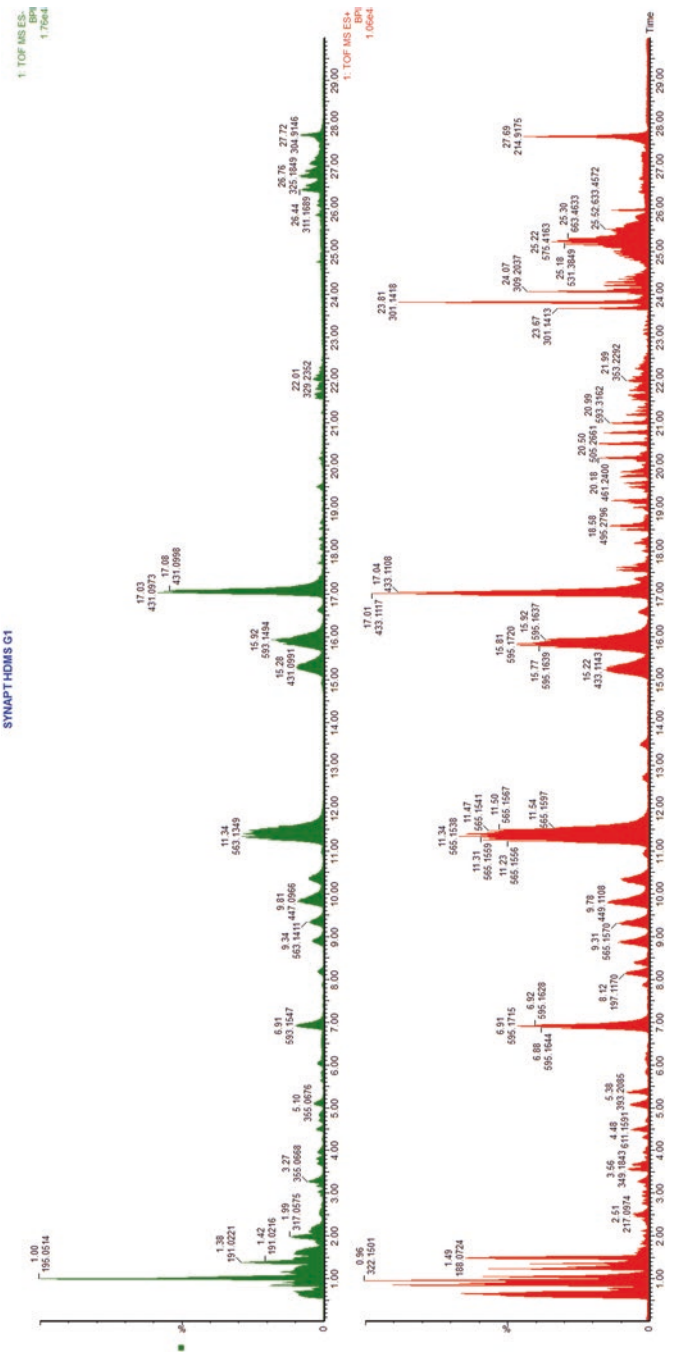

Fig. 10.5 ESIPos and ESINeg chromatographic separation of the 60% MeOH *Passiflora* extract

Table 10.3 Compounds detected in the 60% methanol extract of *Passiflora incarnata*

Compound	Empirical formula	Expected mass (Da)		Rt	Accurate mass (Da)
		ESI Pos	ESINeg		
1-Methyl-β-carboline	$C_{12}H_{10}N_2$	X	X	X	182.0843
7-Hydroxy-1-methyl-β-carboline	$C_{12}H_{10}N_2O$	X	X	X	198.0793
7-Hydroxy-1-methyl-β-carboline; 3,4-dihydro	$C_{12}H_{12}N_2O$	X	X	X	200.0950
7-Hydroxy-1-methyl-β-carboline; 3,4-dihydro, methyl ether	$C_{13}H_{14}N_2O$	X	X	X	214.1106
Orientin	$C_{21}H_{20}O_{11}$	449.1085	447.0927	Tentative	448.1006
Isoorientin	$C_{21}H_{20}O_{11}$	449.1092	447.0934	Tentative	448.1006
Isoorientin-2″-O-glucopyranoside	$C_{27}H_{30}O_{16}$	611.1473	609.1603	Tentative	610.1534
Schaftoside	$C_{26}H_{28}O_{14}$	565.1553	563.1417	Tentative	564.1479
Isoshaftoside	$C_{26}H_{28}O_{14}$	565.1563	563.1403	Tentative	564.1479
Vitexin-2″-O-β-glucopyranoside	$C_{27}H_{30}O_{15}$	595.1671	593.1522	Tentative	594.1585
Isovitexin-2″-O-β-glucopyranoside	$C_{27}H_{30}O_{15}$	595.1662	593.1513	Tentative	594.1585
Chlorogenic acid	$C_{16}H_{18}O_9$	X	X	X	354.0951
Hyperoside	$C_{21}H_{20}O_{12}$	X	463.0894	18.05	464.0955
Vitexin	$C_{21}H_{20}O_{10}$	433.1132	431.0994	17.03	432.1056
Isovitexin	$C_{21}H_{20}O_{10}$	433.1137	431.0993	15.35	432.1056
Caffeic acid	$C_9H_8O_4$	X	X	X	180.0422
Quercetin	$C_{15}H_{10}O_7$	X	X	X	302.0427
Luteolin	$C_{15}H_{10}O_6$	X	285.0421	20.70	286.0477
Rutin	$C_{27}H_{30}O_{16}$	611.1615	609.1467	4.54/8.79	610.1534
Scutellarin	$C_{21}H_{18}O_{12}$	X	X	X	462.0798
Vicenin-2	$C_{27}H_{30}O_{15}$	595.1625	593.1508	6.97	594.5181
Swertisin	$C_{22}H_{22}O_{10}$	X	445.1129	18.13	446.1213
Vitexin-4′-O-α-L-rhamnopyranoside	$C_{27}H_{30}O_{14}$	579.1717	577.1544	13.20	578.1636
Vitexin-2-O-rhamnoside (Tentative)	$C_{27}H_{32}O_{14}$		579.1716	19.14	580.1792

Among the leaf samples the Maracuja sample visually appeared "different" to the other samples. The flower sample (SA Flower) clearly displayed significant differences to the leaf samples as two known compounds, Vitexin-4′-O-α-L-rhamnopyranoside and Vitexin-2-O-rhamnoside were only detected in the flower sample. Two unknown compounds with *m/z* 610.1533 ($C_{27}H_{30}O_{16}$) and *m/z* 610.1897 ($C_{28}H_{34}O_{15}$) could only be detected in the flower extract. Table 10.4 is a summary of the compounds detected in the various Passiflora samples. Most of the compounds could be detected in both ionisation modes except for hyperoside, luteolin, swertisin and vitexin-2-O-rhamnoside which could only be detected in ESINeg ionisation.

Table 10.4 Results of compounds tentatively identified and their distribution in different Passiflora samples

Compound	Empirical formula	Expected mass (Da)		Leaves PL	Flowers SA	Leaves Marcuja	Leaves USA	Leaves France	Leaves Italy
		ESIPos	ESINeg						
Orientin	$C_{21}H_{20}O_{11}$	449.1085	447.0927	Tentative	Tentative	Tentative	Tentative	Tentative	Tentative
Isoorientin	$C_{21}H_{20}O_{11}$	449.1092	447.0934	Tentative	Tentative	X	X	Tentative	Tentative
Isoorientin-2″-O-glucopyranoside	$C_{27}H_{30}O_{16}$	611.1473	609.1603	Tentative	Tentative	Tentative	Tentative	Tentative	Tentative
Schaftoside	$C_{26}H_{28}O_{14}$	565.1553	563.1417	Tentative	Tentative	Tentative	Tentative	Tentative	Tentative
Isoshaftoside	$C_{26}H_{28}O_{14}$	565.1563	563.1403	Tentative	Tentative	Tentative	Tentative	Tentative	Tentative
Vitexin-2″-O-β-glucopyranoside	$C_{27}H_{30}O_{15}$	595.1671	593.1522	Tentative	X	Tentative	Tentative	Tentative	Tentative
Isovitexin-2″-O-β-glucopyranoside	$C_{27}H_{30}O_{15}$	595.1662	593.1513	Tentative	Tentative	Tentative	Tentative	Tentative	Tentative
Chlorogenic acid	$C_{16}H_{18}O_9$	X	X	X	X	X	X	X	X
Hyperoside	$C_{21}H_{20}O_{12}$	X	463.0894	Yes	Substructure	Substructure	Yes	Yes	Yes
Vitexin	$C_{21}H_{20}O_{10}$	433.1132	431.0994	Tentative	Tentative	Tentative	Tentative	Tentative	Tentative
Isovitexin	$C_{21}H_{20}O_{10}$	433.1137	431.0993	Tentative	Tentative	Tentative	X	Tentative	Tentative
Caffeic acid	$C_9H_8O_4$	X	X	X	X	X	X	X	X
Quercetin	$C_{15}H_{10}O_7$	X	X	X	X	X	X	X	Substructure
Luteolin	$C_{15}H_{10}O_6$	X	285.0421	Yes	Yes	Yes	Yes	Yes	Yes
Rutin	$C_{27}H_{30}O_{16}$	611.1615	609.1467	Yes	Yes	Yes	Yes	Yes	Yes
Scutellarin	$C_{21}H_{18}O_{12}$	X	X	X	X	X	X	X	X
Vicenin-2	$C_{27}H_{30}O_{15}$	595.1625	593.1508	Tentative	Tentative	Tentative	Tentative	Tentative	Tentative
Swertisin	$C_{22}H_{22}O_{10}$	X	445.1129	Yes	X	X	Yes	Yes	Yes
Vitexin-4′-O-α-L-rhamnopyranoside	$C_{27}H_{30}O_{14}$	579.1717	577.1544	X	Yes	X	X	X	X
Vitexin-2-O-rhamnoside (Tentative)	$C_{27}H_{32}O_{14}$	X	579.1716	X	Tentative	X	X	X	X
?	$C_{27}H_{30}O_{16}$	611.1605	609.1447	X	Detected	X	X	X	X
?	$C_{28}H_{34}O_{15}$	611.1931	609.1832	X	Detected	X	X	X	X

As visual comparisons tend to miss the smaller chemical differences between samples, MarkerLynx software was utilised to highlight similarities and differences between the plant material listed in Table 10.4. Figure 10.6 represents the Scores Plot comparing the ESIPos data while Fig. 10.7 depicts the ESINeg data of the various plant materials analysed. In both Scores Plots the SA Flower sample was positioned on its own while the leaf samples were loosely grouped together. The Maracuja sample displayed greater similarities to the SA Flower sample than the other leaf samples. The Scores Plot based on the ESINeg data resulted in a "leaf" cluster and a "flower" cluster with the Maracuja and France leaf samples displaying the greatest difference compared to the other leaf samples. Exclusion of the SA Flower sample and Maracuja leaf sample resulted in a tight cluster of the remaining samples (USA Leaves, Italy Leaves and PL Leaves) indicating significant similarity between these samples.

Methanol and methanol:water extracts of the dried leaves of *Passiflora incarnata* produced extracts rich in flavonoids and flavonoid glycosides but void of alkaloids. The unavailability of reference standards complicated the identification of compounds. The high mass accuracy of the TOF data allowed for the tentative identification of 14 compounds. By using MassFragment software and a systematic bond-disconnection approach based on collisionally activated dissociated mass spectra, the identity of the 14 compounds could be tentatively confirmed. The compounds include Orientin, Isoorientin, Isoorientin-2″-O-glucopyranoside, Schaftoside, Isoschaftoside, Vitexin-2″-O-β-glucopyranoside, Isovitexin-2″-O-β-glucopyranoside, Hyperoside, Vitexin, Isovitexin, Luteolin, Rutin, Vicenin-2, Swertisin, Vitexin-4′-O-rhamnopyranoside and Vitexin-2-O-rhamnoside. The base peak intensity (BPI) full scan mass spectral chromatograms of the 60% methanol extracts produced complex chromatograms that could be simplified by applying a selected masses filter to the raw data (XIC) (Fig. 10.8).

10.3.4 Boswellia serrata

Due to the insolubility of boswellic acids and related triterpenoids in water, the percentage water was limited to 20% (v/v). Extracts of the Boswellia resin were analysed directly after preparation and filtered (0.2 μm) prior to analysis. The extract became turbid upon standing due to the evaporation of the methanol and resulted in the formation of a cream-coloured, off-white precipitate. An initial evaluation of the data was made in an attempt to identify some of the compounds listed in literature, but it became clear that without reference standards, the true identity of these compounds as well as their chromatographic retention times could not be established. This is especially true for the structural isomers that display similar mass spectra as well as MS/MS fragmentation. Due to the similar mass spectral data observed for the isomers, MassFragment software could not distinguish between them based on the systematic bond-disconnection approach utilised by the software. This dilemma is clearly illustrated by extracting an ESI negative mass ion chromatogram (XIC) at 455.352 Da which relates to the mass of α-boswellic acid, β-boswellic acid and lupeolic acid. By selecting those compounds displaying a 455.35 Da

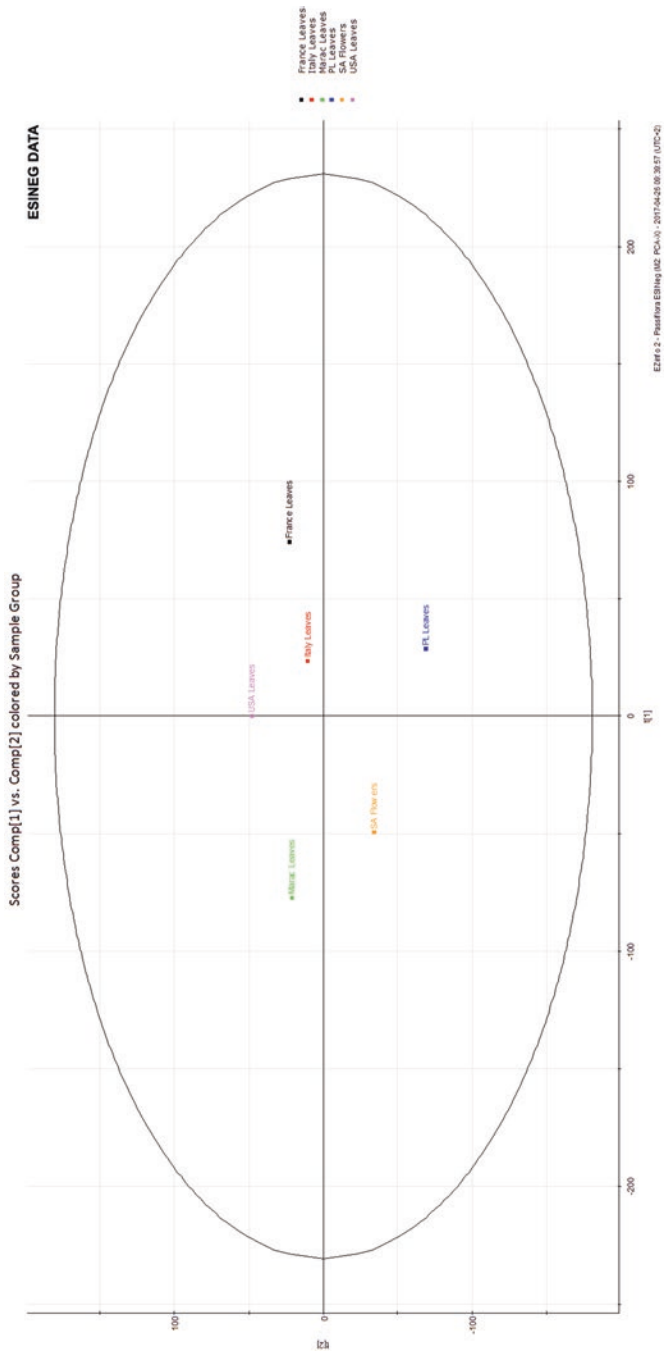

Fig. 10.6 ScoresPlot (ESIPos data) of the plant material listed in Table 10.4

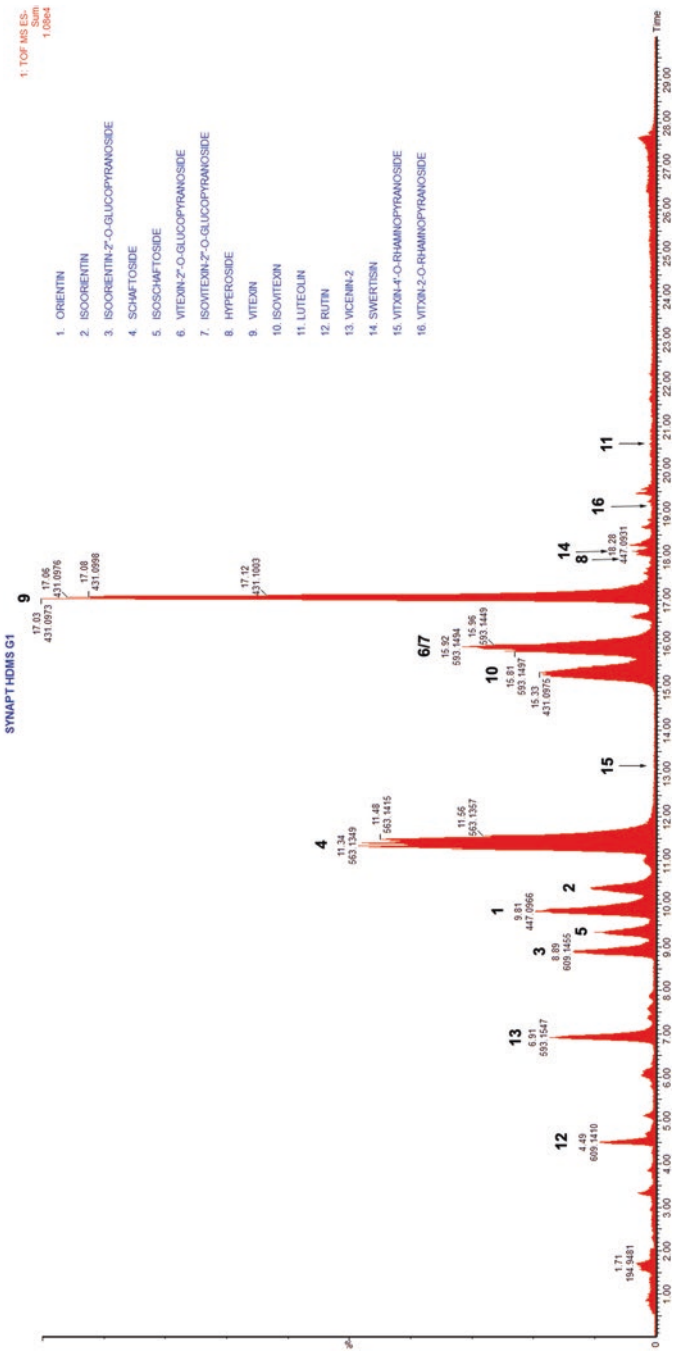

Fig. 10.7 ScoresPlot (ESINeg data) of the plant material listed in Table 10.4

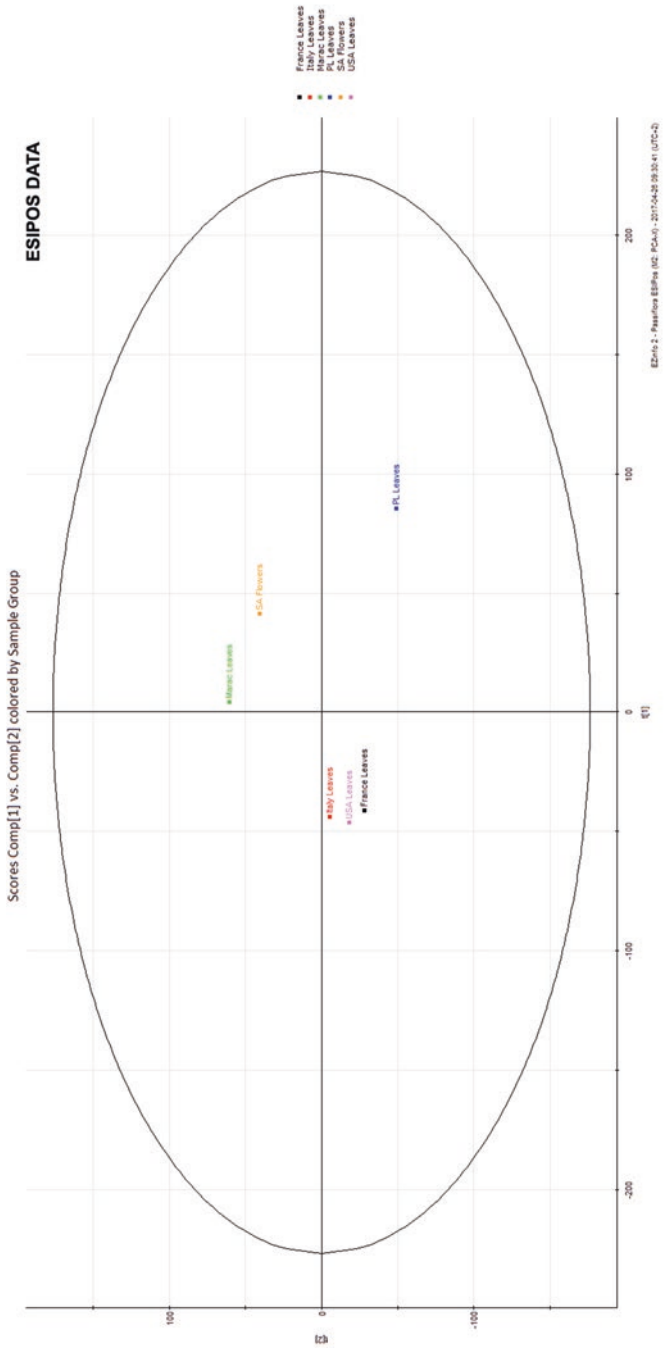

Fig. 10.8 Annotated XIC mass chromatogram of the 60% MeOH *Passiflora* extract

Table 10.5 Compounds tentatively identified in the *Boswellia serrata* extract (PL TPA 70-12-1)

Compound	Empirical formula	Expected mass (Da)		Rt (min)	Accurate mass (Da)
		ESIPos	ESINeg		
Cembrenol	$C_{20}H_{34}O$	291.268	289.253	17.76	290.2609
Incensole	$C_{20}H_{34}O_2$	307.263	305.248	12.10	306.2559
Incensole acetate	$C_{22}H_{36}O_3$	349.274	347.258	X	348.2665
12-Ursene-2-diketone	$C_{30}H_{46}O_2$	439.358	437.342	19.83	438.3498
12-Ursene-3,24-diol	$C_{30}H_{50}O_2$	443.389	441.373	19.39	442.3811
9,11-dehydro-α-Boswellic acid	$C_{30}H_{46}O_3$	455.352	453.336	11.19	454.3447
9,11-dehydro-β-Boswellic acid	$C_{30}H_{46}O_3$	455.352	453.336	16.88	454.3447
Lupeolic acid	$C_{30}H_{48}O_4$	457.368	455.352	Tentative	456.3603
α-Boswellic acid	$C_{30}H_{48}O_3$	457.368	455.352	Tentative	456.7003
β-Boswellic acid	$C_{30}H_{48}O_3$	457.368	455.352	Tentative	456.7003
11-Keto-β-Boswellic acid	$C_{30}H_{46}O_4$	471.347	469.331	10.50	470.3396
Acetyl-9,11-dehydro-α-Boswellic acid	$C_{32}H_{48}O_4$	497.363	495.347	24.85	496.3552
Acetyl-9,11-dehydro-β-Boswellic acid	$C_{32}H_{48}O_4$	497.363	495.347	25.40	496.3552
Acetyl-α-Boswellic acid	$C_{32}H_{50}O_4$	499.378	497.363	25.09	498.3709
Acetyl-β-Boswellic acid	$C_{32}H_{50}O_4$	499.378	497.363	25.23	498.3709
Acetyl-lupeolic acid	$C_{32}H_{50}O_4$	499.378	497.363	24.93	498.3709
Acetyl-11-keto-β-Boswellic acid	$C_{32}H_{48}O_5$	513.358	511.342	18.26	512.3502

pseudo-molecular ion, the similarity of the observed mass spectra can be clearly seen (Fig. 10.9). A closer inspection of the mass spectral data suggested that two related compound classes might give rise to the mass spectra depicted in Fig. 10.9. Extracting a XIC mass chromatogram at 497.36, which relates to the expected pseudo-molecular ions of acetyl-α-boswellic acid, acetyl-β-boswellic acid and acetyl-lupeolic acid produced a chromatogram with three compounds (results not shown). A closer inspection of the fragmentation pattern observed for these compounds (results not shown), indicated that two of the compounds displayed similarities and can probably be assigned to acetyl-α-boswellic acid and acetyl-β-boswellic acid. The remaining chromatographic peak could therefore by default be tentatively assigned to acetyl-lupeolic acid.

From the mass spectral analysis of the *Boswellia serrata* extract it appears that this species produces numerous isomers which make chromatographic peak assignment extremely difficult. The results are summarised in Table 10.5 for the compounds identified in Boswellia.

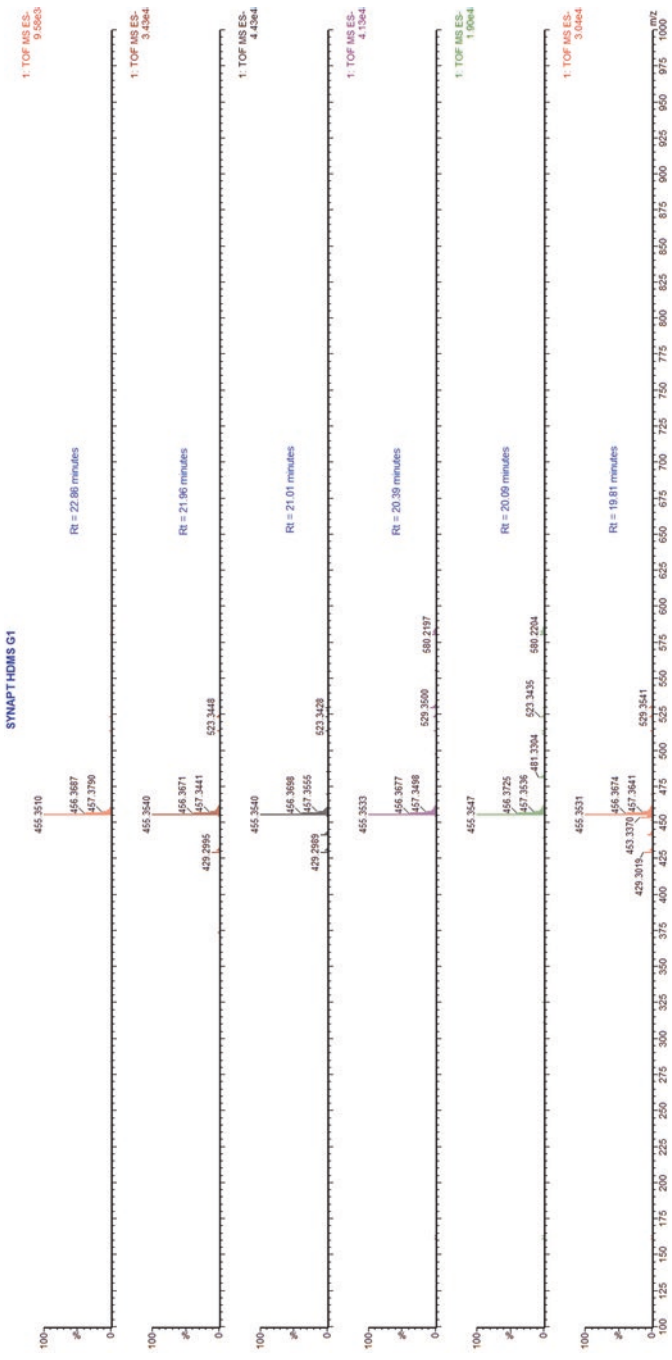

Fig. 10.9 ESINeg mass spectra of those compounds with a 455.35 Da pseudo-molecular ion

Two *Boswellia serrata* samples were received from PlantLIBRA. The first sample (Batch TPA 70-12-1) was a medium fine resin sample and has been discussed in detail above and used as a reference point. The second sample (Batch PL 109) was a coarse resin sample. A third *Boswellia serrata* extract sample was a formulated nutritional product in capsule form (SAS 1) (South Africa). The samples were prepared as described above and analysed using the optimised method. From the ESIPos (Fig. 10.10) and ESINeg (Fig. 10.11) chromatographic data it is clear that the samples differ in chemical composition. This could be a severe challenge in the authentication of *Boswellia serrata* food supplements and formulations.

10.3.5 Valeriana officinalis

An analysis of the mass spectrometric data of the initial data (80% methanol extract) revealed that at least 20 compounds could be tentatively identified and this included alkaloids (Table 10.6). The identity of detected compounds could be more positively confirmed by a process that analyses collisionally activated dissociated (CAD) mass spectra, through the use of a systematic bond-disconnection approach.

By selectively extracting the chromatographic data of the compounds listed in Table 10.6, an extracted mass chromatogram (XIC) can be generated for each ionisation mode. Figures 10.12 and 10.13 represent the ESIPos and ESINeg XIC chromatograms for the 80% (v/v) methanol extract respectively. The XIC chromatograms present a more simplified picture and can easily be used to selectively monitor a selection of compounds present in *Valeriana officinalis* plant material. The base peak intensity (BPI) display format however would provide a superior data format to compare plant material for similarities or differences in chemical composition.

The unavailability of a full set of reference standards complicated the identification of compounds. The high mass accuracy of the TOF data allowed for the tentative identification of 21 compounds. Four of the 21 compounds could be confirmed with reference standards and included valerenic acid, hydroxyvalerenic acid, acetoxyvalerenic acid and hesperidin.

10.3.6 Cinnamomum verum

Products derived from *Cinnamomum verum* and *Cinnamomum cassia* are freely available. Two samples obtained through the PlantLIBRA network was extracted with 60% methanol but produced significantly different chemical profiles. A commercially available product was purchased and a sample extracted using the same extraction protocol. Figures 10.14 and 10.15 represent the chemical diversity observed in ESIPos and ESINeg modes for the 60% methanol extracts of the three samples, respectively. Although the three samples displayed similarities when

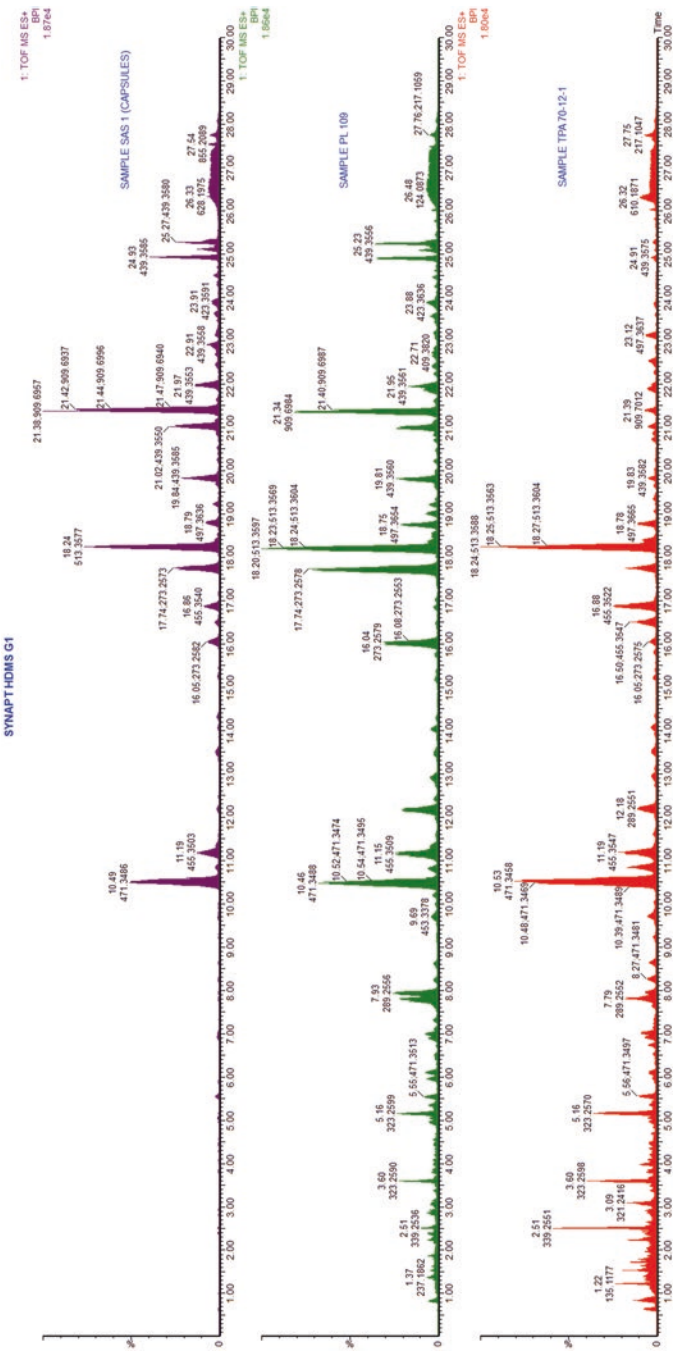

Fig. 10.10 ESIPos mass chromatograms of the three Boswellia samples evaluated

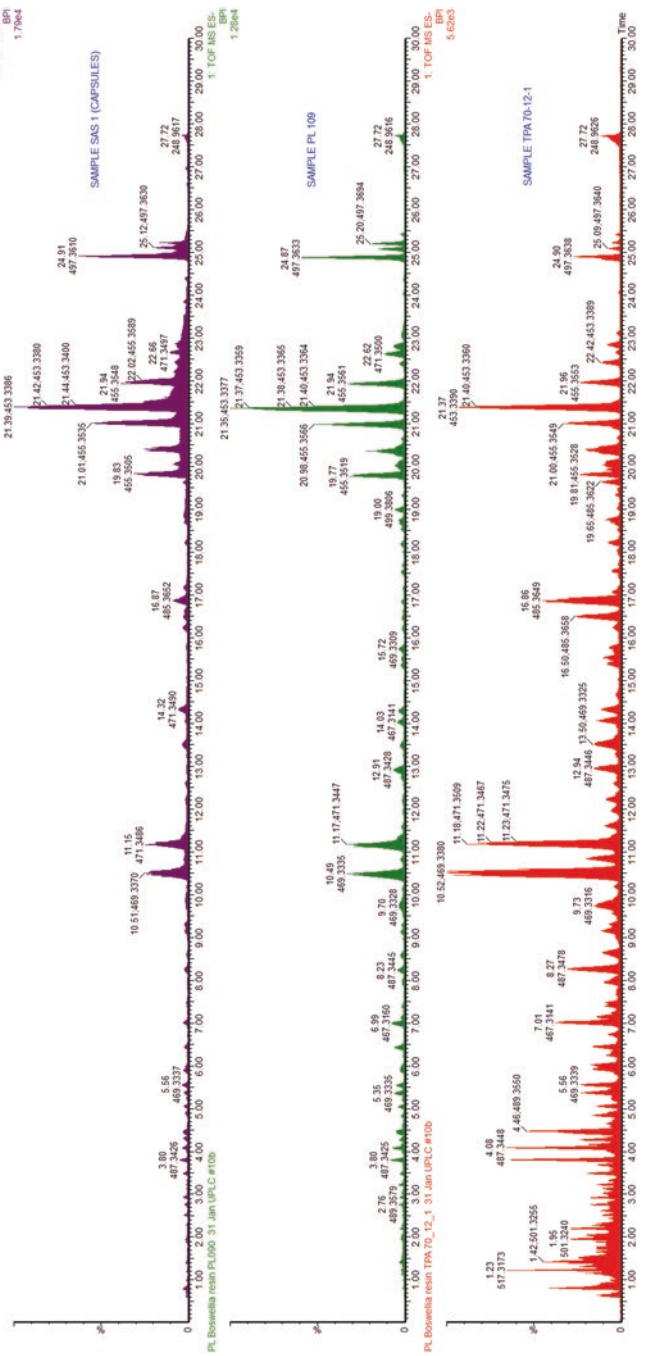

Fig. 10.11 ESINeg mass chromatograms of the three Boswellia samples evaluated

Table 10.6 Compounds tentatively detected in the 80% methanol extract of *Valeriana officinalis* PL

Compound	Empirical formula	Expected mass (Da)		Detected and Rt	Accurate mass (Da)
		ESIPos	ESINeg		
Actinidine; (S)-form	$C_{10}H_{13}N$	148.1126	X	3.17	147.1048
3-Acetyl-2,7-naphthyridine	$C_{10}H_8N_2O$	173.0796	X	1.54	172.0637
2-Hydroxy-3-methylbutanoic acid	$C_{10}H_{18}O_4$	X	201.1055	19.73	202.1205
Tamariscene	$C_{15}H_{24}$	205.1956	X	23.18(T)	204.1878
1(9),10-Pacifigorgiadiene (+)	$C_{15}H_{24}$	205.1956	X	?	204.1878
1(9),10-Pacifigorgiadiene (−)	$C_{15}H_{24}$	205.1956	X	?	204.1878
Valerenol	$C_{15}H_{24}O$	221.1905 220.1298	X X	X 4.28(T;*)	220.1827
Valerenic acid	$C_{15}H_{22}O_2$	X	233.1542	28.01	234.1620
Isoeugenol isovalerate	$C_{15}H_{20}O_3$	249.1491	247.1334	17.90	248.1412
Valerenolic acid	$C_{15}H_{22}O_3$	X	249.1491	22.57	250.1569
N-(p-Hydroxyphenethyl) actinidine	$C_{18}H_{22}NO$	269.1780	X	5.80	268.1701
6,7-Dihydro-4-(hydroxymethyl)-2-(p-hydroxyphenethyl)-7-methyl-5H-2-pyrindinium	$C_{18}H_{22}NO_2$	284.1622	X	3.74	284.1651
Acetylvalerenoic acid	$C_{17}H_{24}O_4$	293.1753	X	25.88	292.1675
Deacetylisovaltrate	$C_{20}H_{28}O_7$	X	379.1757	10.46(T;*)	380.1835
Valtrate	$C_{22}H_{30}O_8$ $C_{22}H_{28}O_8$	X 421.1862	X X	X 21.90	422.1941 420.1784
Kanokoside A	$C_{21}H_{32}O_{12}$	X	475.1816	9.05	476.1894
Berchemol-4-O-glucioside	$C_{26}H_{34}O_{12}$	X	537.1972	8.97	538.2050
Hesperidin	$C_{28}H_{34}O_{15}$	611.1976	609.1820	10.56	610.1898
Kanokoside D	$C_{27}H_{44}O_{16}$ $C_{27}H_{42}O_{16}$	X 623.2551*	X X	X 27.14	624.2629 622.2473
Kanokoside C	$C_{21}H_{18}O_{12}$	639.2500	X	19.45	638.2422
		Possible molecules to be confirmed			
Valerianine	$C_{11}H_{15}NO$ $C_{11}H_{13}NO$	X 175.0997*	X X	X 0.76	177.1154 175.0997
Madolin	$C_{15}H_{22}O_2$	–	233.1454	?	234.1620
Volvalerenal B/Volvalerenal D/Volvalerenic acid A	$C_{15}H_{22}O_2$	–	233.1454	?	234.1620
Volvalerenal B	$C_{15}H_{20}O_3$	X	247.1266	17.87	248.1412
Volvalerenic acid B	$C_{17}H_{24}O_4$	X	291.1525	24.57	292.1675
Volvalerenic acid C	$C_{15}H_{22}O_3$	X	249.1426	22.39	250.1569
Volvalerenal E	$C_{17}H_{24}O_3$	277.1748	X	25.3–25.9(T)	276.1725
Volvalernal A/Volvalerenal C	$C_{17}H_{24}O_4$	X	291.1511	24.44	292.1675

T tentative, * empirical formula calculation inconclusive

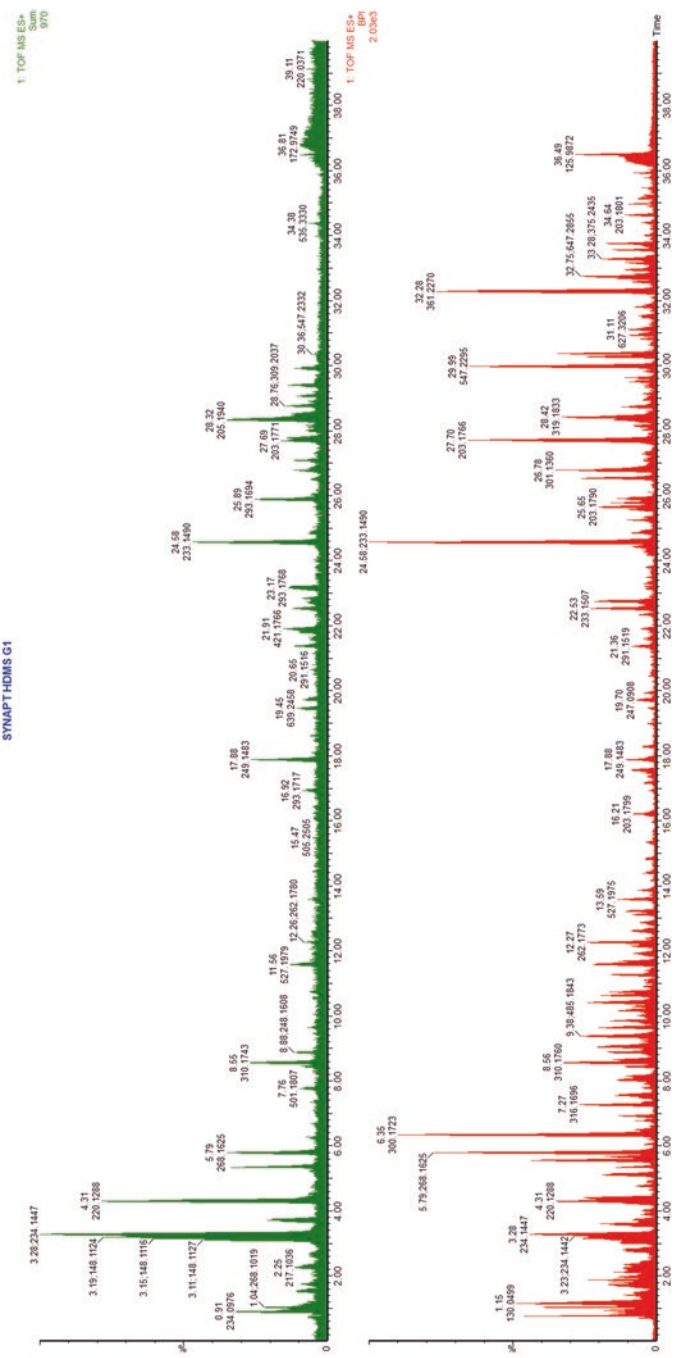

Fig. 10.12 ESI positive BPI (*Red*) and XIC (*Green*) chromatographic separation of the 80% MeOH *Valeriana officinalis* extract

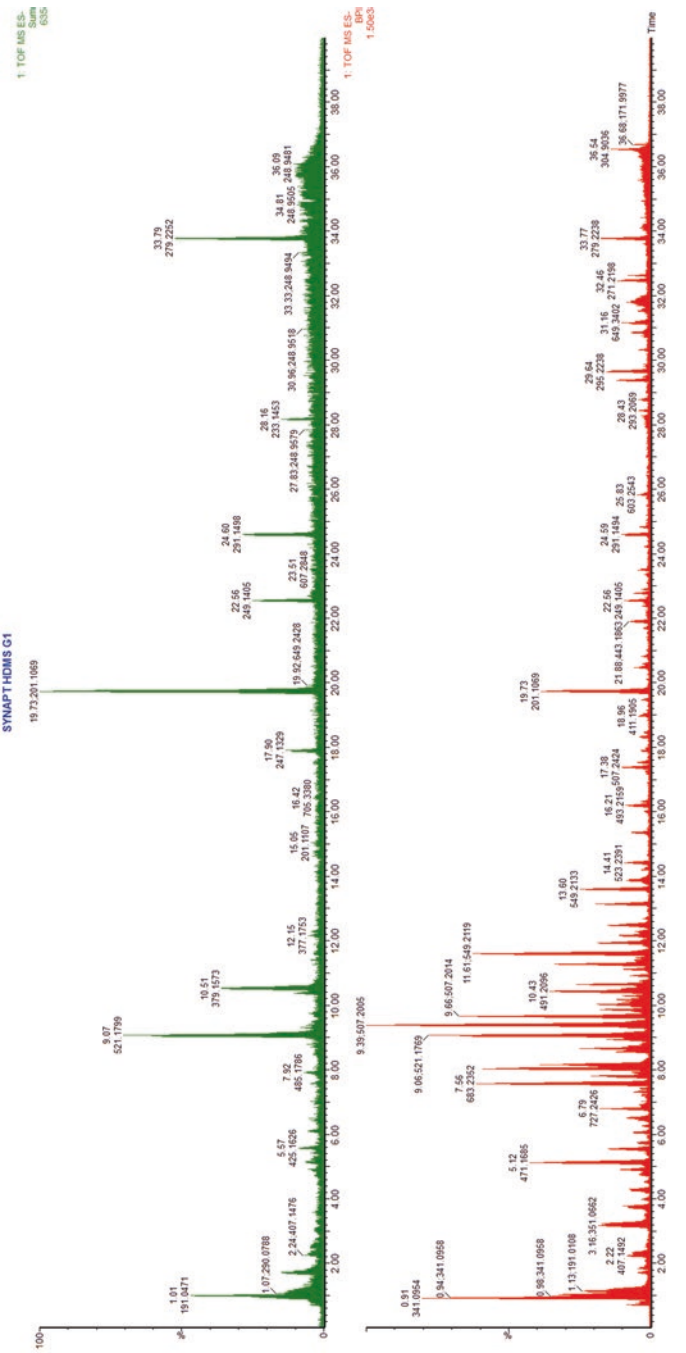

Fig. 10.13 ESI negative BPI (*Red*) and XIC (*Green*) chromatographic separation of the 80% MeOH *Valeriana officinalis* extract

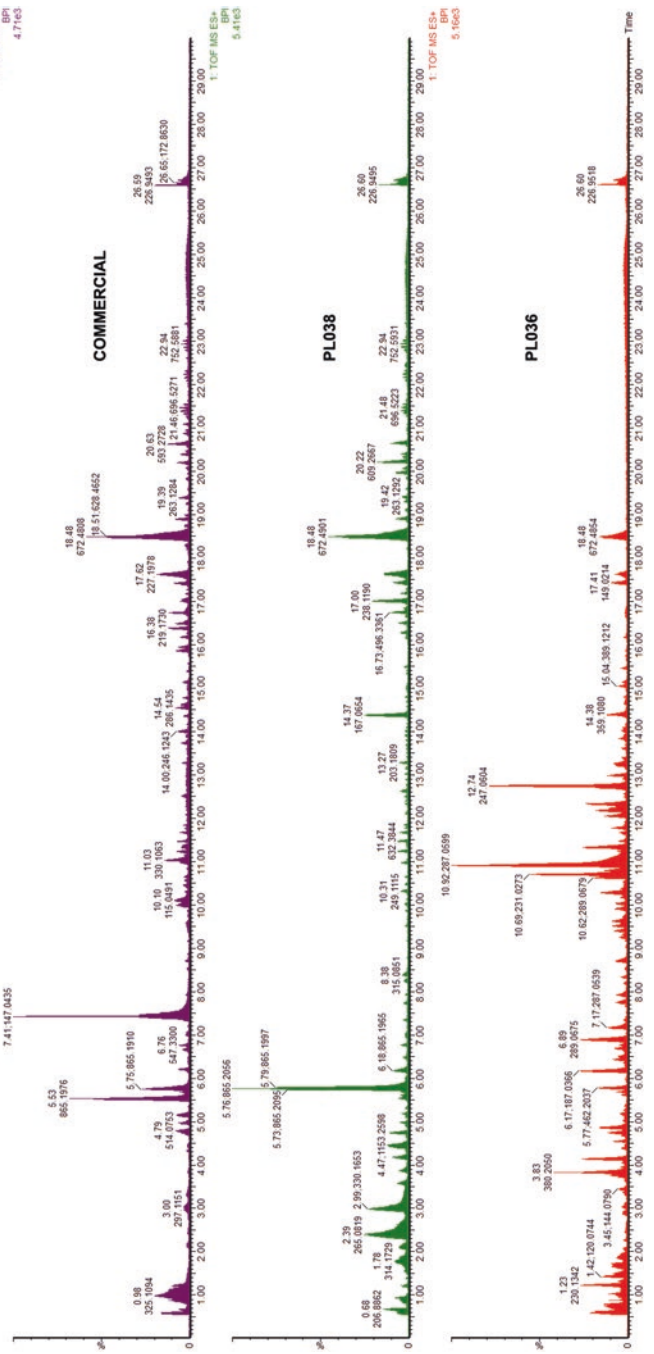

Fig. 10.14 Comparison of the ESI positive BPI chromatograms of three *Cinnamomum* samples extracted with 60% MeOH

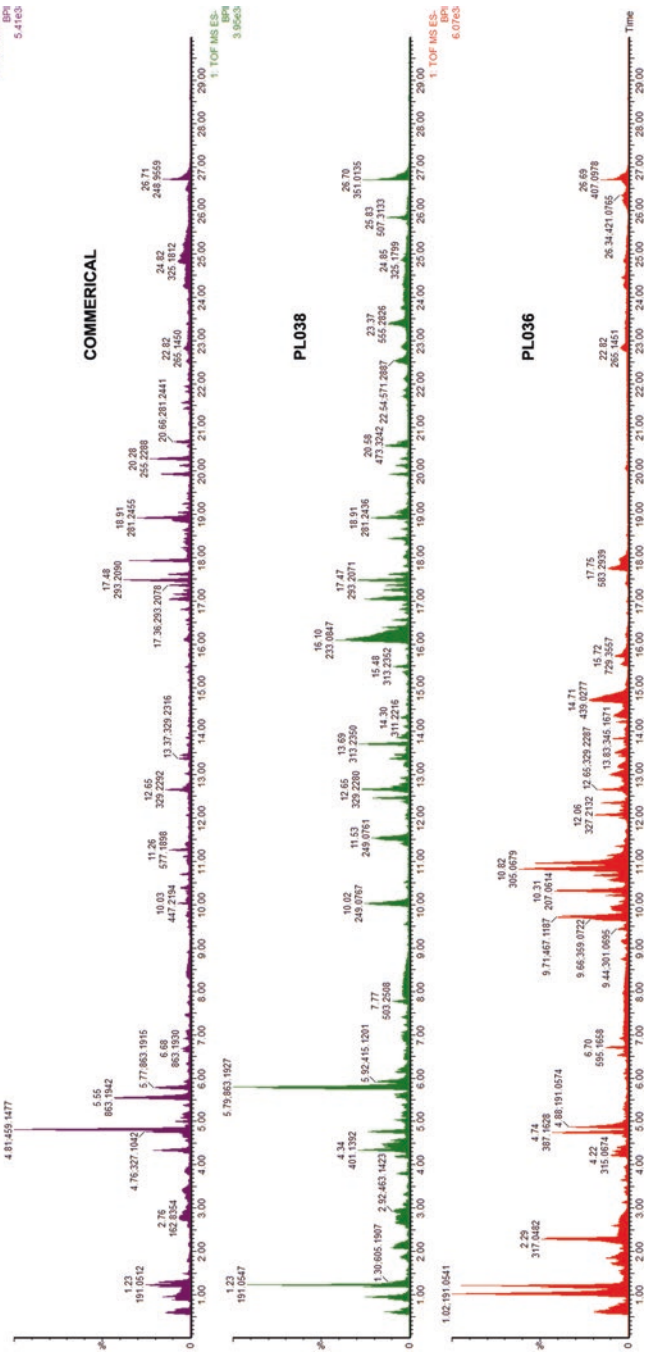

Fig. 10.15 Comparison of the ESI negative BPI chromatograms of three *Cinnamomum* samples extracted with 60% MeOH

Table 10.7 Compounds tentatively detected in the 60% methanol extracts of *Cinnamomum* samples

Compound	Formula	Accurate mass (Da)	Detected mass (Da)		RT (min)
			ESIPos	ESINeg	
Cinnamaldehyde	C_9H_8O	132.0575	X	X	X
Cinnamyl alcohol	$C_9H_{10}O$	134.0732	X	X	X
2-Methoxybenzaldehyde	$C_8H_8O_2$	136.0524	X	135.0358	4.40
Coumarin	$C_9H_6O_2$	146.0368	147.0488	X	6.80
Cinnamic acid	$C_9H_8O_2$	148.0524	X	147.0370	8.11
p-Menth-4(8)-en-1-ol	$C_{10}H_{18}O$	154.1358	X	X	X
Methyl eugenol	$C_{11}H_{14}O_2$	178.0994	X	X	X
Epiafzelechin	$C_{15}H_{14}O_5$	274.2687	X	X	X
Epicatechin	$C_{15}H_{14}O_6$	290.0790	291.0884	289.0712	Multi
Epigallocatechin	$C_{15}H_{14}O_7$	306.0739	X	305.0620	10.94
Cinnzeylanol	$C_{20}H_{32}O_7$	384.2148	X	X	X
Cinnzeylanine	$C_{22}H_{34}O_8$	426.2254	X	X	X
Epicatechingallate	$C_{22}H_{18}O_{10}$	442.0900	X	X	X
Procyanidin A1 or Procyanidin A2	$C_{30}H_{24}O_{12}$	576.1268	577.1346	575.1187	6.64
Unknown	$C_{30}H_{24}O_{12}$	576.1268	577.1380	575.1187	7.05
Unknown	$C_{30}H_{24}O_{12}$	576.1268	577.1311	575.1187	8.03
Procyanidin B1	$C_{30}H_{26}O_{12}$	578.1424	579.1412	577.1312	3.84
Procyanidin B2	$C_{30}H_{26}O_{12}$	578.1424	579.1412	577.1312	4.77
Epicatechin(2β → 7,4β → 8) epicatechin(4β → 8)epicatechin	$C_{45}H_{36}O_{18}$	864.1902	865.1839	863.1890	5.35
Epicatechin(2β → 7,4β → 8) epicatechin(4α →8)epicatechin (Pavetannin B2; Cinnamtannin B1)	$C_{45}H_{36}O_{18}$	864.1902	865.1810	863.1910	5.45
Unknown	$C_{45}H_{36}O_{18}$	864.1902	865.1810	863.1902	6.15
Procyanidin C1	$C_{45}H_{38}O_{18}$	866.2058	867.1992	865.2019	4.81
Epicatechin(4β → 6) epicatechin(2β → 7,4β → 8) epicatechin(4β → 8)epicatechin (Pavetannin C1)	$C_{60}H_{48}O_{24}$	1152.2536	1153.2266	1151.2609	4.42
Epicatechin(4β → 8) epicatechin(2β → 7,4β → 8) epicatechin(4β → 8)epicatechin (Cinnamtannin B2)	$C_{60}H_{48}O_{24}$	1152.2536	1153.2280	1151.2667	5.70
Procyanidin D	$C_{60}H_{50}O_{24}$	1154.2692	1155.2522	1153.2853	5.07
[Epicatechin(4β → 8)]2epicatechin(2β → 7,4β → 8) epicatechin(4β → 8)epicatechin (Pavetannin D1)	$C_{75}H_{60}O_{30}$	1440.3170	1442.2297	1439.3428	4.79
Unknown	$C_{75}H_{60}O_{30}$	1440.3170	1442.2430	1439.3339	4.99
Unknown	$C_{75}H_{60}O_{30}$	1440.3170	1442.2273	1439.3428	5.19

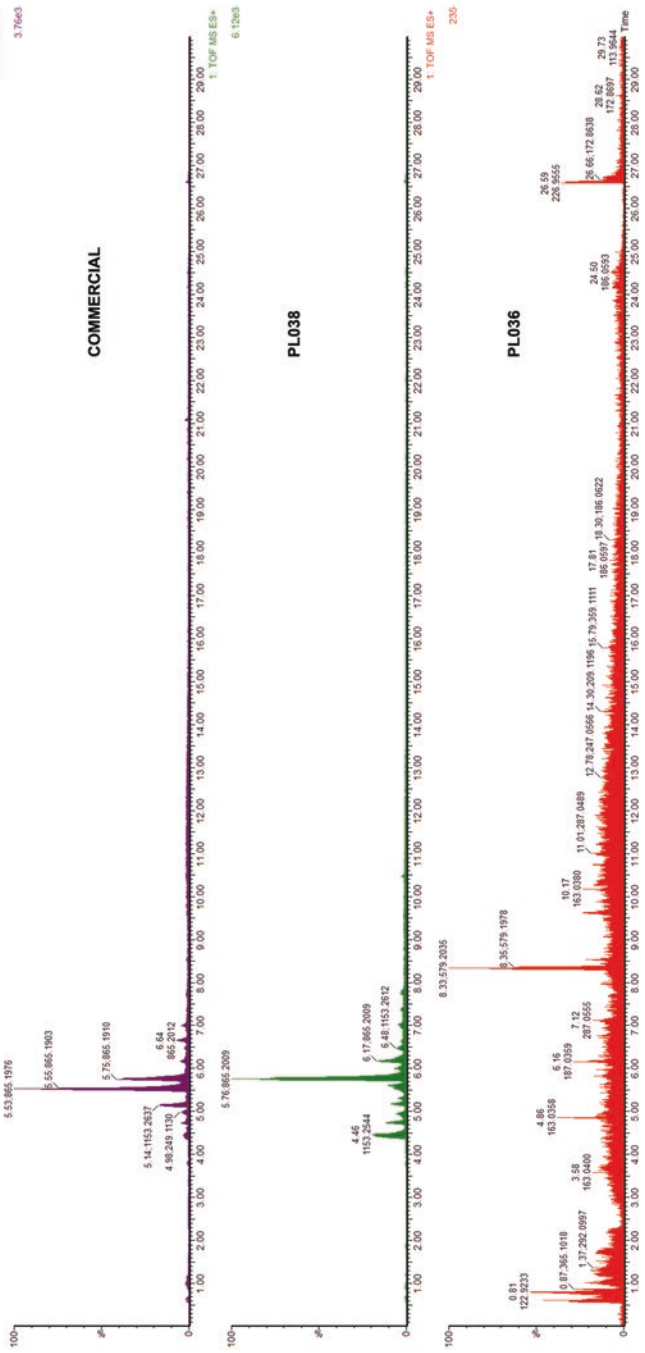

Fig. 10.16 Comparison of the ESI positive XIC chromatograms of three *Cinnamomum* samples extracted with 60% MeOH

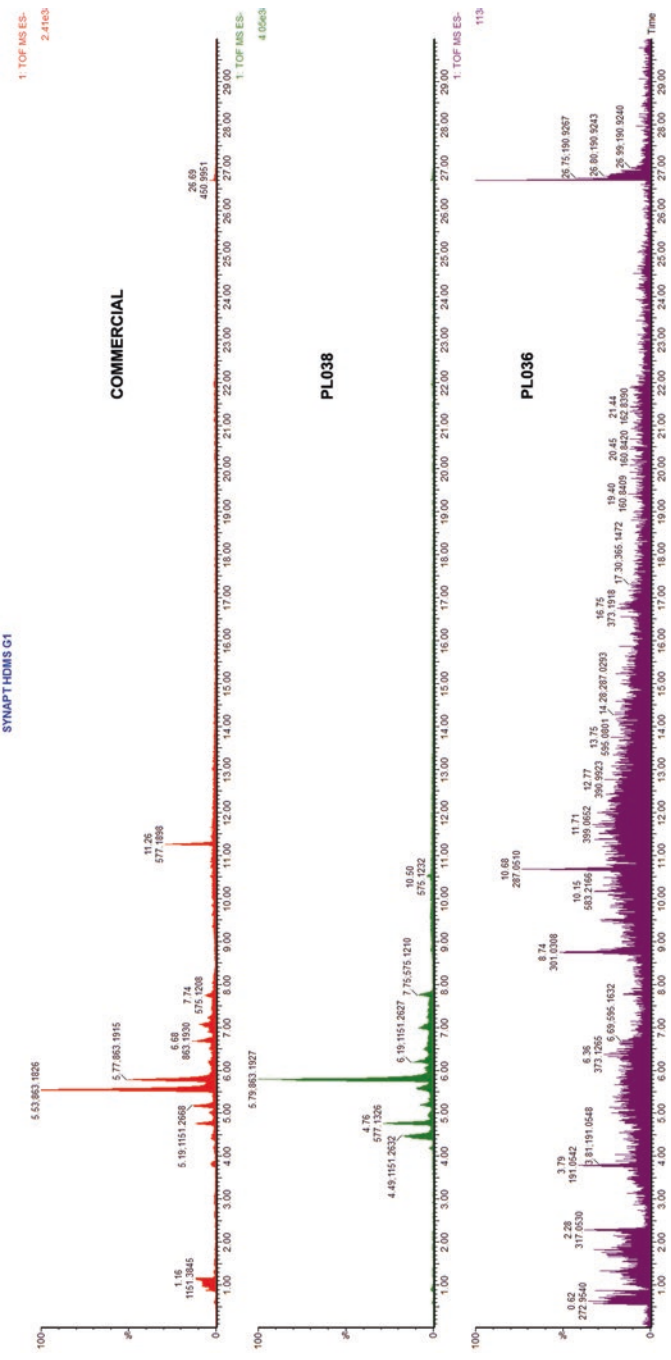

Fig. 10.17 Comparison of the ESI negative XIC chromatograms of three *Cinnamomum* samples extracted with 60% MeOH

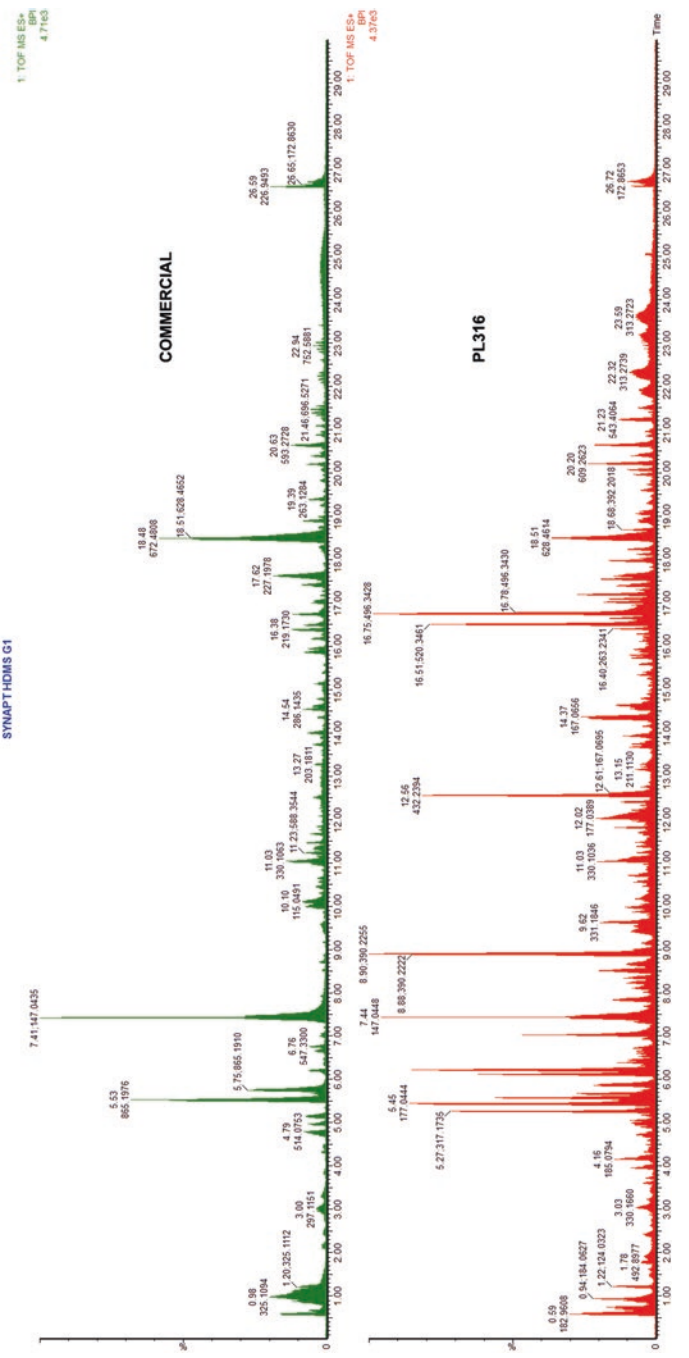

Fig. 10.18 ESIPos BPI chromatograms of the commercial samples (PL316 and Commercial)

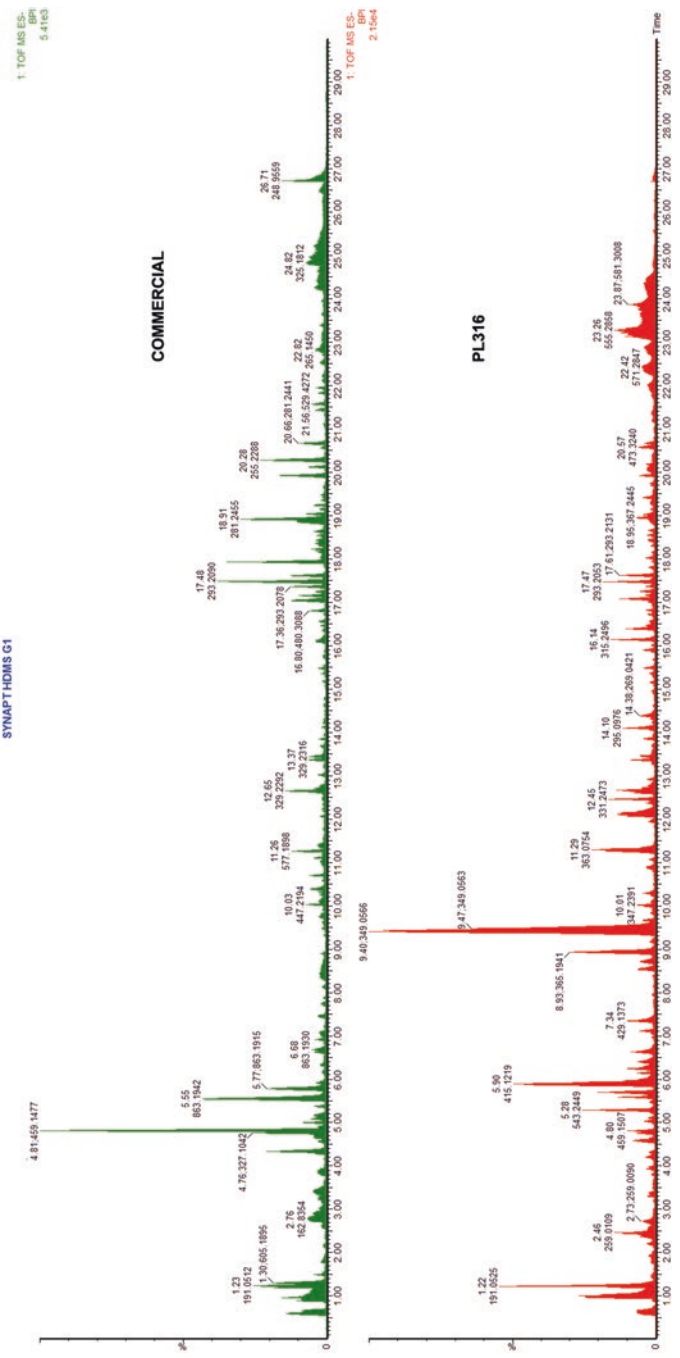

Fig. 10.19 ESINeg BPI chromatograms of the commercial samples (PL316 and Commercial)

comparing the detected compounds in the two ionisation modes, it was clear that PL036 and PL038 differed from the Commercial sample. An analysis of the mass spectrometric data of Figs. 10.14 and 10.15 (60% methanol extracts) revealed that a significant number of known compounds could be detected and tentatively identified (Table 10.7).

The evaluation of the three samples by extracting the masses listed in Table 10.7 and larger than 570 Da, produced even more evidence that the samples differed significantly. PL038 and the Commercial sample displayed the greatest similarity while PL036 did not display any of the marker compounds observed in the other two samples (Figs. 10.16 and 10.17).

Due to the significant differences observed between PL036, PL038 and the Commercial sample, another "commercial" sample (PL316) was obtained from the PlantLIBRA network. The analysis of the two commercial products (PL316 and Commercial) produced interesting results (Figs. 10.18 and 10.19). These two samples were supposedly derived from *Cinnamomum* plant material but displayed significant differences in chemical composition. The observed differences might be the result of different processing protocols employed by the manufacturers of these commercial products.

10.4 Conclusion

Using UPLC-MS for the authentication of food supplements, botanical formulations, or extracts, and detecting the presence of new or possible contaminants, represents a challenging task due to the complexity of the plant-based products. It however provides improved capabilities as it is possible to separate the different compounds from each other and the high mass accuracy of the MS can aid in the tentative identification of the different compounds in the absence of reference standards.

The use of formulated samples, as illustrated in some of the examples, or even plant extracts from different global locations, highlights the challenges encountered during the chemical profiling of plant-based material. Some of the main reasons include the absence of marker compounds, the presence of new and unexpected compounds, or significant differences in concentration. The use of a number of major compounds always present in a specific species can be used as evidence of a specific plant extract being used. Careful examination of new peaks will be necessary to determine if they are contaminants such as pesticides or if they are merely due to the location or origin of the plant extract. The power of accurate mass can also be extended to detect the presence of adulteration of plant material. The growth of spectral libraries in the UPLC-MS area will also be invaluable for the authentication of food supplements and herbal formulations and the detection of contaminants in plant-based products and formulations. The applicability of profiling plant extracts using UPLC-MS has been illustrated through the six plant examples

described and can be expanded by creating detailed dossiers of plants and plant extracts used as food supplements or in herbal formulations.

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Chapter 11
Classic/Recommended Methods and Development of new Methods to Control Residues and Contaminants of Botanicals

Mihaela Badea, Laura Floroian, Angela Marculescu, Liviu Gaceu, Marius Moga, Laura Gaman, Codruta Cobzac, Qi Chang, Jian Xue, and Patrizia Restani

Abstract A remarkably high number of analytical methods concerning bioactive compounds, contaminants and biomarkers of exposure in Plant Food Supplements (PFS) have been developed by different scientific groups.

The work present an update of classic and new developed methods for detection of several heavy metals, pesticides and mycotoxins from different samples. The advantages, the characteristics of different analytical procedures, as well as the possible interferences, were underlined.

Some of the novel methods, in particular biosensors, for heavy metal pesticide and mycotoxin analysis in PFS and microchip based tools (Screen Printed Electrodes) seems to be available for routine analytical tools in the near future.

Keywords Contaminants • Residues • Pesticides • Heavy Metals • Mycotoxins • Radiations

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11.1 Introduction

The observed substantial increase in the use of herbs/botanicals and their products, especially extracts for plant food supplements (PFS) over the last two decades was not always adequately accompanied by quality and safety control (Garcia-Alvarez et al. 2014). Food supplements, first of all, belong to the food sector and must therefore comply with all relevant requirements of EU food legislation concerning composition, quality and safety. This includes in particular identity/authenticity, contaminants, residues (of e.g. pesticides) and other undesired substances (Sanzini et al. 2011).

Considering the multi-component nature and differentiated bioactivity of PFS, a proper and meaningful quality control must also include the analysis of a broad spectrum of potential contaminants or undesired substances in plants. This chapter will consider the most common contaminants and residues interesting the botanical market: heavy metals, pesticides and mycotoxins.

11.2 Heavy Metals

The scientific reviews performed for analysis of heavy metal from plants, showed that most data concerning the detection methods are available on cadmium, lead and mercury. Few data were available from systematic review for detection of thallium (Tl), chromium (Cr) and antimony (Sb) in plants and plant derivatives.

Because the concentration of heavy metals in plants (roots, leaves) are in the trace and ultra-trace range, the World Health Organization (WHO) established maximum concentration limits for these residues in botanicals, in order to be safety used. Suitable methods are necessary for their evaluation and most appropriate sample preparation methods are required. Zeiner and Cindric (2017) preformed an intensive review of the available reported methods underlining the inductively coupled plasma atomic/optical emission spectrometry—ICP-AES/OES; inductively coupled plasma mass spectrometry—ICI-MS which are considered multi-elemental methods used to obtain good analytical data in a short time. Other representative methods are recommended: (flame) atomic absorption spectrometry ((F)AAS), electrochemical methods, charged particle induced activation analysis (CPAA), neutron activation analysis (NAA), X-ray fluorescence methods (total reflection, energy dispersive or particle induced X-ray emission), thin layer chromatography TLC), ion chromatography (IC) and Mossbauer spectrometry.

11.2.1 Detection by Atomic Absorption Spectrometry (AAS)

On the basis of the literature search, the most used methods for heavy metals detection in plant food supplements are atomic absorption and emission methods. These are very sensitive methods, but very expensive, both to be acquired and exploited and need well trained personnel.

11.2.1.1 Working Procedure

Sample preparation for analysis: The sample is processed properly (chopped, minced, grinded, homogenized) in order to obtain a uniform and homogeneous mass. To prepare the sample for analysis must be available a sample mass of at least 200 g of edible parts of laboratory sample. Processed sample to be stored in plastic bags or hermetically sealed cans. Samples used for the experiments were obtained from a local market from Brasov: fresh and frozen sea-buckthorn—*Hippophae rhamnoides*; fresh bilberry—*Vaccinum myrtillus*. Some samples were purchased from Naturist shops from Brasov, Romania: Tinctura de senna- tincture of senna (*Cassia angustifolia*) (manufacturer Steaua Divina) and the leaves of senna (*Cassia angustifolia*) commercialized as Frunze Senna (manufacturer Phares Bio Vital)—tea from medicinal plants. All chemicals used for the preparation of stock and standard solutions were of analytical reagent grade and purchased from Sigma-Aldrich or Merck. Standard solutions were of 1000 mg/L of metal concentrations.

Dry mineralization: The product (10–20 g) was dried and burned slowly on a hotplate until the stage of coal In the final phase of coal combustion ignition can occur. Attention should be given to the burning products or splash inflation tended to avoid losses during these operations. After completing of previous step (no smoke in the crucible), the crucible is inserted into furnace set at 450° ± 10 °C, where will remain a convenient time to complete calcination (10–16 h). The ash must have uniform colour (white or grey) and black dots contain no carbon. After cooling, the crucible is placed on the site of asbestos and expects them completely cool in order to add hydrochloric acid. If the sample is not completely moisten, the ash is treated with 1–3 mL of deionized water or hydrogen peroxide. It will be placed back in the oven at no more than 200 °C and gradually increase the temperature up to at 450° ± 10 °C for 1–2 h or more.

For liquid samples the amount taken is about 100–200 mL, which will be weighted, evaporated to dryness and then will be used working protocol previously presented.

Sample mineralization: In the crucible with ash will be added 5 mL HCl 6 M, so that all ashes to contact with acid. Evaporate the acid using bath sand. Residue is then dissolved in an exact volume of nitric acid 0.1 M (10–30 mL). Cover with a watch glass and allow standing from 1–2 h. There were stirred the solution well in the crucible with a wand, filtered, moving the contents into a glass bowl, which will be used for direct determinations. If, in the analyzed sample the content of element is greater than the maximum standard calibration solution, then the solution is diluted page properly. Mineralized sample solution can be used to determine: Pb, Cd, Cu, Zn, Fe.

Determination of heavy metals from samples obtained by dry mineralization, using AAS. According to the specific program of the device (specific for each AAS equipment) are performed the steps of initiating, calibration and after reading analysed samples. The AAS analysis was performed using the collaboration of Sanitary Veterinary Direction and for Food Safety Direction of Brasov, Romania. Correction factor in drawing standard curve must be at least 0.9850. After reading each sample,

Table 11.1 Instrumental parameters for detection of Cd and Pb using AAS

	Detection of Cd	Detection of Pb
System type	Flame	Flame
Flame type	Air-acetylene	Air-acetylene
Fuel flow	1.0 L/min	1.0 L/min
Oxidant flow	10 L/min	10 L/min
Lamp current	3.0 mA	5.0 mA
Wavelength	228.8 nm	217.0 nm
Read time	3.0 s	3.0 s
Replicated	3	3

Table 11.2 Concentration of Cd and Pb in tested samples

Sample type	Concentration of Cd (μg/mL)	Concentration of Pb (μg/mL)
Dried sea-buckthorn—*Hippophae rhamnoides*	0	0.034
Frozen sea-buckthorn—*Hippophae rhamnoides*	0	0.031
Fresh bilberry fruits—*Vaccinum myrtillus*	0	0.028
Tincture of senna—*Cassia augistifolia*	0	0.053
Leaves of senna—*Cassia augistifolia*	0	0.013

the aspiration route is washed with acid previous used to dilute sample (nitric acid). Instrumental parameters are listed in Table 11.1.

Calibration lines were obtained. Using interpolation procedure, there were analysed real samples (plants, fruits, tea and tincture) (Table 11.2).

No level of Cd and low concentrations of Pb were detected in all samples. This procedure could be used for detection of heavy metals from different matrices and also for validation step for new developed methods for heavy metals detection.

11.2.2 Detection by Electrochemical Detection

New methods used in environmental monitoring use sensors and biosensors. Sensors are translating chemical or physical information into a measurable signal (electrical one). The most used sensors are electrochemical which translate redox reactions that are produced at surface of the working electrode immersed into an electrochemical cell. These methods are intended to be used for sensors development for heavy metals detection in plant extracts and plant food supplements as new methods (Buzea et al. 2012; Florescu et al. 2009).

Scientific literature indicated the use of biosensing systems based on well-known interactions between heavy metals and biomolecules (e.g. proteins, peptides, enzymes, antibodies, whole cells, and nucleic acids) (Mehta et al. 2016).

Enzyme-based biosensors are also indicated by scientific literature as possible method for detection of heavy metals as mercury (Wang et al. 2012), chromium (Michel et al. 2006) cadmium (David et al. 2011).

Specific studies were done to optimize the detection of heavy metals using free and immobilized *Penaeus merguiensis* alkaline phosphatase on gold nanorods (Homaei 2017). Reliable results were obtained for pH11.0 and a temperature close to 60 °C.

Recent review studies highlights the major advances of DNA-based electrochemical biosensors for the detection of heavy metal ions such as Hg2+, Ag+, Cu2+ and Pb2+ (Saidur et al. 2017; Zhan et al. 2016).

11.2.3 Detection by Thin Layer Chromatography (TLC)

Scientific studies indicated Thin Layer Chromatography (TLC) as a simple and cheap method for heavy metals detection (Badea et al. 2009).

Other studies (Agarwal and Behari 2007) applied the method for screening the mercury in environmental samples (water and aqueous industrial effluent samples) and urine. Mercury was detected by complexation with dithizone followed by TLC, also in the presence of other heavy metals, including arsenic, cadmium, lead, copper, iron, zinc, and nickel.

11.3 Mycotoxins

There is an increasing concern for mycotoxin contamination in foods and feeds, because they can be found in a wide range of commodities including cereals, spices, dried fruits, apple products, wine and coffee. Previous studies (Efuntoye 1999) have demonstrated that aromatic and/or medicinal herbs are susceptible to mycotoxin contamination. Fungal contamination may occur pre-harvest or as a result of poor production practices. The treatments used for reducing microbial load (irradiation or steam treatment) may not be suitable for mycotoxins destruction, if present. Nowadays, the consumption of medicinal and aromatic herbs is increasing, either for their therapeutic or natural properties, which may lead to an increase in the intake of mycotoxins (Sanzini et al. 2011).

The most important mycotoxins are aflatoxins (AFs), ochratoxin A (OTA), zearalenone (ZEA), deoxynivalenol (DON), T-2 toxin and fumonisins (FBs), produced by different genus of fungi (Table 11.3).

Only few data on the occurrence and levels of mycotoxins in herbs and plant food supplements have been published. For EU regulated mycotoxins in food samples, are identified chromatographic and immunochemical methods for their detec-

Table 11.3 Fungi producing mycotoxins from (Santos et al. 2009)

Mycotoxin	Fungi producing mycotoxins
Aflatoxins	*Aspergillus flavus*
	Aspergillus parasiticus
	Aspergillus nomius
Ochratoxin A (OTA)	*Penicillium verrucosum*
	Aspergillus ochraceus
	Aspergillus carbonarius
Zearalenone (ZEA)	*Fusarium*
Deoxynivalenol (DON)	
T-2 toxin	
Fumonisins (FBs)	
Citrinin	*Penicillium citrinum*
	Penicillium expansum
	Penicillium verrucosum

tion, with good analytical performances. Some new methods (fast, reliable, low costs) would be optimised and recommended. The EFSA Panel on Contaminants in the Food Chain (CONTAM Panel) carries out risk assessment on contaminants in food and feed and recommends intensive scientific research in order to identify if their presence could be associated with adverse health effects in the European population. The presence of hazardous chemical contaminants or undesirable substances in food and feed is often unavoidable as these substances may occur ubiquitously or are of natural origin. Therefore, human and animal exposure to such substances is also unavoidable. The Rapid Alert System for Food and Feed (RASFF) (http://ec.europa.eu/food/safety/rasff_en), managed by The Health and Consumers Directorate-General of the European Commission, consists essentially of clearly identified contact points in the Commission, EFSA, EEA and at national level in member countries, exchanging information in a clear and structured way by means of an online system, iRASFF. According to the seriousness of the risks identified and the distribution of the product on the market, the RASFF notification is classified after verification by the Commission contact point as alert, information or border rejection notification before the Commission contact point transmits it to all network members.

According with recently published RASFF report (http://ec.europa.eu/food/safety/docs/rasff_annual_report_2015.pdf), in 2015 there were 475 notifications on mycotoxins in food, most related to the presence of aflatoxins (421 notifications).

11.3.1 Detection by Chromatographic Methods

The detection and continuously monitoring of mycotoxins is important for the safety reason (Habibipour et al. 2016). Several procedures were optimized and recommended by the scientific research teams.

Depending on the detection systems used after HPLC separation, there were successfully tested: quadrupole linear ion trap mass spectrometry (Xing et al. 2016). MS-MS (Li et al. 2016a, b), fluorescence detector (Chen et al. 2016), UV diode array detection (Urraca et al. 2016).

Different procedures for extraction and cleaning samples were tested also: clean-up methods-with BondElut Mycotoxin and MycoSep 227 columns (Bernhardt et al. 2016), multiple antibody immunoaffinity columns (Zhang et al. 2016), magnetic molecularly imprinted polymers for selective extraction (Urraca et al. 2016), online solid phase extraction (Campone et al. 2016), solid bar microextraction (Al-Hadithi et al. 2015), liquid-liquid extraction (Kwaśniewska et al. 2015), enzyme-assisted extraction (Pietri et al. 2016).

A simple and efficient method for determining multiple mycotoxins was developed using a QuEChERS (quick, easy, cheap, effective, rugged and safe)-based extraction procedure in vegetable oils. Following this extraction step, high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) was used for the quantification and confirmation of 16 chemically diversified mycotoxins, in 62 vegetable oil samples (Zhao et al. 2017). Zearalenone (ZEN), aflatoxin B1 (AFB1), aflatoxin B2 (AFB2), aflatoxin G1 (AFG1) and α-zearalenol (α-ZOL) were detected, with maximum concentrations of 0.59 (AFG1)-42.5 (ZEN) ng/g. The method developed has the advantages of high sensitivity, accuracy and selectivity, and it can be applied to the target screening of mycotoxins in real samples.

LC, HPLC and LC-MS/MS procedures offered the advantage of the quantification of individual toxins in contrast to ELISA technique. HPLC and LC-MS/MS methodologies are able to identify greater number of contaminated samples in comparison to TLC and ELISA techniques. Furthermore, HPLC and LC-MS/MS techniques offer an added advantage for the detection of aflatoxins in low concentration in cereals samples (Iqbal et al. 2014).

11.3.2 Detection by Sensors-Biosensors (Enzyme-Based Biosensors and Immunosensors)

In order to quantify the OTA concentration in beverage samples such as beer and wine, there were used fluorescence detection based on the analysis of the brightness and a, b for the color-opponent dimensions (L*a*b) and Hue, Saturation, Value (HSV) tests (Bueno et al. 2016).

Using their advantages of high sensitivity and rapidity, advanced sensors based on antibodies or aptamers are used in the mycotoxin detection. Having the possibility of miniaturization, low costs, these sensors are applicable to high-throughput modes. Optical and electrochemical sensing modes were used and it was underline in several studies the challenges and the future of antibody or aptamer-based sensors (Rhouati et al. 2016; Xu et al. 2016).

Impedimetric immunosensors for ochratoxin A (OTA) (Badea et al. 2016a) and respectively for aflatoxin B1 (Badea et al. 2016b) detection, were developed via the immobilization of monoclonal specific antibody on bovine serum albumin modified gold electrodes. A four-step reaction protocol was tested to modify the gold electrode and obtain the sensing substrate. All the steps of the immunosensor elaboration and also the immunochemical reaction between surface-bound antibody and ochratoxin A were analyzed using cyclic voltammetry and electrochemical impedance spectroscopy. Modification of the impedance due to the specific antigen-antibody reaction at immunosensor surface, was used in order to detect ochratoxin A. Linear proportionality of the charge transfer resistance to the concentration of OTA allows ochratoxin A detection in the range of 2.5–100 ng/mL (Badea et al. 2016a).

The steps of the procedures for obtaining impedimetric immunosensor for aflatoxin B1 detection were followed using atomic force microscopy (AFM) and electrochemical impedance spectroscopy (EIS) (Badea et al. 2016b). The resistance to charge transfer (Rct) was the most sensitive parameter to changes induced to the interfacial properties of the immunosensor by the incubation with aflatoxin and varied linearly with aflatoxin concentration in the range 1–20 ng/mL. The immunosensor was applied for the detection of aflatoxin in spiked liquorice extracts with good recovery factors.

Other type of biosensors used recombinant cell fluorescence sensor was reported simple and rapid of DON and ZEN (Ji et al. 2016).

11.3.3 Detection by Surface Plasmon Resonance (SPR)

In Surface Plasmon Resonance the glass prism is coated with a gold film. In conducting metals, such as Au, the free conduction electrons form periodic oscillations, called plasma waves (https://nicoyalife.com/technology/surface-plasmon-resonance/how-surface-plasmon-resonance-works/). Like every periodic electromagnetic wave, this can also be described in a particle fashion. Like photons and phonons are the particle names for light and sound waves, respectively, a plasmon is the particle name for the plasma wave (Vermeeren et al. 2009).

The binding of target by immobilized aptamers or antibodies determines the changes in the composition of the material at the interface between the Au and the buffer and will alter the momentum of the surface plasmons, and their associated evanescent wave. As a consequence, SPR no longer occurs at the previous incidence angle, and a SPR shift takes place. The shift in the resonance angle is directly proportional to the change in mass at the Au surface (Fig. 11.1).

Recently, multiplex surface plasmon resonance biosensing were developed (Joshi et al. 2016a). Preliminary in-house validation of a portable nanostructured imaging surface plasmon resonance (iSPR) instrument showed that DON, T-2, ZEA and FB1 can be detected at the European Union regulatory limits, while for OTA and AFB1 sensitivities should be improved.

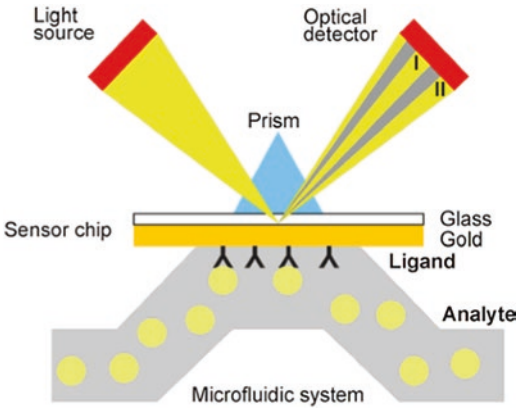


Fig. 11.1 Principle of detection using SPR (modified from Hodnik and Anderluh (2009))

Analysis of mycotoxins were tested by different research teams which recommend their detection in beer sample (Joshi et al. 2016b), wheat (Sanders et al. 2016), milk (Karczmarczyk et al. 2016), red yeast rice (Atar et al. 2015), wine and peanut oil (Zhu et al. 2015).

Commercially available, the analytical systems have exploited high affinity polyclonal monoclonal and recombinant antibodies, conducting to rapid, accurate and sensitive means of determining of several mycotoxins. Combination of surface plasmon resonance with enzyme-derivatised sensors, molecularly imprinted polymers, fluorescence spectroscopy and the use of gold nanoparticles for signal enhancement are indicated in several applications (Meneely and Elliott 2014).

11.3.4 Detection by Immunoassay

Different immunochemical strategies were optimised to detect contaminants and residues from food matrices and biological samples (Badea et al. 2010)

The four strains of *Aspergillius* and *Penicillium* isolated from plants matrices were evaluated for their ability to produce AFB1 and AFB2 and OTA. *A flavus* revealed production of 3.5 µg/kg AFB2 and 3.8 µg/kg OTA. *A Flavus* produced 7.45 µg/kg AFB1 and *A ochraceus* produced 21.7 µg/kg AFB2 and 7.25 µg/kg OTA (Alwakel 2009).

Three detection methods were compared for OTA detection from soybeans: lateral flow strip assay using strip with mimotope peptide, ELISA and lateral flow strip assay using strip with OTA–BSA (Lai et al. 2009).

A method using an immunochromatographic kit has been adopted as the official screening method in Japan, and criteria for the kit have been set (Yoshinari et al. 2016). In order to confirm whether commercial immunochromatographic kits for detecting AFM1 satisfy these criteria, the performance of four kits was evaluated by performing spike-and-recovery experiments using AFM1-free milk samples and milk samples spiked at seven levels (100–700 ng/kg).

A multiplex lateral flow immunoassay (LFA) was developed for the simultaneous on-site determination of three mycotoxins (aflatoxin B1, zearalenone and ochratoxin A) in corn, rice and peanut (Chen et al. 2017). There were optimised the preparation of antibody-gold nanoparticle conjugates, the size of gold nanoparticle and the position of capture antigen. This developed LFA can obtain a visual detection limit of 10 μg/kg for aflatoxin B1, 50 μg/kg for zearalenone and 15 μg/kg for ochratoxin A. For quantitative analysis, the limits of detection were 0.10–0.13 μg/kg for aflatoxin B1, 0.42–0.46 μg/kg for zearalenone, and 0.19–0.24 μg/kg for ochratoxin A, which were far below the regulatory limits set by the European Commission.

Advantages of enzyme-linked immunosorbent assays with chemiluminescent (CL-ELISA) were demonstrated by comparison with ELISA with colorimetric detection (COL-ELISA) (Yu et al. 2011).

Some of the methods recommended by scientific literature for detection of aflatoxins and ochratoxin in soybeans, fennel, cinnamon and orange are presented in Table 11.4.

Cleaning procedures are sometimes reported in the scientific literature, using different columns for separations steps (Fig. 11.2).

Immunoaffinity columns (IAC) (Fig. 11.2a, b) are based on a specific antibody-analyte binding technology. Immunoaffinity columns contain a gel bed with toxin-specific antibodies coupled to the gel particles. These antibodies will capture a specific mycotoxin present in a sample and release them again after an elution step.

Molecularly imprinted polymers (MIPs) are highly stable crosslinked polymers that possess selective molecular recognition properties for specific molecules considered as template (imprint) molecules. The cavity which remains after the molecule removal is used for a selective separation/clean-up/pre-concentration of molecule of interest from complex matrices (Saini and Kaur 2013). The method using MIP (molecularly imprinted solid phase extraction, MISPE) has the advantages to be not only highly selective and specific, but also chemically and thermally stable, compatible with all solvents and cost effective.

Aptamers are short single-stranded oligonucleotides chains are synthesized by SELEX (systematic evolution of ligands by exponential enrichment) (McKeague et al. 2014). Aptamers columns are able to recognize and bind to targets with high affinity and selectivity through non-covalent interactions.

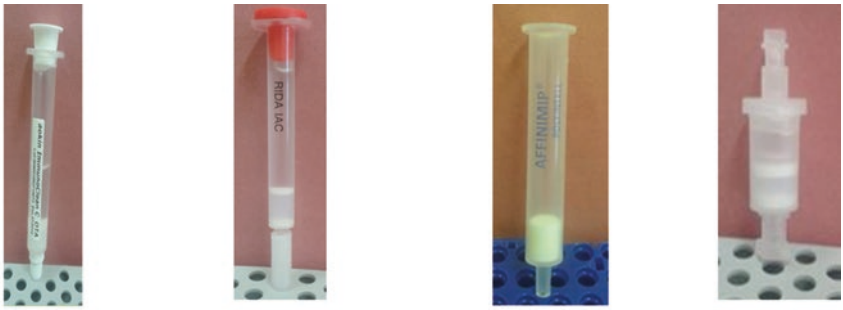
11.4 Pesticides

The effects of acute and chronic exposure to pesticides determine the scientists to propose and to optimize different detection methods for their analysis (Farcas et al. 2013).

In 2015, the number of RASFF notifications for pesticide residues decreased slightly further to 402 (http://ec.europa.eu/food/safety/docs/rasff_annual_report_2015.pdf) and only 34 notifications are about products of EU origin. Seven of these notifications concerned feed. The possible explanation of the decrease of RASFF notifications is that entry points to the EU have reinforced border checks.

Table 11.4 Characteristics of immunochemical methods used for the detection of aflatoxins and ochratoxins from soybeans, fennel, cinnamon and orange samples

Sample		Mycotoxin	Method	Characteristics of the detection	References
Glycine max (*L.*) *Merr.*	Soybean	AFB1	CLEIA	LOD of AFB1 was 0.01 ng/g Linear range was 0.05–10.0 ng/g, Cross reaction rates of DON, OTA, ZEA, AFB2 and AFG2 were less than 1%, the average recovery was 79.8–115.4%, Sensitivity was 0.01 ng/g, which was one-fifth of ELISA	Fang et al. (2011)
Glycine max (L.) Merr.	Soybean	AFB1	Immunoenzymatic assay on membrane; in situ sample cleanup	LOD 5 μg/kg The average recoveries from different noninfected food samples spiked with AFB1 at concentrations of 5 to 100 g/kg were between 99 and 105% Good correlation with HPLC tests	Pal et al. (2005)
Cinnamomum verum J. Presl	Cinnamon	AFB1, AFB2	Elisa—HELICA	μg/kg	Alwakel (2009)
Foeniculum vulgare L.	Fennel	AFB1, AFB2	Elisa–HELICA	μg/kg	
Citrus sinensis (L.) Osbeck	Orange	OTA	ELISA—Ochra kit	LOD range—Ng/g	Marino et al. (2009)
Glycine max (*L.*) *Merr.*	Soybean	OTA	Lateral flow strip assay using strip with mimotope peptide	LOD—10 ng/mL	Lai et al. (2009)
Glycine max (L.) Merr.	Soybean	OTA	ELISA	LOD—0.1 ppb	
Glycine max (*L.*) *Merr.*	Soybean	OTA	Lateral flow strip assay using strip with OTA–BSA	LOD—10 ng/mL	
Glycine max (L.) Merr.	Soybean	OTA	CL-ELISA—Competitive chemiluminescent enzyme-linked immunosorbent assay	Levels from 0.96 to 4.64 ng/g The values of IC10, IC50, and working range (IC20–IC80) for CL-ELISA were 0.01, 0.08, and 0.02–0.3 ng/mL respectively	Yu et al. (2011)
Glycine max (L.) Merr.	Soybean	OTA	COL-ELISA - ELISA with colorimetric detection	Ng/g The values of IC10, IC50, and working range (IC20–IC80) for COL-ELISA were 0.08, 0.58, and 0.17–2.2 ng/mL, respectively	



a) Aokin immunoclean C | b) RIDA immunoaffinity column Ochratoxin A | c) Affinimip SPE | d) Aptamers column

Fig. 11.2 Immunoaffinity columns used for cleaning samples containing mycotoxins. (**a**) Aokin immunoclean C. (**b**) RIDA immunoaffinity column Ochratoxin A. (**c**) Affinimip SPE. (**d**) Aptamers column

The number of alerts varied significantly from year to year. It can be observed that even if some of the pesticides are not allowed in the EU, there is evidence of their use (due to their report of RASFF). According to the percentage of citation in 2015, these molecules are listed in Table 11.5. RASFF reported also the distribution of the reported pesticides residues in 2015 in different types of products (http://ec.europa.eu/food/safety/docs/rasff_annual_report_2015.pdf).

In 2015 were reported 401 notifications of pesticides residues, with different distribution per country (Fig. 11.3) (http://ec.europa.eu/food/safety/docs/rasff_annual_report_2015.pdf).

It was observed that the most reported pesticides residues were reported in 2015 in Italy (77), France (38), Belgium (33), The Netherlands (28).

11.4.1 Detection by Chromatographic Methods

Several chromatographic methods were found in scientific literature. The detection of pesticides has been widely determined using various liquid chromatography-mass spectrometry (LC–MS) techniques. However, many pesticides found to cause residue problems are often only amenable to single residue methods (for example phenoxy-acetic acids) so this should also be borne in mind when only multi-screening methods are considered.

In Table 11.6 are listed some of the references dealing with detection of pesticides in different botanicals – tea, lemon, orange, soybean.

Using the advantages of a new vacuum ultraviolet (VUV) detector which was coupled with a gas chromatograph, qualitative and quantitative information for multiclass pesticide identification was obtained (Fan et al. 2015). Using a spectral acquisition in a wavelength range of 115–240 nm, a number of 37 pesticides across

Table 11.5 Decreasing order of RASFF notification in 2015 for different pesticides

No	Pesticide citation in RASFF, in 2015
1	Chlorpyrifos
2	Carbendazim
3	Acetamiprid
4	Dimethoate
5	Dichlorvos
6	Formetanate
7	Carbofuran
8	Imidacloprid
9	Profenofos
10	Antraquinone
11	Cypermethrin
12	Biphenyl
13	Ethophon
14	Methomyl
15	Malathion
16	Dithiocarbamates
17	Fipronil
18	Methamidophos
19	Omethoate
20	Acephate
21	Triazophos
22	Propargite
23	Monocrotophos
24	Hexaconazole
25	Clorfentazine
26	Ethion
27	Methidathione

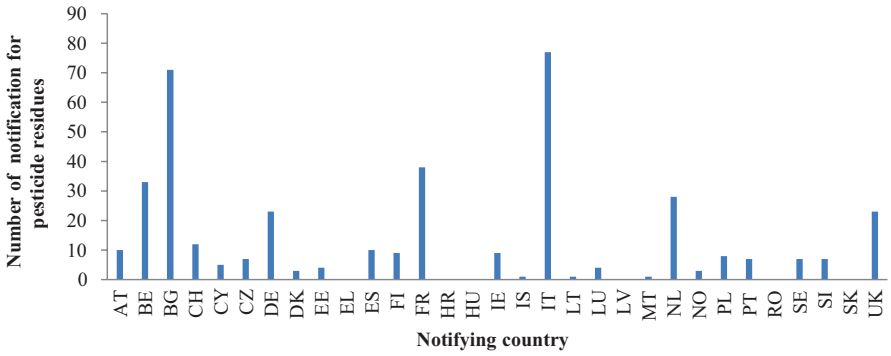


Fig. 11.3 Notification of pesticides residues per country in 2015, according RASFF report (http://ec.europa.eu/food/safety/docs/rasff_annual_report_2015.pdf)

Table 11.6 Analytical methods for detection of pesticides in tea, lemon, orange and soybean samples using chromatographic methods

Plant name (scientific and common names)	Purpose of the method	Analytical method	Class of analytical method	Level	References
Unspecified Tea	Analysis of pesticides in tea	HS-SPME-GC × GC/TOF MS (head-space solid-phase microextraction coupled to comprehensive two-dimensional GC-time-of-flight MS)	2DGC-MS	ND-14 μg/kg (36 target pesticides)	Schurek et al. (2008)
Camellia sinensis Tea	Study on the residue and degradation of fluorine-containing pesticides in oolong tea	GC-MS	1DGC-MS	0.20–55.1 μg/g (10 target pesticides)	Chen et al. (2012)
Camellia sinensis Tea	Analysis of PCDD/PCDF, chlorinated pesticides and PAH in Chinese teas	USEPA methodSW-846	Standard method	0–137.6 μg/kg	Fiedler et al. (2002)
Unspecified Lemon	Pesticide analysis in water based commodities	Liquid-liquid microextraction and analysis by GC-MS	GC-TofMS	ND-230 μg/kg (140 target pesticide)	Dasgupta et al. (2011)
Unspecified Lemon	Analysis of 150 pesticide residues in fruits	Low-pressure gas chromatography-tandem mass spectrometry (LP-GC/MS-MS) using a triple quadrupole instrument	QuEChERS	No details	Koesukwiwat et al. (2011)
Unspecified Lemon	Analysis of 140 pesticides from conventional farming foodstuff samples (including lemon)	105 pesticides with GC/SQ-MS and 46 pesticides with HPLC/IT-MS after extraction with the QuECheRS method	QuEChERS GC/MS and HPLC/MS analysis	No details	Lesueur et al. (2008)

Unspecified Lemon	Determination of 4,6-dinitro-o-cresol (DNOC) in soil and lemon	HPLC	Single residue method	1 mg/L	Uzer et al. (2006)
Unspecified Lemon	Determination of 52 non gas chromatography-amenable pesticides and metabolites in different food commodities	LC-MS-MS	Multiresidue method	No details	Hernández et al. (2006)
Unspecified Lemon juice	Determination of 24 new pesticide residues concentrated lemon juice	LC-ESI-MS-MS	Multi-residue method	None of samples contained residues higher than 0.010 mg/kg	Sannino et al. (2004)
Unspecified Lemon	Determination of several pesticides present in crude extracts from a variety of fruit and vegetables	LC-MS-MS	Multi-residue method	ND-2.10 mg/kg	Taylor et al. (2002)
Unspecified Lemon	Determination of imazalil in cirtus fruits	HPLC	Single residue method	1.32 ppm	Watanabe et al. (2001)
Unspecified Lemon	Determination of pesticide multiresidues in vegetable and fruit extracts	GC-MS	Multi-residue method	ND	Sojo et al. (1997)
Unspecified Orange	Determination of imazalil in cirtus fruits	HPLC	Single residue method	1.83 ppm	Watanabe et al. (2001)
Unspecified Orange	Analysis of pesticide residues in fruits and vegetables	GC–TOF MS	Multi-residue method QuEChERS	No details	Cervera et al. (2012)
Unspecified Orange	Multi-residue analysis with QuEChERS	GC/MS-sim	Multi-residue method QuEChERS	No details	Zhao et al. (2012)

(continued)

Table 11.6 (continued)

Plant name (scientific and common names)	Purpose of the method	Analytical method	Class of analytical method	Level	References
Unspecified Orange	Multiresidue determination of pesticides in industrial and fresh orange juice	LC-electrospray-tandem MS	Multi-residue method	No details	Bedendo et al. (2012)
Unspecified Orange	Dilutions of the orange matrix were tested to study the signal suppression of 53 pesticides	LC–MS/MS;LC-Q-TOF-MS	QuEChERS method;multi-residue method	No details	Ferrer et al. (2011a)
Unspecified Orange	Extraction pesticides in water based commodities (orange)	Liquid-liquid microextraction and analysis by gas chromatography–mass spectrometry	GC-TofMS	ND	Dasgupta et al. (2011)
Unspecified Orange	Pesticide residue analysis of fruit juices	LC–MS/MS direct injection	LC-MS/MS direct injection	No details	Ferrer et al. (2011b)
Unspecified Orange	Analysis of 150 pesticides in fruits	LP-GC/TOFMS	QuEChERS	No details	Koesukwiwat et al. (2010)
Unspecified Orange	Hollow fiber-liquid-phase microextraction of fungicides from orange juices	LC/MS	Multiresidue method	LOD $\leq$ 0.1 μg/L	Barahona et al. (2010)
Unspecified Orange	Analysis of pesticides in fruit and vegetables	GC-MS	Multiresidue method	No details	Guan et al. (2010)
Unspecified Orange	Surveillance of pesticide residues in fruits	GC-NPD;GC-ECD;GC-MS	Standard method	No details	Berrada et al. (2010)
Unspecified Orange	Monitoring of selected pesticide classes in fruits	GC-qMS; GC × GC-ECD	Comparison of different methods	No details	Ramos et al. (2009)

Unspecified Orange	Analysis of pesticide residues	GC-NCI-MS	Multi-residue method; QuEChERS	No details	Húškovaá et al. (2009)
Unspecified Orange	Compensation for matrix (orange)effects	GC–MS/MS	Multi-residue method	ND	Garrido Frenich et al. (2009)
Unspecified Orange	Study of a comprehensive list of 160 pesticide residues in multi-class vegetables	LC-ESI-MS/MS	Multi-residue method; QuEChERS	Lower than 100 μg/kg	Kmellár et al. (2008)
Unspecified Orange	Extraction of organophosphorus pesticides from orange juice	GC-NPD	Multi-residue method	ND	Ravelo-Perez et al. (2008)
Unspecified Orange	Fast determination of pesticides in fruit juices	UPLC-MS/MS	Multi-residue method; QuEChERS	Thiabendazol (9.2–24.9 μg/L)	Romero-Gonzàlez et al. (2008)
Unspecified Orange	Rapid determination of carbamate pesticides in food	Dual counter-current chromatography (dual CCC)–tandem mass spectrometry (MS/MS)	Analysis of residual carbaryl, fenobu-carb and methomyl	No details	Ito et al. (2008)
Unspecified Orange	Fast screening of pesticide residues in fruit juice	GC-MS/MS	Screening method	48 μg/L of the mentioned organo-phosphorus insecticides	Cortes-Aguado et al. (2008)
Unspecified Orange	Multi-residue method for the target analysis of pesticides in crops	LC-MS/MS	Multi-residue method	No details	Hiemstra and de Kok (2007)
Unspecified Orange	Determination of pesticide residues in foods	UPLC-MS/MS	Multi-residue method	No details	Leandro et al. (2007)
Unspecified Orange	Measuring multiresidual pesticides in agricultural products	GC-MS	Multi-residue method	No details	Liu et al. (2007)

(continued)

Table 11.6 (continued)

Plant name (scientific and common names)	Purpose of the method	Analytical method	Class of analytical method	Level	References
Unspecified Orange	Evaluation of 10 pesticide residues	LC-MS	Multi-residue method	ND-1100 μg/kg	Blasco et al. (2006)
Unspecified Orange	Determination of organochlorine and pyrethroid pesticides in fruit and vegetables	GC–ECD	Multi-residue method	No details	Sharif et al. (2006)
Unspecified Orange	Analysis of multiresidual pesticides in agricultural products	GC-MS	Multi-residue method	No details	Liu et al. (2006a)
Unspecified Orange	Analysis of organophosphorus pesticides in juice	GC-FPD	Multi-residue method	No details	Zhao et al. (2006)
Unspecified Orange	Determination of organophosphorus pesticides (OPPs) in orange juice	GC-TSD	Multi-residue method	No details	Liu et al. (2006b)
Unspecified Orange	Analysis of pesticides in fruits	(LC-it-MS3	Multi-residue method	0.02–0.69 mg/kg	Blasco et al. (2005)
Unspecified	Multi-residue pesticide analysis in fruits	LC–TOF-MS	Multi-residue method	No real samples analysed	Ferrer et al. (2005)
Unspecified Orange	Detection of pesticides	LC-TQ/MS and LC-QIT/MS	Multi-residue method	0.01–5.01 mg/kg	Soler et al. (2005)
Unspecified Orange	Analysis of pesticides in fruit, vegetables and cereals	LC-MS-MS	Multi-residue method	No real samples analysed	Granby et al. (2004)
Unspecified Orange	Analysis of six pesticides	LC-MS/MS	Multi-residue method	ND-0.60 mg/kg	Blasco et al. (2004)

Unspecified Orange	Determination of organophosphorus pesticide multiresidues in food	GC-FPD	Multi-residue method	10.2 ng/g fenitrothion, 2.1 ng/g triazophos	Yu et al. (2004)
Unspecified Orange	Determination of abamectin and azadirachtin residues	LC-ESI-MS-MS	Fast-run method	ND	Pozo et al. (2003)
Unspecified Orange	Determination of pesticides	LC-MS	Multi-residue method	0.008–10 mg/kg	Blasco et al. (2002)
Unspecified Orange	Analysis of pesticides in fruit	GC–MS	Multi-residue method	No details	Kristenson et al. (2001)
Unspecified Orange	Determination of imazalil in citrus fruits	HPLC	Single residue method	1.83 ppm	Watanabe et al. (2001)
Unspecified Orange	Detection of pesticide residues in citrus fruit	HPLC	Multi-residue method	No details	Valenzuela et al. (1999)
Unspecified Orange	Determination of thirteen fungicide and insecticide residues in fruit and vegetables	GC-ECD;GC-MS	Multi-residue method	ND-18.73 mg/kg	Torres et al. (1997)
Unspecified Orange	Analysis of multiple pesticides in fruits and vegetables	GC-MS	Multi-residue method	LOD 1–304 ng/g	Lehotay and Valverde-García (1997)
Glycine max (L.) Merrill Soybean	Analysis of 169 pesticides in soybean grain	(LC-MS/MS	Multi-residue method	0–8 µg/kg	Pizzutti et al. (2009)
Unspecified Soybean	Analysis of 169 pesticides in soybean grain	GC–MS(/MS) and LC–MS/MS	Multi-residue method	No real samples analysed	Pizzutti et al. (2007)

(continued)

Table 11.6 (continued)

Plant name (scientific and common names)	Purpose of the method	Analytical method	Class of analytical method	Level	References
Unspecified Soybean	Determination of 95 pesticides in soybean oil	GC-MS	Multi-residue method	All compounds were below the maximum residue limits (MRLs) established by the Korean legislations for soybean oil	Nguyen et al. (2010)

Legend of abbreviations: *GC-MS* gas chromatography with mass spectrometry, *GC-ECD* gas chromatography with electron capture detector, *GC-TSD* gas chromatography with thermionic sensitive detection, *GC-FPD* gas chromatography with flame photometric detector, *GC-NPD* gas chromatography-nitrogen phosphorous detector, *GC-qMS* gas chromatography triple quad mass spectrometry, *GC × GC-ECD* two-dimensional gas chromatography- gas chromatography with electron capture detector, *GC-NCI-MS* gas chromatography using negative chemical ionization mass spectrometry, *GC/SQ-MS* gas chromatography coupled to single quadruple mass spectrometers, *HPLC/IT-MS* high performance liquid chromatography-ion trap mass spectrometry, *HPLC* high performance liquid chromatography, *HS-SPME-GC × GC/TOF MS* head-space solid-phase microextraction coupled to comprehensive two-dimensional GC-time-of-flight MS, *LC-ESI-MS-MS* liquid chromatography with tandem electrospray ionization - mass spectrometry- mass spectrometry, *LC–TOF-MS* liquid chromatography with time-of-flight mass spectrometry, *LC-TQ/MS* liquid chromatography with triple-quadrupole mass spectrometer, *LC-QIT/MS* liquid chromatography-quadrupole ion trap/mass spectrometry, *LC-MS* liquid chromatography with mass spectrometry, *LC-MS/MS* liquid chromatography with tandem mass spectrometry- mass spectrometry, *LC-IT-MS3* liquid chromatography with ion trap mass spectrometry, *LP-GC/TOF-MS* low-pressure gas chromatography with time-of-flight mass spectrometry, *QuECheRS* Quick Easy Cheap Effective Rugged Safe, *UPLC-MS/MS* ultra performance liquid chromatography—tandem mass spectrometer- mass spectrometer

different classes were recorded. As a universal detector, VUV provides both. It offers high specificity, sensitivity (pg on-column detection limits), and a fast data acquisition rate, making it a powerful tool for multiclass pesticide screening when combined with gas chromatography.

11.4.2 Detection by Spectrophotometric Methods

It was demonstrated that Vis/NIR spectroscopy could be an appropriate, fast and non-destructive technology for safety control of intact cucumbers by the absence/presence of diazinon residues (Jamshidi et al. 2015). The samples were analysed at the range of 450–1000 nm, using partial least squares-discriminant analysis (PLS-DA) models, which were developed based on different spectral pre-processing techniques.

Based on the inhibitory effect of acetylcholinesterase (AChE) induced by inhibitors, including organophosphorus and carbamates pesticides, a colorimetric analysis was used for detection of OPs with computer image analysis of color density in CMYK (cyan, magenta, yellow and black) color space and non-linear modeling (Li et al. 2016a, b). The quantitative analysis of dichlorvos was achieved by Artificial Neural Network (ANN) modeling, and the results showed that the established model had a good predictive ability between training sets and predictive sets. Accuracy, precision and repeatability and good correlation between colorimetry and gas chromatography (GC) detection of dichlorvos from real cabbage samples was obtained.

A non-separative, fast and inexpensive spectrofluorimetric method based on the second order calibration of excitation-emission fluorescence matrices (EEMs) made possible to identify unequivocally three pesticides (carbaryl, carbendazim and 1-naphthol) in dried lime tree flowers (Rubio et al. 2014).

Differentiation of aldrin, 1,2,3,4,6,7,8-heptachlorodibenzo-p-dioxin, chlorpyrifos, and dieldrin in the complex matrices of tissue fats and rendering oils is described using the advances of laser-induced breakdown spectroscopy (LIBS) (Multari et al. 2013). The technique of laser-induced breakdown spectroscopy (LIBS) consists in a series of powerful laser pulses, which are directed at a surface to form microplasmas from which light is collected and spectrally analyzed to identify the surface material In most cases, no sample preparation is needed, and results can be automated and made available within seconds to minutes. The pesticide concentrations in the tested samples ranged from 0.005 to 0.1 μg/g.

Ellman method is useful for detection of inhibition of free acetylcholinesterase in presence of different inhibitors as organophosphorus and carbamates pesticides. Depending on the sources of AChE, are indicated to be used commercial enzymes (from Electric eel) (Molchanova et al. 2016) or mutants AChE from Drosophila melanogaster (Dm) (Badea et al. 2007).

11.4.3 Detection by Sensors and Biosensors

Recently, a colorimetric sensor array consisting of citrate-capped 13 nm gold nanoparticles (AuNPs) has been proposed for the detection and discrimination of several organophosphate pesticides (OPs) (Fahimi-Kashani and Hormozi-Nezhad 2016).

Qualitative and semiquantitative estimation with naked eyes and quantitative organophosphorus pesticide through image analysis was developed using a new paper-based biosensing approach has been developed for sensitive and rapid detection of acetylcholinesterase (AChE) inhibitors (Wu et al. 2017). The biomolecule of acetycholinesterase (AChE) was immobilized into two layers of biocompatible sol-gel-derived silica ink with a "sandwich" form. Indoxyl acetate (IDA) was used as a chromogenic substrate, which is colorless and can be catalytically hydrolyzed into blue-colored indigo dipolymer. The concentrations of paraoxon in apple juice samples were detected using this method, the results being confirmed with high-performance liquid chromatography, indicating the advantage for on-site detection of OPs in practical application.

There were developed an immunosensor was used for the conductometric sensing of atrazine. The detection of atrazine was achieved with a high sensor sensitivity (limit of detection = 0.01 nM) and specificity in the presence of diverse pesticides (e.g., endosulfan, parathion, paraoxon, malathion, and monochrotophos) (Bhardwaj et al. 2015).

Determination of organophosphate and carbamate pesticides in spiked samples of tap water and fruit juices (orange) a biosensor with photothermal detection was optimized for a LOD =2.8 ng/mL paraoxon in orange juice (Pogaanik and Franko 1999.

Enzyme based biosensors using different types of acetylcholinesterase (commercial or mutants of Dm) were optimized by our research group for detection of different orgnophosphorus pesticides. There were used different immobilization methods as PVA and sol-gel method, and kinetical parameters of immobilized enzyme were compared with the similar ones of the free enzymes. After the biosensor stability test, calibrations curves were constructed (using different concentration of acetylthiocholine chloride – substrate for the enzyme), and the influence of the inhibitors were tested (different incubation times, different pesticides, different concentrations of the pesticide) (Badea et al. 2006, 2008a, b; Nunes et al. 2014).

Good results were obtained and the method could be successfully used as cheap and easy to do test for qualitative and quantitative data monitoring. Future studies are still in progress in order to try to use the detection systems for telemonitoring of different samples (Badea et al. 2011).

11.4.4 Immunoassay

Immunoassays are often used as an analytical method for the screening of several samples or quantitative analysis; detection is associated with enzymatic activity (Enzyme ImmunoAssay-EIA, Enzyme Linked ImmunoSorbent Assay-ELISA).

Analysis of azinphos-methyl in fruit juices (orange) used a single residue method with a monoclonal antibody in ELISA format (Mercade and Montoya 1997).

The development of a new multiplex immunoassay (microarray chip) for simultaneous detection of seven pesticides (triazophos, methyl-parathion, fenpropathrin, carbofuran, thiacloprid, chlorothalonil, and carbendazim) used seven antigens immobilized on a nitrocellulose membrane (Lan et al. 2016). Nanogold was employed for labeling and signal amplification to obtain a sensitive colorimetric immunoassay. The direct and indirect detection formats were further compared using primary antibody-gold and secondary antibody-gold conjugates as tracers.

Recently were presented in scientific literature different immunoassays for simple, rapid and quantitative detections of phytoavailable neonicotinoid insecticides in cropland soils (Watanabe et al. 2016).

Sensitivity, affinity and matrix effect for detection of deoxynivalenol in wheat and wheat dust were compared using three different methods - ELISA, Surface Plasmon Resonance (SPR) and Biolayer Interferometry (BLI) (Sanders et al. 2016). The preferred ELISA and BLI methods were validated according to the criteria established in Commission Regulation 519/2014/EC and Commission Decision 2002/657/EC.

Simultaneous detection of imidacloprid and parathion by the dual-labeled time-resolved fluoroimmunoassay was developed (Shi et al. 2015). Europium (Eu(3+)) and samarium (Sm(3+)) were used as fluorescent labels. Comparing the obtained results with chromatographic methods, it was demonstrated that dual-labeled TRFIA is convenient and reliable to detect parathion and imidacloprid simultaneously in food and environmental matrices.

11.5 Conclusions

The issue of the presence of undesirable contaminants in botanicals is a critical point both for raw material and final commercial products. The diffusion of the use of botanicals in developed countries requires new research and the development of analytical methods to ensure efficacy and safety of the most frequently used plants. Products must guarantee the highest quality standards to allow the consumers to receive benefits without toxicological risk. Nowadays, due to the market globalization, most botanicals do not come from the country of origin but from third countries (WHO 2007). This raises new concerns about the presence of environmental contaminants (chemicals and heavy metals), pesticides and mycotoxins, which must be strictly controlled. Traditional and novel methods are useful tools to detect and quantify these molecules, producing data necessary for the elaboration of a suitable risk and benefit assessment.

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Chapter 12
Classic/Recommended Methods and Development of new Methods to Control Adulteration and Counterfeits

Chiara Di Lorenzo, Francesca Colombo, Francesca Orgiu, Gianfranco Frigerio, Donatella Caruso, and Patrizia Restani

Abstract The control of adulterations and counterfeits requires suitable analytical methods, capable to identify and quantify illicit additions. Methods should be selected according to the objectives: in the first steps fast and simple methods are necessary for the screening, and Thin-Layer Chromatography (or High-Performance Thin-Layer Chromatography) can be the suitable approaches. To confirm the results obtaining during the screening, advanced techniques (GC/HPLC with different detectors including mass spectrometer) are required for a precise quantification. The reliability of analytical methods is also essential to guarantee the quality of results. Method application to real samples is not always simple due to the complexity of matrix, as for example in food supplements. This chapter illustrates some cases of illicit additions and the analytical approaches used to identify the class of molecules involved.

Keywords Food supplements • Counterfeit • Adulteration • Screening by HPTLC

12.1 Introduction

Adulterations and counterfeits are quite common problems in the market of food supplements; the reasons of this phenomenon are numerous but the main objective of these illicit activities is always the increase of profits. Due to the large number of products on the market, it is objectively difficult to control the quality of all of them; moreover, parallel markets (internet, gym, etc.) escape easily to any kind of quality surveillance. Adulterations and counterfeits include: (1) the use of ingredients of lower quality and economical value (discussed in Chaps. 8–10); (2) the addition of

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ingredients to enhance the physiological/pharmacological activity (addition of active amines, conventional drugs, hormones, etc.); and (3) the inclusion of molecules helping in speeding the expected objective (drugs used for body weight loss). Obviously, the illicit addition is not declared in the label, so that their identification can be difficult due to the presence of hundreds of potential adulterants.

12.2 General Aspects

In search for prohibited substances, analytical approaches could be in some cases limited to qualitative tests; however, in most cases the quantitation of illicit molecules is required in order to assess the risk for consumers. In fact, a suitable risk assessment is based on the estimation of potential human exposure and, as a consequence, the development of reliable and validated analytical methods is a crucial step to reach the goal. Even though the adulterations and counterfeits have been described in all categories of food supplements, some of them are more frequently object of illicit additions due to the specific results expected by the consumers: food supplements used for physical activity (body building, sport), for the body weight loss and for sexual performances.

Working in the area of illicit additions, the first decision to take is related to the class/classes of molecules to search for. In this step, screening methods are welcome, even though any analytical approach is normally insufficient to detect/exclude the presence of thousands of possible chemical substances (Restani et al. 2014; Sanzini et al. 2011). Thin-Layer Chromatography (TLC) and the more recent High-Performance Thin-Layer Chromatography (HPTLC) are often the best analytical approaches to screen a large number of samples, as in the case of actions against criminality performed by police or other institutional bodies.

TLC and HPTLC are simple, flexible, relatively inexpensive techniques and offer an efficient separation for qualitative, semi-quantitative or quantitative analysis. The evolution of the technique in its "High performance variant" has allowed an optimization of the separation efficiency, thanks to the standardization of all steps in the procedure: a precise sample deposition, reproducible chromatographic separation and computerized data analysis.

Other analytical techniques have been developed and validated to confirm and quantify adulterants, e.g. high-performance liquid chromatography (HPLC) coupled with UV or fluorimetric (FL) detector or HPLC plus mass spectrometry (HPLC-MS) using different ion sources and analyzers, including single quadruple, triple quadrupole, linear trap, Orbitrap (Fourier transform MS) and time of flight (TOF), among others. In addition, MS can be rearranged to a tandem (MS/MS) or a multistage (MSn) MS, improving its accuracy and resolution (Patel et al. 2014).

Other techniques used in detection of adulterants in plant food supplements (PFS) are based on spectroscopic methods (e.g. NMR) and bioassays (e.g. estrogen and mammalian reporter gene assays, ELISA test, etc.) (Rocha et al. 2016).

The next sections illustrate some examples of cases faced by the authors of the chapter, where general and specific analytical strategies used in each problem will be described.

12.3 Food Supplements for the Improvement of Physical Performances (Products for Athletes)

12.3.1 Food Supplements Containing Active Amines

Active amines—mainly ephedrine and octopamine—are among the most usual compounds added as adulterants to food supplements aimed at improving physical performances and/or reducing body weight.

In 2004, FDA (US Food and Drug Administration) banned the use of ephedrine in food supplements (FDA 2003; FDA 2004), due to the severe adverse cardiovascular effects occurred to several athletes (Haller and Benowitz 2000). The ban was then extended to other continents, including Europe. This led to an increased use of *Citrus aurantium* as an alternative to *Ephedra* derivatives, with possible new risks for consumers. The most important active ingredients of *C. aurantium* are amines having adrenergic activity: synephrine, octopamine, tyramine and N-methyl-tyramine. Since *Citrus aurantium* is an allowed ingredient, synephrine and other correlated amines are present in food supplements containing this botanical, but their level is regulated by national and international legislations. For example, in Italy, food supplements containing *C. aurantium* must contain a daily dose of synephrine lower than 30 mg, while the sum of other amines (including octopamine) must be approximately 1/8 (12.5%) of synephrine. Very high amounts of octopamine are associated with the addition of the purified molecule; this molecule is allowed as an ingredient in certain countries but it is considered doping in others. It is not rare the presence of ephedrine or high quantity of octopamine in food supplements for athletes, coming from the parallel markets, such as gyms or internet shops. For this reason, the development of analytical protocols is important to have an efficient tool in challenging the criminality and protect the health of both athletes and general consumers.

12.3.1.1 Screening Analysis

At the beginning of the analytical control, methods useful for a quick screening are welcome and, as said above, HPTLC is usually suitable to reach this goal (Di Lorenzo et al. 2014). TLC and HPTLC have long tradition in the characterization of medicinal plants. This technique is usually applied for the preliminary identification of adulterations of herbal products with conventional drugs (Rocha et al. 2016; Sanzini et al. 2011).

Table 12.1 LOD of active amines at visible light

Analyte	LOD (ng)[a]	LOD (μg/g)[b]
Ephedrine	150	60
Pseudoephedrine	200	80
Octopamine	40	16
Norephedrine	80	32
Synephrine	50	20

[a]Amount of analyte detectable on the plate
[b]Concentration calculated as μg/g of starting sample

One of the possible analytical protocols for the separation/semi-quantitation of active amines is described below.

Standard preparation. 5 mg of each standard - ephedrine, synephrine, octopamine, norephedrine, and pseudoephedrine (Sigma-Aldrich, Chemie, Schnnelldorf, Germany)—are solubilized in 5 mL of methanol in order to obtain a final concentration of 1 mg/mL.

Sample preparation. Ten tablets (or equivalent quantity) of food supplement suspected for a possible illicit addition of active amines are randomly selected and then carefully mixed; 0.5 g of the resulting powder are added to 15 mL of methanol and the resulting solution is stirred with a magnetic bar for 15 min and filtered. Then, the solution is concentrated to 1 mL under dry nitrogen and loaded on the chromatographic plate in parallel to the standard solutions (1 mg/mL).

Analytical procedure. The HPTLC equipment is from Camag (Muttenz, Switzerland); it consists of an automatic applicator Linomat 5 and of a TLC Visualizer. The Vision Cat software Camag is used for data acquisition and processing. Plates of silica gel with fluorimetric indicator 60-F254 are from Merck (Darmstadt, Germany).

The analytical procedure is based on the semi-automatic application on the plate of 5 μL of methanolic solution of sample or 10 μL of the standard solutions. The mobile phase contains chloroform:methanol:32% ammonia, 60:18:1.5 (v/v/v). The elution chamber is saturated with mobile phase for 20 min before each run. At the end of the chromatographic run, the plate is exposed at 254 nm; after derivatization with ninhydrin (0.2% in ethanol), the plate is dried in extractor wood at 110 °C for 5 min. The compounds are then visualized at 366 nm and at visible light.

Validation. The limit of detection (LOD) is calculated by applying decreasing concentrations of the standard solution on the plate. Compared to those at 254 and 366 nm, the exposure at visible light is the most sensitive detection mode for active amines. Table 12.1 shows the limit of detection at visible light of molecules included in the assay.

Precision was evaluated loading onto the plate the same standard solutions in three different days and comparing the Rf (Ratio frontis) values obtained (Table 12.2).

Sample analysis. Figure 12.1 illustrates the results of the screening assay usually performed on food supplements aimed to improve physical activity and suspected for the presence of adrenergic amines either banned (ephedrine, pseudo-ephedrine,

Table 12.2 Results of the precision test performed for active amines in three different days

Analyte	Rf Day 1	Rf Day 2	Rf Day 3	Mean	SD[a]
Ephedrine	0.29	0.24	0.23	0.25	0.03
Pseudoephedrine	0.32	0.29	0.25	0.29	0.03
Octopamine	0.17	0.14	0.12	0.14	0.02
Norephedrine	0.43	0.38	0.35	0.39	0.04
Synephrine	0.12	0.09	0.08	0.10	0.02

[a]Standard Deviation
Since standard deviation values were always below ±0.05, the method is precise

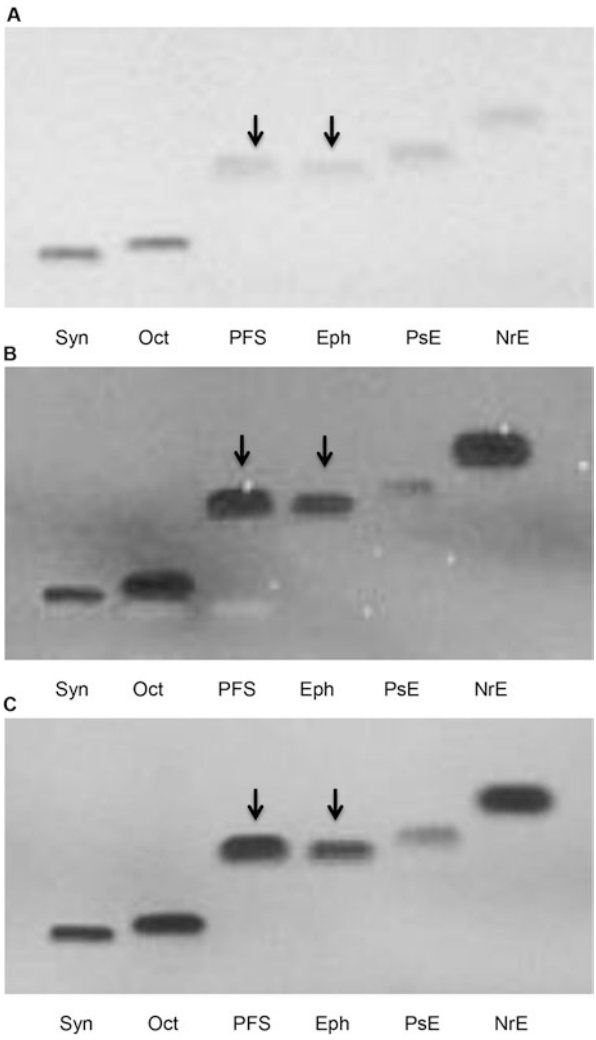

Fig. 12.1 HPTLC of food supplement (PFS) and standards of amines exposed at 254 nm (panel (**a**)); after derivatization with ninhydrin and exposure at 366 nm (panel (**b**)) or visible light (panel (**c**)). Arrows indicate bands at Rf = 0.26 corresponding to ephedrine. **Legend**: Syn = Synephrine, PFS = Food supplement, PsE = Pseudoephedrine, Oct = Octopamine, Eph = Ephedrine, PsE = Pseudoephedrine

nor-ephedrine) or in concentrations above the limits (synephrine, octopamine, and other *Citrus aurantium* compounds).

The sample, used as an example, shows a band having the same *Rf* (0.26) of ephedrine (at any light exposure); this molecule can be used in medicinal products but it is prohibited in food supplements. It is important to underline that also molecules having a very similar molecular structure, such as ephedrine, pseudoephedrine and norephedrine, are well separated and easily identified by HPTLC.

12.3.1.2 Liquid Chromatography-Mass Spectrometry (LC-MS)

To confirm the presence of ephedrine, the sample was further analyzed by HPLC coupled with mass spectrometry (MS).

Standard preparation. 10 mg of each standard are solubilized in 10 mL 0.1 N HCl to obtain a final concentration of 1 mg/mL.

Sample preparation. 0.5 g of the homogenated sample are added to 50 mL of 0.1 N HCl and the resulting solution is stirred with a magnetic bar for 10 min, filtered with a 0.45 μm filter (VWR International, Fontenay Sous-Bois, France) and injected into the chromatoghraphic system.

Chromatographic conditions and MS parameters. The equipment includes a HPLC Surveyor MS Pump Plus coupled to an ion trap mass spectrometer LCQ Deca XP MAX (Thermo Electron Co, San Jose, CA, USA) and a Surveyor Autosampler Plus (Thermo Electron Co, San Jose, CA, USA). The software Excalibur® Release 2.0 SR2 (Thermo Electron, San Jose, CA, USA) is used for integration. The column used is an ODS2 2.1 × 150 mm, particle size 5 μm, maintained at 24 °C.

The analysis is performed using a gradient elution at a flow rate of 0.2 mL/min in which mobile phases are (A) 0.1% formic acid in water (v/v) and (B) 0.1% formic acid in methanol. The gradient is programmed as follows: 0–13 min, from 90 to 0% A; 13–14 min, from 0 to 90% A.

The mass spectrometric technique uses ESI-IT-MSn (ESI-Ion Trap Multistage Tandem Mass Spectrometry); the source is an electrospray with positive (ESI+); nitrogen is the nebulized gas, vaporization temperature is set at 450 °C; helium is the collision gas, collision energy is set at 20 V and capillary temperature at 275 °C.

The identification process started by acquiring the mass spectrum of ephedrine, pseudoephedrine and norephedrine with ESI sources operating both in MS mode and in tandem mass spectrometry. The ESI+ ionisation has provided the most reliable results, so that analyzes were carried out with this type of ionization.

Pseudoephedrine and ephedrine have an identical spectrum with the molecular ion at m/z 166 and an ion at m/z 148, due to the loss of a molecule of water. The norephedrine, as expected, has the molecular ion at m/z 152 and an ion at m/z 134 corresponding to the loss of a molecule of water.

Figure 12.2 shows the results obtained from the analysis of a mixture of the three standard molecules. Panel A shows the total ion current (TIC) profile, where the

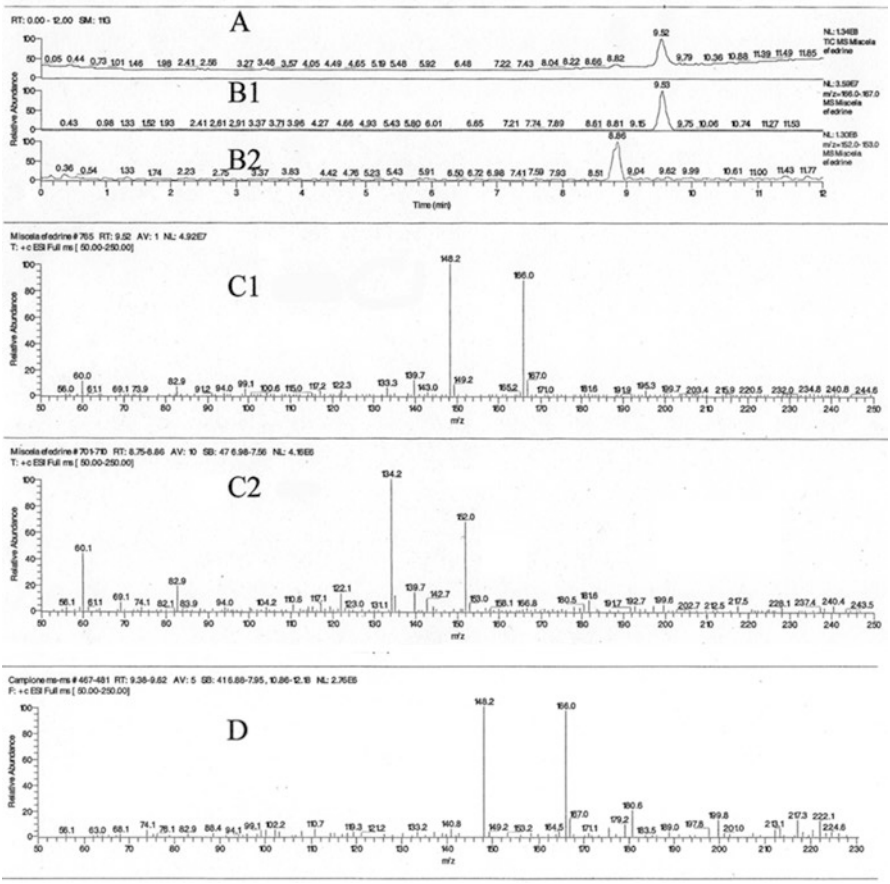

Fig. 12.2 Analysis of the mixture of the three standard compounds (ephedrine, pseudoephedrine and norephedrine). A = total ion current profile. B1 and B2 = chromatograms of the ions at m/z 166, characteristic of ephedrine and pseudoephedrine and at m/z 152, characteristic of norephedrine. C1 and C2 = corresponding mass spectra. D = mas spectrum of the ion at m/z 166 identified in the sample of food supplement

whole range of masses are detected. Panel B1 shows the chromatogram of the characteristic ion of ephedrine/pseudoephedrine at m/z 166, and panel B2 that of norephedrine. Panel C1 and C2 illustrate the mass spectra of ions shown in B1 and B2, respectively. Panel D illustrates the mass spectrum of the ion at m/z 166 identified in the sample chromatogram, confirming the presence of ephedrine or pseudoephedrine. Taking into consideration the different *Rf*s observed in HPTLC, the compound present in the sample was definitively identified as ephedrine. The amount quantified by HPLC technique showed that the ephedrine concentration was high enough to produce a pharmacological activity with a possible risk for unaware consumers (data not shown).

12.3.2 Food Supplements Added with Steroid Hormones

Steroid hormones (e.g. androstenedione, nandrolone, stanozolol, testosterone, testosterone enanthate) are often added illicitly to PFS to enhance physical performances. The use of performance-enhancing drugs in sports (doping) is quite common, even though the practice is considered unethical by all international organizations and it can be seriously detrimental to athletes' health (Restani et al. 2014). Among the adverse effects, associated with the use of anabolic steroids, there are cardiovascular and SNC effects, with an increased risk of cancer (Maravellas et al. 2005). An example of this kind of adulteration and a relative analytical protocol is described below.

12.3.2.1 Screening Analysis

Also in the case of anabolic steroids, HPTLC is a useful method to screen samples under control.

Standard preparation. 10 mg of testosterone, androstenedione, dehydroepiandrosterone, methandrostenolone, nandrolone (Sigma-Aldrich Chemie, Schnnelldorf), stanozolol and testosterone enanthate (Steroid S.p.A, Cologno Monzese, Milan) are solubilized in 10 mL of methanol to obtain a final concentration of 1 mg/mL.

Sample preparation. Ten tablets (or equivalent representative quantity) of the food supplement suspected of adulteration are mixed; 0.2 g of the resulting powder are added to 10 mL of methanol and the solution is stirred with a magnetic bar for 15 min and filtered with a paper filter. Then, the solution is concentrated to 1 mL under nitrogen stream.

*Analytical procedure***.** 5 μL of the sample methanolic solution or 10 μL of standard solutions are applied on silica gel plate 60-F254 (Merck, Darmstadt, Germany). The mobile phase contains chloroform:acetone 85:15 (v/v). The elution chamber is saturated with mobile phase for 20 min before each run. At the end of the chromatographic run, the plate is sprayed with sulphuric acid (5% in ethanol, v/v), dried and heated at 110 °C until development of spot staining. The compounds are visualized at 254 nm, 366 nm and at visible light.

Validation. The limit of detection (LOD) is evaluated by applying decreasing concentration of the standard compounds on the plate.

Comparing the three exposure options, that at 366 nm shows the highest sensitivity; as a consequence only LODs calculated at this exposure light are reported in Table 12.3.

Precision was evaluated loading onto the plate the same standard solutions in three different days and comparing the Rf (Ratio frontis) values obtained (Table 12.4).

Sample analysis. Figure 12.3 illustrates the results of the assay performed to screen the presence/absence of forbidden hormonal compounds in the food supplements under control. The sample, used as an example, shows a band having the same *Rf* (0.35) and staining of methandrostenolone (at any light exposure). The molecule is

Table 12.3 LOD of steroid hormones separated by HPTLC and exposed at 366 nm

Analyte	LOD (ng)[a]	LOD (μg/g)[b]
Testosterone	6	6
Androstenedione	6	6
Dehydroepiandrosterone	9	9
Methandrostenolone	8	8
Nandrolone	9	9
Stanozolol	9	9
Testosterone enanthate	9	9

[a]Amount of analyte detectable on the plate
[b]Concentration calculated as μg/g of starting sample

Table 12.4 Results of the precision test performed by HPTLC for anabolic hormones

Analyte	Rf Day 1	Rf Day 2	Rf Day 3	Mean	SD
Testosterone	0.37	0.35	0.37	0.36	0.01
Androstenedione	0.58	0.58	0.57	0.58	0.01
Dehydroepiandrosterone	0.43	0.43	0.43	0.43	0.001
Methandrostenolone	0.33	0.35	0.34	0.34	0.01
Nandrolone	0.34	0.34	0.33	0.34	0.01
Stanozolol	0.12	0.13	0.12	0.12	0.01
Testosterone enanthate	0.74	0.71	0.71	0.72	0.02

Since standard deviation values were always below ±0.05, the method is precise

considered doping and, as a consequence, cannot be used in food supplements. The sample was further analyzed by HPLC technique coupled with mass spectrometry (MS), to confirm the identification of forbidden molecule.

12.3.2.2 LC-MS

Standard preparation. 10 mg of each standard are solubilized in 10 mL of methanol to obtain a concentration of 1 mg/mL. The solutions are diluted (1:40 v/v) with methanol in order to obtain a final concentration of 25 μg/mL.

Sample preparation. 0.2 g of the homogenated tablets are added to 10 mL of methanol and the resulting solution is stirred with magnetic bar for 15 minutes, filtered with a 0.45 μm filter (VWR International, Fontenay Sous-Bois, France), diluted (1:10 v/v) in methanol and injected into the chromatoghraphic system.

Chromatograpich condition and mass spectrometer parameters. The equipment includes a HPLC Surveyor MS Pump Plus coupled to an ion trap mass spectrometer LCQ Deca XP MAX (Thermo Electron Co, San Jose, CA, USA) and a Surveyor Autosampler Plus (Thermo Electron Co, San Jose, CA, USA).

The software Excalibur® Release 2.0 SR2 (Thermo Electron, San Jose, CA, USA) is used for integration. The column used is a Hypersil GOLD Aq 2.1 × 100 mm, particle size 3 mm, maintained at 24 °C.

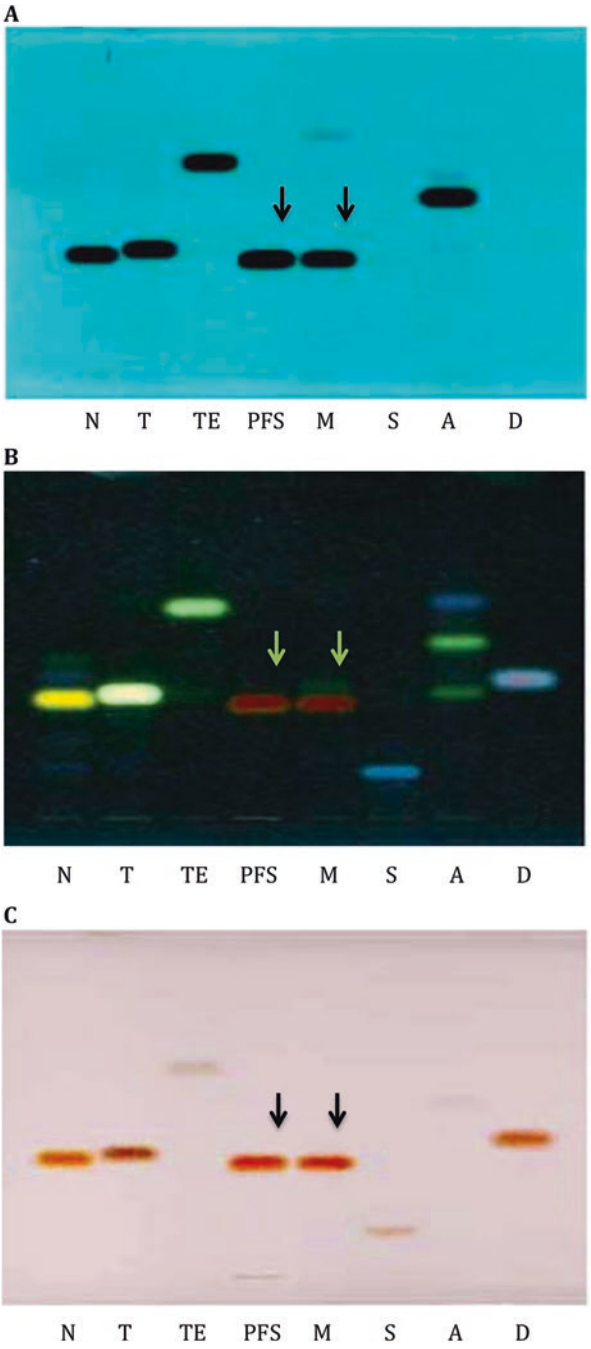

Fig. 12.3 HPTLC of food supplement and standards exposed at 254 nm (**a**), and after dervatization with sulphuric acid at 366 nm (**b**) and visible light (**c**). Arrows indicate the bands at Rf = 0.35. **Legend**: N = Nandrolone, T = Testosterone, TE = Testosterone Enanthate, PFS = Food Supplement, M = Methandrostenolone, S = Stanozolol, A = Androstenedione, D = Dehydroepiandrostenedione

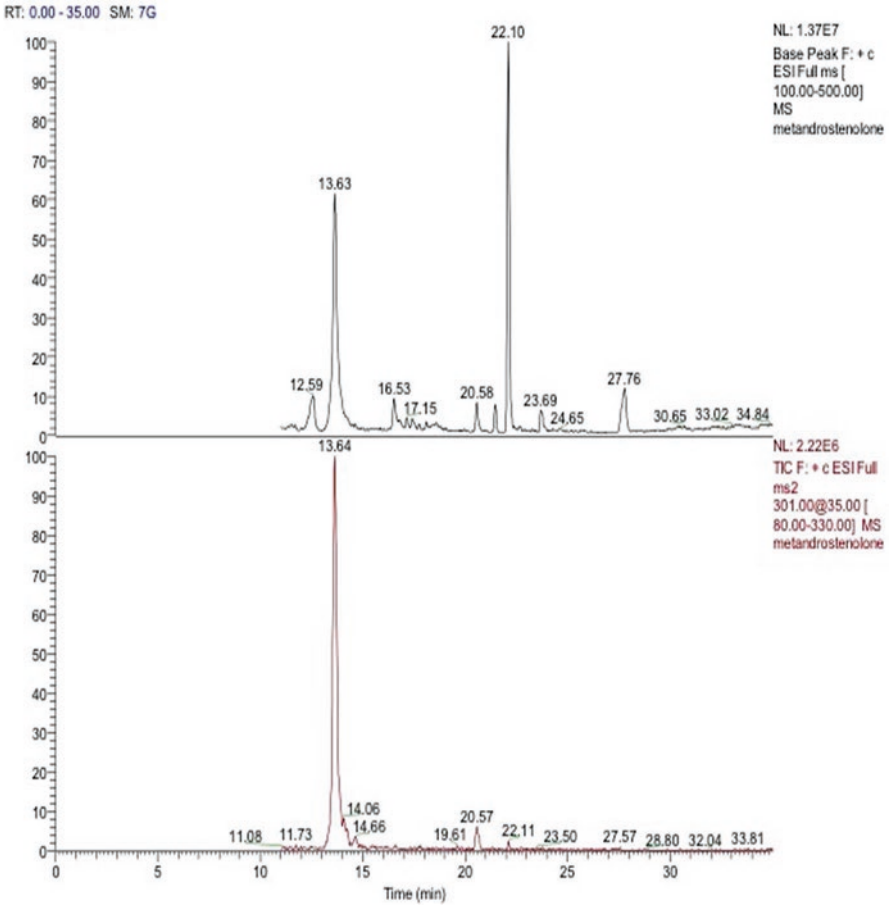

Fig. 12.4 Chromatogram of methandrostenolone standard analyzed in TIC (Total Ion Chromatography) (panel (**a**)) and chromatogram of the molecular ion at at *m/z* 301 (panel (**b**)). The retention time of the molecular ion is 13.63 min

The analysis is performed using a gradient elution at a flow rate of 0.25 mL/min; mobile phases were (A) 0.1% formic acid in water (v/v) and (B) 0.1% formic acid in methanol (v/v). The gradient program is: 0–1.5 min, 70% A; 1.5–10 min, from 70 to 36% A; 10–20 min, from 36 to 1% A; 20–25 min, 1% A; 25–25.1 min, from 1 to 70% A; 25.1–40 min, 70% A. Injected volume is 10 µL.

The mass spectrometric technique used is ESI-IT-MSn (ESI-Ion Trap Multistage Tandem Mass Sprectrometry); the source is an electrospray with positive ionization (ESI+), nitrogen is the nebulized gas, vaporization temperature is set at 450 °C, helium is the collision gas; collision energy is set at 20 V and capillary temperature at 275 °C.

Figure 12.4 shows the chromatogram of methandrostenolone standard analyzed in TIC (Total Ion Chromatography) (panel A) and the chromatogram of the molecular ion at *m/z* 301 (panel B). The retention time of the molecular ion is 13.63 min.

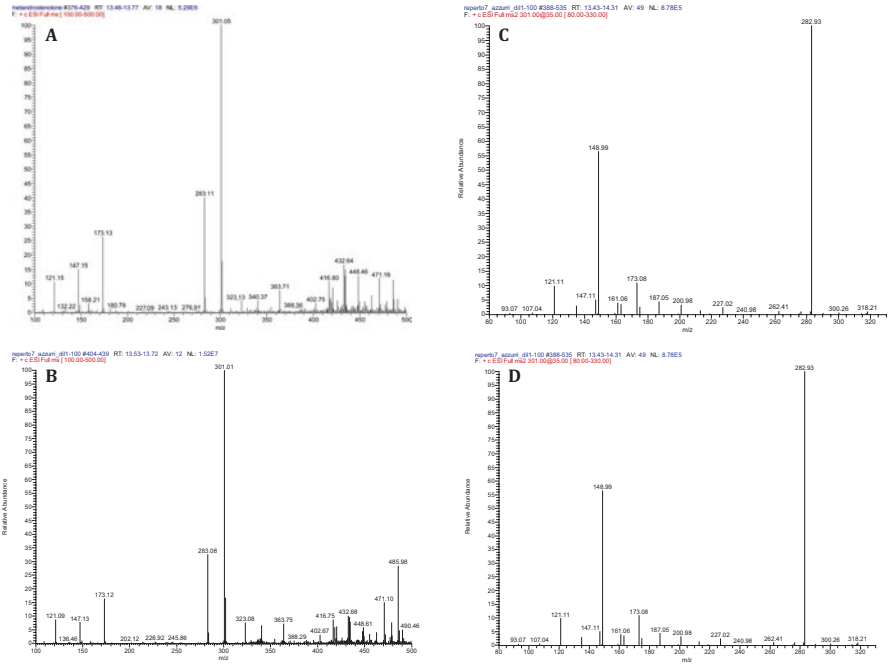

Fig. 12.5 MS and MS^2 spectra of molecular ion with *m/z* 301, corresponding to methandrostenolone. Panel (**a**) and (**c**) illustrates the results obtained with the standard hormone, panel (**b**) and (**d**) the corresponding spectra of methandrostenolone in PFS under analysis. The most abundant ions in MS^2 were *m/z* 121, 149, 173 and 282, in both standard and sample

Figure 12.5 shows the MS and MS^2 spectra of the molecular ion (m/z 301) of the standard methandrostenolone (panel A and C, respectively) and the spectra of the methandrostenolone present in PFS sample (panel B and D). The identity was easily confirmed.

12.4 Food Supplements Added with Conventional Drug Aimed at Improving Sexual Performances

During the last years, the demand for phosphodiesterase type-5 enzyme (PDE-5) inhibitors (e.g. sildenafil, vardenafil, tadalafil) has been increasing worldwide to enhance the sexual performance. Even though these substances must be considered as prescription drugs, they are often used in improper ways. The reasons of the phenomenon are: (1) the lack of correct information, (2) the higher cost of conventional drugs, (3) the availability from "private" accessible sources, such as Internet (Campbell et al. 2013). As a consequence, several recent studies have shown the illegal presence of PDE-5 inhibitors and/or its analogs in PFS and in parallel, Internet and television started advertising such products, as a "natural" resolution

for sexual problems (Strano-Rossi et al. 2015). From 2010, FDA has reported more than 200 public alerts for dietary supplements due to the detection of approved PDE-5 inhibitors or its analogs (Rocha et al. 2016). A protocol for the identification and quantification of this class of molecules is reported below.

12.4.1 Screening Analysis

Standard preparation. One tablet of tadalafil (Cialis; Eli Lilly, Indianapolis; USA) containing 5 mg of active compound is homogenated and extracted with 5 mL of methanol. Then, the solution is filtered with a 0.45 μm filter (VWR International, Fontenay Sous-Bois, France).

Aliquots of 5 mg of pure sildenafil (Sequoia Research Products Inc., Pangbourne, UK) and vardenafil (Bayer HealthCare, Leverkusen, Germany), are solubilized in 5 mL of methanol, in order to obtain a final concentration of 1 mg/mL.

Sample preparation. The food supplement, used here as an example is a liquid product reporting in the label the claim "all natural". The sample is applied on the plate after suitable dilution. In order to verify the efficacy of the method and to confirm the presence of PDE-5 inhibitors, the sample is loaded onto the HPTLC plate, as such or spiked with the standard compounds at the final concentration of 0.1 mg/mL.

Analytical procedure. Sample or standard solutions (10 μL) are applied on the silica gel plate (60-F254, Merck, Darmstadt, Germany). The mobile phase contains chloroform:methanol:32% ammonia 95:3:2 (v/v/v). The elution chamber is saturated with mobile phase for 20 minutes before each run. At the end of the chromatographic run, the plate is exposed at 254 and 366 nm, and then sprayed with sulphuric acid (5% in ethanol, v/v), dried and heated at 110 °C until development of colour. The image of the plate is finally acquired after exposure at 366 nm.

Validation. The limit of detection (LOD) is obtained by applying onto the plate decreasing concentration of the standards (500 ng, 300 ng, 100 ng, 50 ng, 25 ng, 12.5 ng, 6.2 ng, 3.1 ng)**.** Results obtained after exposure at 254 nm, where the sensitivity is higher, are listed in Table 12.5.

Figure 12.6 shows the HPTLC chromatografic separation of the food supplement without or with the internal addition of the standard compounds. A band with Rf

Table 12.5 HPTLC chromatographic run (*Rf*) and LODs of different analytes included in the study

Standard	Rf	UV 254 nm (μg)[a]	LOD (μg/g) (UV 254 nm)[b]
Sildenafil	0.13	0.12	12
Tadalafil	0.20	0.25	25
Vardenafil	0.10	0.12	12

[a]Amount of analyte detectable on the plate
[b]Concentration calculated as μg/g of starting sample

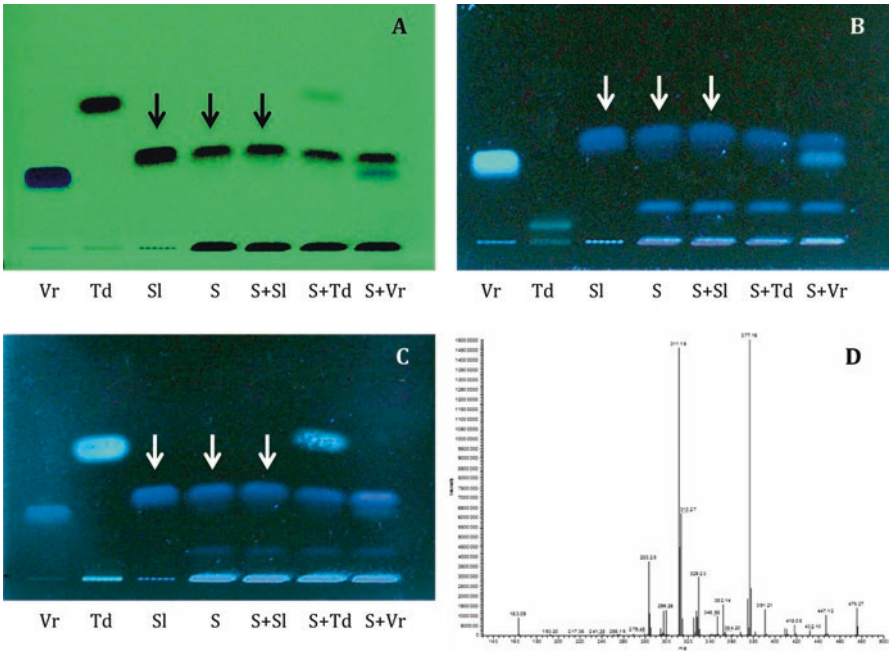

Fig. 12.6 HPTLC of sample and standard compounds exposed at: 254 nm (panel A), 366 nm (panel B), and 366 nm after spraying sulphuric acid (panel C). Arrows indicate the band with Rf = 0.13 corresponding to sildenafil. Panel D shows the MS^2 spectrum of sildenafil, which was confirmed in the sample. **Legend**: Vr = Vardenafil, Td = Tadalafil, Sl = Sildenafil, S = Food supplement, S + Vr = Sample spiked with vardenafil, S + Sl = Sample spiked with sildenafil, S + Td = Sample spiked with tadalafil

corresponding to that of the sildenafil (0.13) was found in the food supplement, with and without internal addition. When the plate is exposed to the light at 366 without pre-derivatization the molecule of tadalafil is not visible (panel B), while it appears at the same wavelength after spraying the plate with 5% sulphuric acid in ethanol (panel C). In order to confirm the identification, the sample was further analyzed by HPLC coupled with mass spectrometry (MS).

12.4.1.1 LC-MS

Standard preparation. Two tablets of tadalafil (Cialis; Eli Lilly, Indianapolis; USA) are homogenated and extracted with 10 mL of 0.1 N HCl, obtaining a solution at 1 mg/mL. Standard compounds (10 mg) of sildenafil and vardenafil are solubilized in 10 mL of 0.1 N HCl.

Sample preparation. Food supplement (0.250 g) is weighed and added to 5 mL of 0.1 N HCl. The resulting solution is stirred with a magnetic bar for 30 min, centri-

Table 12.6 Parameters used for MS^2 analysis

Analyte	Transition (m/z)	Collision energy (%)
Acetildenafil*	467 → 297	58
Homosildenafil*	489 → 283	60
Hydroxyhomosildenafil*	505 → 487	35
Sildenafil	475 → 377; 311; 313; 283	52.7
Tadalafil	390 → 262; 268; 250; 135	22
Vardenafil	489 → 376; 169; 377; 299; 312	50

Data were obtained from Zhou et al. (2006)

fuged at 12,000 rpm for 5 min. The supernatant is filtered with a 0.45 μm filter (VWR International, Fontenay Sous-Bois, France) and injected into the chromatoghraphic system.

Chromatographic conditions and MS parameters. The equipment is a HPLC Surveyor MS Pump Plus coupled with an ion trap mass spectrometer LCQ Deca XP MAX (Thermo Electron Co, San Jose, CA, USA) and a Surveyor Autosampler Plus (Thermo Electron Co, San Jose, CA, USA).

The software Excalibur® Release 2.0 SR2 (Thermo Electron, San Jose, CA, USA) is used for integration. The column used is a Hypersil GOLD 3 × 100 mm, particle size 3 μm. The analysis is performed using an isocratic elution at a flow rate of 0.5 mL/min; mobile phase contains 0.01 M ammonium formiate/acetonitrile 62:38 (v/v).

The mass spectrometric technique used is ESI-IT-MSn (ESI-Ion Trap Multistage Tandem Mass Spectrometry); the source is an electrospray with positive ionization (ESI+); nitrogen is the nebulized gas, helium the collision gas and capillary temperature is set at 300 °C. The parameters used for MS^2 analysis are listed in Table 12.6, and were described by Zou et al. (2006).

Figure 12.6 (panel D) shows the MS^2 spectra of the molecular ion of standard sildenafil. The LC-MS analysis of the food supplement, here used as an example, identified a compound, characterized by m/z 475 (not shown), having a MS^2 and MS^3 fragmentation pattern corresponding to that of sildenafil, confirming the previous identification by HPTLC.

12.5 Conclusions

According to the current legislation in EU countries and the U.S., dietary supplements (including PFS) can be commercialized without any specific control on chemical composition or clinical study. Safety is guaranteed taking into consideration the declaration by the producer and/or by dossiers containing data from the literature. This can facilitate criminal actions normally associated with the parallel market (mainly internet), where food supplements adulterated with pharmaceutical drugs are prepared to increase product effectiveness. The request by consumers of quick results without strict diet (loss of body weight) or medical recommendation (improvement

of physical and sexual performances) makes consumers suitable victim of "natural" remedies". As a consequence, there is an increased need for more effective control of possible adulterations and the development of new and/or improved analytical methodologies is critical to protect public health and ensure the quality of dietary supplements. The recent evolution of screening methods, such as TLC and HPTLC, gives to laboratories involved in food control suitable tools for a rapid and relatively cheap identification of several classes of adulterants. The use of more expensive and sensitive techniques, such as HPLC coupled with different detectors, can be limited to the samples positive at screening, allowing wider control of the market.

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Chapter 13
Detection of Irradiated Herbal Ingredients of Plant Food Supplements by Thermoluminescence Technique

Concetta Boniglia, Brunella Carratù, Raffaella Gargiulo, and Emanuela Bortolin

Abstract In EU the treatment with ionizing radiation is allowed for dried aromatic herbs, spices and seasonings, but not for plant food supplements and their ingredients. Nevertheless, checks carried out in EU show a large number of irradiated plant food supplements and herbal ingredients. Several methods to detect the radiation treatment have been standardized by European Committee for Standardization (CEN) and among them the EN 1788 Thermoluminescence (TL) based method appears to be the most reliable to detect irradiation in herbal materials. In this paper the applicability of the thermoluminescence method to plant food supplements ingredients has been discussed on the basis of data reported in literature and the results obtained by the study carried out in the framework of the European project PlantLIBRA on twelve different not irradiated and irradiated raw materials. The data confirmed that the EN 1788 method can be successfully applied to PFS ingredients, but has to be used with caution for the identification of plant food supplements containing different components which could have been separately irradiated.

Keywords Irradiation • Thermoluminescence Method • Plant Food Supplements

13.1 General Aspects

The popularity of Plant Food Supplements (PFS) is on the rise in Europe and other parts of the world. They contain as ingredients plants (whole, fragmented or cut) in unprocessed, usually dried, form and/or botanical preparations (herbal extracts):

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their beneficial effects are based on the bioactivity of the complex mixture of phytochemicals. Unfortunately, plants are frequently contaminated and/or infested with organisms and their metabolites, which are harmful to public health. The treatment of food with ionizing radiation has emerged as a leading method against microbial deterioration of foodstuffs, to preserve hygienic quality, ensure shelf life and hence to reduce public health threat (Arvanitoyannis et al. 2009). The safety and wholesomeness of irradiated foods have been extensively studied and international expert groups concluded that foods irradiated up to 10 kGy are both safe for consumption and nutritionally adequate (WHO 1981, 1999; EFSA 2011). On these basis, an increasing number of countries all over the world has approved the irradiation of different food items, ranging from spices and grains to fruit and vegetables, meat, poultry and seafood (Kume et al. 2009). Within the EU a large variety of plant origin foods (dried aromatic herbs, spices, potatoes, onions, garlic, fresh and dried fruits) has been authorized for irradiation up to10 kGy, with the exception of plant food supplements and their ingredients (Directive 1999/2/EC and 1999/3/EC). On the contrary in some extra European countries (USA, New Zeland, India, Argentina and others), the irradiation of some plant materials up to 30 kGy is accepted. European legislation states also that checks to detect products treated with ionizing radiation have to be performed by each member state every year; in particular PFS and their ingredients are strongly recommended to be controlled as they have been found non-compliant with the directives during the surveys (Food Safety Authority of Ireland 2004; Boniglia et al. 2009) and the checks performed by the member states during the last years (http://ec.europa.eu/food/food/biosafety/irradiation/reports_en).

Several methods to detect the radiation treatment have been standardized by European Committee for Standardization (CEN), each one applicable to a specific group of products (https://ec.europa.eu/food/safety/biosafety/irradiation/legislation_en).

On the basis of data reported in literature, the EN standards, which might be used to detect irradiation treatment in plants are: EN 1787:2000-Detection of irradiated food containing cellulose by ESR spectroscopy, EN 1788:2001-Thermoluminescence detection of irradiated food from which silicate minerals can be isolated, EN 13783:2001-Detection of irradiated food using Direct Epifluorescent Filter Technique/Aerobic Plate Count (DEFT/APC) and EN 13751:2009-Detection of irradiated food using photostimulated luminescence. Indeed, these methods have been standardized for aromatic dried herbs, spices but their application to PFS and their ingredients is not always effective.

Two of the mentioned methods, EN 13751 based on photostimulated luminescence (PSL) and EN 13783 (DEFT/APC) are screening methods and require a second analysis, by other standardized methods, to confirm all the samples screened as not negative. Both methods can give a large number of incorrect responses when applied to specific groups of products: PSL method fails in detecting irradiated herbal extracts due to their low content of radiation markers (Bortolin et al. 2009), whereas EN 13783 (DEFT/APC) method can give false negatives if the same threshold value (4log units) is set for all the herbs (Wirtanen et al. 1993; Oh

et al. 2003). Furthermore, DEFT/APC, which is a not specific method able to detect also viable microorganism reduction due to causes other than irradiation, can provide a large number of false positive results (about 40%) when applied to PFS and their ingredients, as found by Leth et al. (2006) in a survey on 106 products. EN 1787 (ESR) method is limited by the content of cellulose and the life-time of the radiation-induced signals (often shorter than the shelf-life of the product) (Bortolin et al. 2006). Moreover, the ESR spectra of many herbs are complex and difficult to interpret: they often show several components related to different, radiation-induced as well as intrinsic, radicals (Yordanov et al. 2009; Ahn et al. 2014). EN 1788 Thermoluminescence (TL) based method appears to be the most reliable to detect irradiation in herbal materials: it is applicable to raw materials as well as their extracts and allows the detection of irradiation treatment for long time (over the shelf-life of the product).

13.1.1 The Method

13.1.1.1 Principle

EN 1788 Standard specifies a method for the detection of irradiation treatment of food or food ingredients based on the luminescence properties of silicate minerals contaminating food. Silicates, in fact, largely present in the natural environment (dust, air particulate, soil, water), can keep track of the ionizing radiation exposure through charge trapping processes in the crystal defects and detect irradiation through the emission of thermally stimulated luminescence (Fig. 13.1).

In principle the method can be applied to all the agricultural products (vegetables, fruit, cereals, plants, herbs, spices, etc.) which grow up being exposed to air (wind), as well as to seafood such as clams, mussels, shrimps and prawns, which hold sand in their intestine.

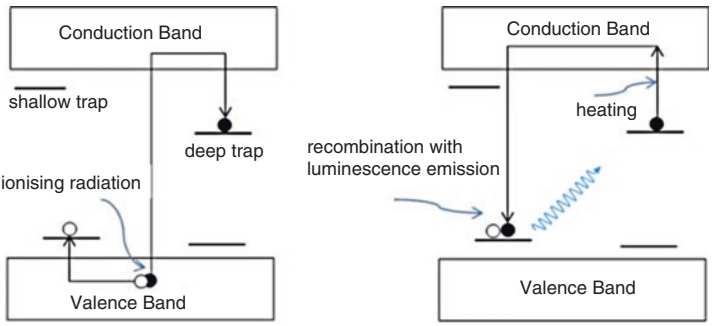

Fig. 13.1 Illustration of the principle of TL phenomenon. In the *left side*: formation of an electron–hole pair and trapping of the unpaired electron (hole) in a deep trap. In the *right side*: recombination, after heating, of the electron and the hole leading to thermoluminescence. The traps (metastable levels) are due to the presence of impurities or defects in the crystalline lattice

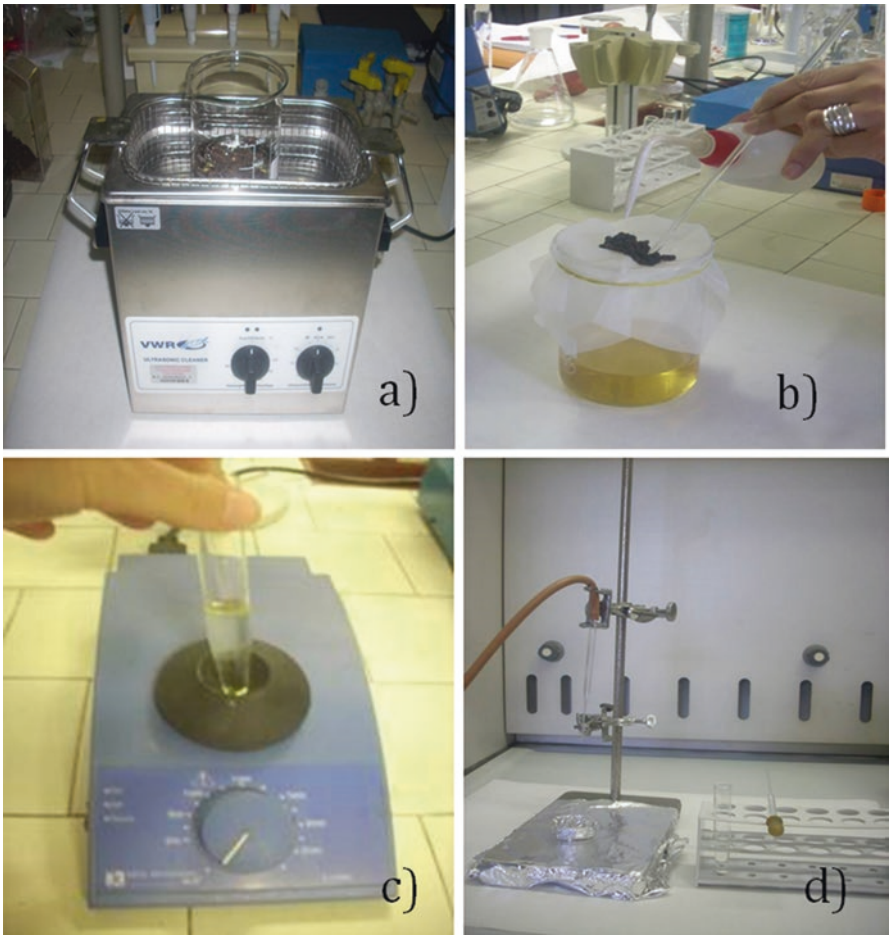

Fig. 13.2 Silicate extraction in water from a sample of black pepper: (**a**) ultrasonic treatment to loosen the adhering minerals; (**b**) sieving to concentrate the minerals; (**c**) density separation with sodium polytungstate to eliminate the organic residuals; (**d**) silicate transfer, under nitrogen flux, in a stainless steel cup

To avoid spurious signals due to the heating of organic materials, the silicate minerals have to be isolated from the foodstuffs and be as free of organic constituents as possible. Thus, the first step of the analysis is the silicate extraction (Fig. 13.2) which includes a mineral preconcentration step, specific for each food category (herbs and spices, shellfish, fruit and vegetables), followed by a density separation with sodium polytungstate, as described in the EN 1788 Standard.

After the silicate extraction, a first thermoluminescence (TL) signal (Glow1) is recorded by heating the separated mineral extracts up to 350–500 °C (Fig. 13.3). Generally, irradiated samples show glow curves with intense peaks at $T < 250$ °C, well distinguishable from the weak geological residuals, with maximum intensity at

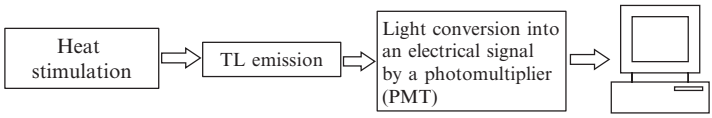

Fig. 13.3 Schematic description of a TL measurement: the light emitted by the sample under heat stimulation is converted by a photomultiplier tube (PMT) into an electrical signal proportional to the luminescence intensity; the electrical signal is finally transmitted to a computer and processed by a specific software. The output is a curve (glow curve) which shows the thermoluminescence intensity vs. time (temperature). Some apparatus can hold a radiation (beta) source for sample calibration

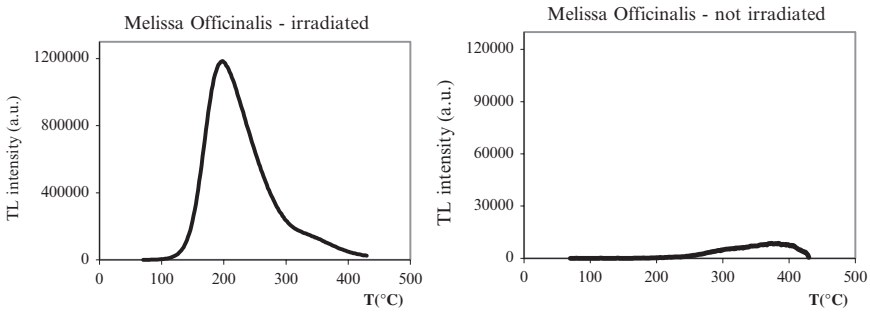

Fig. 13.4 Glow1 curve of Melissa Officinalis irradiated with a dose of 1 kGy and not irradiated

glow curve temperature of about 300 °C, typical of the not irradiated samples (Fig. 13.4). However, since quality and composition (quartz, feldspars etc.) of mineral extracts could exhibit very variable integrated TL intensities after irradiation, a second TL glow curve (Glow2) of the same sample after exposure to a fixed dose of radiation is necessary to normalize the TL response. The TL glow ratio of the integrated Glow1 and Glow2 is used to indicate radiation treatment of the food, as the irradiated samples, on principle, yields higher TL glow ratios than those of not irradiated samples. Sample classification is based on the shape of Glow1 and on the value of glow ratio: the presence of a peak in the $T < 250$ °C region of Glow1 and a glow ratio > 0.1 indicate a radiation treatment. For sample containing only a part of irradiated food, e.g. food supplements containing one or more irradiated ingredients, the TL glow ratio could be below the threshold of 0.1; in this case the shape of Glow1 indicates the status of the sample.

Figure 13.5 represents schematically the method described above applied to an irradiated sample.

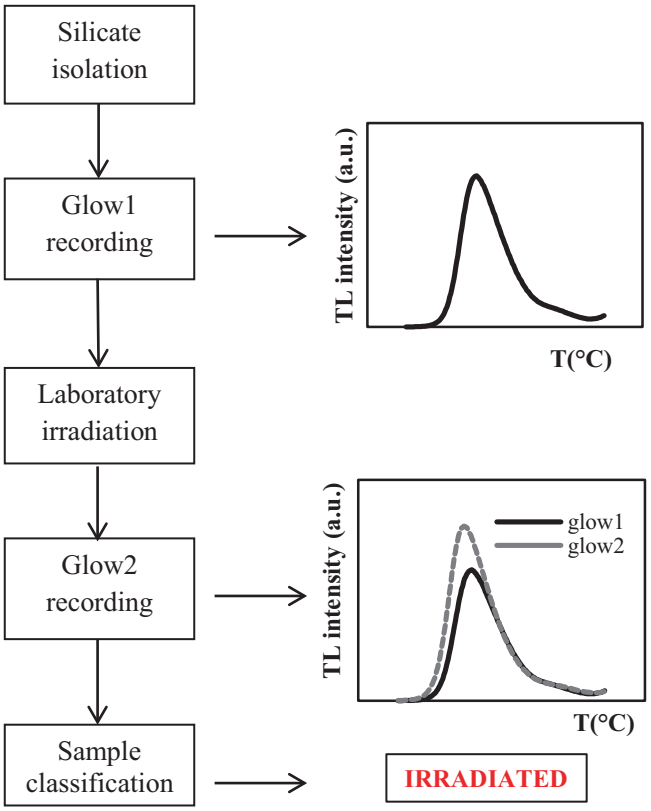

Fig. 13.5 Scheme of the analysis procedure applied to an irradiated sample. A first TL measurement (Glow1) is performed on the silicates isolated from the food. After laboratory irradiation the silicates are measured again (Glow2) for calibration purposes. Sample classification is based on the shape of Glow1 and on the value of glow ratio: the presence of a peak in the T < 250 °C region of Glow1 and a glow ratio, Glow1/Glow2, above the 0.1 threshold indicate a radiation treatment

13.1.1.2 Limitations

The effectiveness of the method relies on the quantity and composition of the silicates isolated from the food; in particular, detection limit and stability depend on the amount and types of silicates collected. Generally, vegetable matrices such as herbs and spices are rich of minerals, however, some spices (pepper, nutmeg) (EN 13751, 2009) and herbal extracts (Bortolin et al. 2009) appear very "clean" and provide limited amount of silicates. The same problem occurred with some fruit and vegetables (Schreiber et al. 1993; Marchioni et al. 1999) as reported in EN 1788 Standard. However, this does not represent a serious limitation since, in practise, it is possible to overcome the problem by using large sample volumes. Indeed, the method has been successfully tested in several inter-laboratory trials with herbs and spices, shellfish, potatoes and several types of fresh and dehydrated fruits and

vegetables, irradiated with different doses and analysed even after long time from irradiation.

In all the trials EN 1788 standard has been validated with samples wholly irradiated or not irradiated but, in principle, it can be used also to detect minor irradiated components, such as few irradiated ingredients in plant food supplements. Beside the reduced content of irradiated silicates, another important limitation in this cases is represented by the presence of the minor geological components in the 200–300 °C region which could hide the weak signal due to low percentages of irradiated component in the sample. The outcome of the analysis depends on the relative sensitivities of the irradiated and not irradiated components.

Concluding, EN 1788 is a reliable method, with very high percentages of success on a wide group of matrices, but it is time-consuming as it requires silicate extraction and a second measurement for calibration purpose. Thus, it is mainly recommended as a confirmatory method after the screening analysis.

13.2 Application of EN 1788 Method to PFS and their Ingredients: State of art

EN 1788 method has been successfully applied to PFS ingredients, raw materials as well as herbal extracts.

Pal et al. (2009) analysed, by different methods including EN 1788, twenty medicinal herbs consisting in root, rhizome, cortex, fruit, peel, flower, spike, ramulus, folium and whole plant, purchased from local wholesale markets in Seul (South Korea). The samples were irradiated in the range (0–50) kGy with a Co-60 irradiator. Due to the different quantity and quality of the silicate contaminants, some differences were observed in the glow curve shape and intensity of the various herbal products, but in any case the responses (Glow1) of irradiated samples appeared very different from those obtained with the not irradiated ones. The glow ratios were below the 0.1 threshold for all the not irradiated samples and above 0.5 for all those irradiated. All the samples were correctly classified. In a more recent work Pal et al. (2010) analysed nineteen different herbs by using TL method and verified the applicability of the method up to twelve weeks from irradiation. Glow1 curves were reproducible after storage under dark condition at about 23 °C and TL glow ratios of irradiated samples (1.223–3.059) were still found to be much higher than those of not irradiated controls (0.001–0.026) for all the nineteen samples.

Another extensive work was carried out by Kwon et al. (2013) on twenty teas (*Camellia sinensis*, *Rosa canina*, *Thymus vulgaris*, *Mentha piperita*, *Ginkgo biloba*, etc.) in both powdered and packed (bag) form, purchased from local wholesale markets in Daegu (South Korea). Not irradiated and irradiated (5, 10 kGy) samples were analysed. All the irradiated samples showed similar glow curves with a maximum at about 180 °C, but different intensities due to the different features of the mineral contaminants. The glow ratios of not irradiated tea samples were all below the 0.1

threshold, whereas the irradiated samples exhibited glow ratios typically higher than 0.1, in accordance with the EN 1788 Standard. The method not only allowed the discrimination between not irradiated and irradiated samples but also showed a clear difference in the intensity of the glow curves obtained at different doses.

Regarding herbal extracts, they are expected to be "clean", e.g. with a reduced content of silicates, as a consequence of the procedure of production at the manufacture stage. This is confirmed by the work of Bortolin et al. (2009), who analysed sixteen different herbal extracts and eight raw plants by both photo-stimulated luminescence (PSL) based EN 13751 and thermoluminescence based EN 1788 methods. In this work the authors also compared the results of eight extracts with those obtained with the corresponding raw materials: the data, especially those of PSL method, which does not require pre-concentration of minerals, clearly indicated a significant reduction of the signal intensity of the extract with respect to the corresponding raw material. Nevertheless, this did not prevent the correct identification of the herbal extracts by TL analysis, as, in this case, the lack of silicate contaminants can be easily overcome by the pre-concentration of minerals during the sample preparation. Indeed, increasing the quantity of product used for the extraction it was possible to collect a sufficient amount of silicates from all the tested herbal extracts.

Detection of irradiated ingredients in PFS appears more complicate since, in this case, the problem is to identify minor irradiated components in a blend. The results depend on the irradiated component percentage as well as on the dose values.

Lee et al. (2010) investigated the applicability of TL method for the detection of different ratios of gamma irradiated turmeric. 1 and 10 kGy irradiated components were detected above 4% blending rate by the analysis of the Glow1 curve as the TL glow ratios were all below the 0.1 threshold.

Kim et al. (2012) report the results of a study carried out on different spice blends with small quantities of irradiated powder spices such as red pepper, garlic or ginger. The glow ratios of the majority of the blends were below the 0.1 threshold. Nevertheless, the method allowed to detect 1% of 1–10 kGy irradiated spices and 0.5% of 10 kGy irradiated garlic by the analysis of the Glow1 which showed the typical features of a positive response.

In the work of Ahn et al. (2012) TL analysis was applied to identify gamma-irradiated garlic powder in Korean barbeque sauce before and after pasteurization (85 °C, 30 min) when blended in different ratios (1, 3 and 5%). The identification of sauce samples were more influenced by blending ratios than by irradiation doses, showing that 3 and 5% irradiated garlic produced the typical glow peaks in the (150–250) °C range. After pasteurization TL glow intensity decreased but did not change its shape or temperature range which still allowed the detection of irradiation.

In a more recent work, Kim et al. (2015) report the results of an intercomparative test to verify the applicability of EN 1788 method for the detection of minor irradiated component in blends. Blends of garlic and ginger containing 0, 0.5, 1, 5 and 10% of 1 or 10 kGy irradiated product, and samples of curry powder and black bean sauce containing 0, 5, 10, 15 and 20% of 10 kGy irradiated garlic or ginger were analysed by four laboratories. The results indicated that the identification of

irradiated ingredients depends on the irradiation dose, food type and proportion of irradiated ingredients mixed with the food. Blends of garlic or ginger containing more than 5% of irradiated product showed positive Glow1, but the detection sensitivity of the method decreased in complicate food matrices such curry powder or black bean. TL glow ratios were not always positive.

All these findings were confirmed by the results of the study recently carried out in the framework of the European project PlantLIBRA. Twelve different raw materials, not irradiated and irradiated with a dose of 1 kGy, were analyzed with EN 1788 method: *Melissa officinalis*, *Peumus boldus*, *Matricaria recutita*, *Passiflora incarnata*, *Foeniculum dulcis* fructus, *Vitex agnus-castus*, *Silybum marianum* fructus, *Citrus aurantium*, *Harpagophytum procumbens*, *Serenoa repens*, *Aloe ferox*, *Plantago ovata*.

The procedure for the extraction of mineral silicates, described in the EN protocol, has proved to be inadequate only for *Aloe ferox* and *Plantago ovata*. In both cases the procedure was modified using ethanol (95%), instead of water, in the first step of extraction. TL measurements were carried out using a Harshaw 3500 TL reader in the 70–430 °C range with a heating rate of 6 °C/s. Samples irradiation was performed by using a Co-60 Gammacell facility. The whole PFS ingredients were packed in polyethylene bags and irradiated at the dose of 1 kGy, which is well below the minimum dose applied for the treatment of similar products such as herbs and spices. The products were irradiated and stored in containers protected from light. The silicates were irradiated, for calibration purpose inside single stainless steel cups used for TL measurement. As regard to irradiated samples, they showed Glow1 curves characterized by intense glow peaks in the range (170–209) °C. The differences observed in the glow curve shape recorded with different samples are to be attributed to the variable composition of the minerals extracted from the plants grown up in different environmental conditions. The glow ratios of the irradiated samples were all above the threshold (0.1) demonstrating the effectiveness of the CEN Standard 1788 protocol in detecting irradiation even at low doses (1 kGy). Not irradiated samples provided TL glow ratios, G1/G2, well below the 0.1 threshold, as required from the CEN Standard 1788 protocol. The Glow1 curves of the not irradiated samples showed a weak peak at about 300 °C due to the natural radiation background (geological signal). In Fig. 13.6, as an example, the glow curves of not irradiated and totally irradiated samples of *Melissa officinalis* are reported. The differences between Glow1 and Glow2 of the irradiated sample are due to the fading effect during the storage: the low temperature portion of Glow1 tends to reduce with time which causes a shift of the whole curve towards the high temperature side. Measurements were also carried out to test the applicability of the method for the detection of PFS containing minor irradiated components. To this purpose, an aliquot of irradiated (5 kGy) raw material was added to an aliquot of not irradiated sample to obtain a blend of 10% of irradiated component. The plants used for the analyzed blends were: *Aloe ferox*, *Citrus aurantium*, *Serenoa repens* and *Melissa officinalis*. All the blends, containing 10% of irradiated product, showed Glow1 curves with a visible glow peak at $T < 250$ °C; this glow peak, well distinguishable from the geological residual

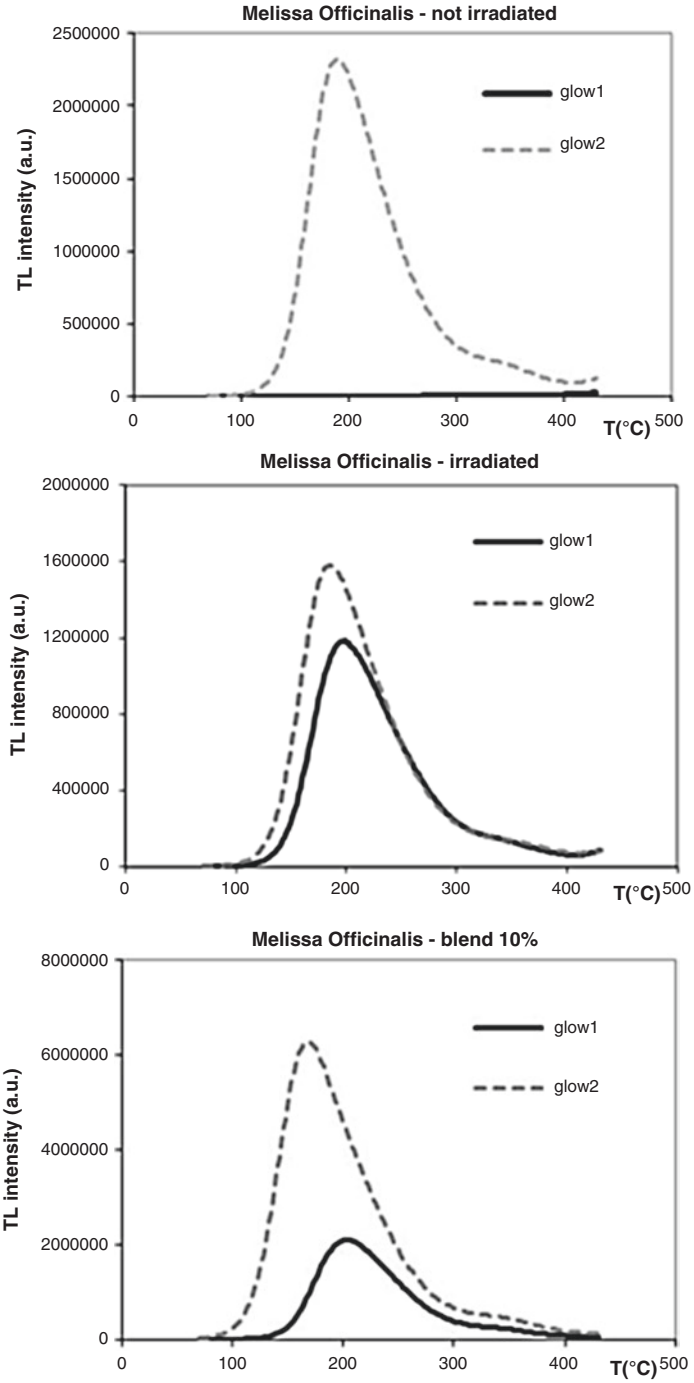

Fig. 13.6 Glow curves of plant samples, not irradiated, totally irradiated and partially irradiated (blend). The peak at about 200 °C in the Glow1 of the blend clearly indicated the radiation treatment. The intensity of the peak appeared reduced with respect to the Glow2 since only a part of the silicates extracted from the sample were irradiated

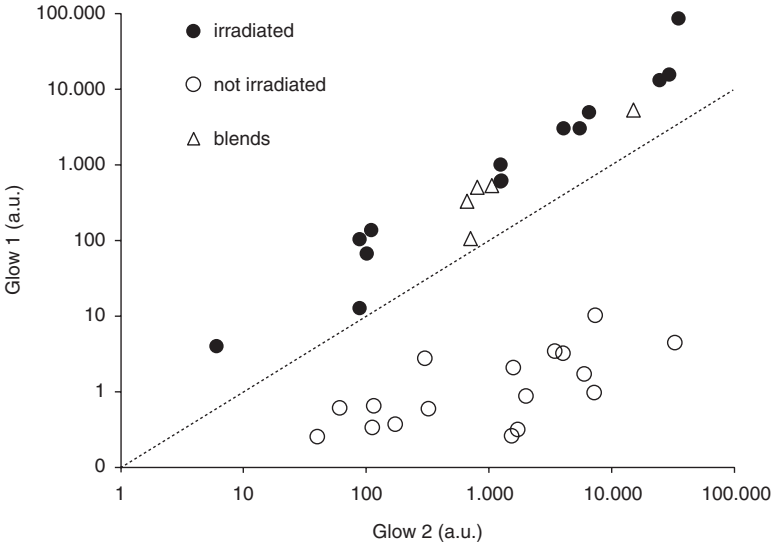

Fig. 13.7 First glow curve (Glow1) vs. second glow curve (Glow2): all the irradiated samples, including the blends, are above the curve corresponding to glow ratios = 0.1 (*dotted line*), whereas the not irradiated ones lie below

visible in the untreated products, was due to the fraction of silicates contaminating the irradiated component (10%) of the product. The presence of this glow peak in the T < 250 °C) allowed to correctly classify the samples. The glow ratios were all above the threshold (0.1). In Fig. 13.6 the glow curves of a blend containing a minor irradiated component is also reported.

The results of the study are summarized in the graph of Fig. 13.7. As can be seen, the two groups of not irradiated and irradiated samples appear well separated: all the irradiated ones, including the blends, lie above the curve corresponding to glow ratios = 0.1, whereas the not irradiated samples are localized below.

Concluding, EN 1788 method can be successfully applied to PFS ingredients, raw materials as well as herbal extracts but has to be used with caution for the identification of herbal supplements containing different components which could have been separately irradiated. On the basis of the results reported above, in fact, the detection of minor irradiated components in a blend is not always possible by TL method, depending on the irradiation dose, food matrix and proportion of irradiated ingredients mixed in the product.

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Part III
The Risk and Benefit Assessment for Consumers' Safety

Chapter 14
Risk and Benefit Assessment: Methodologies and Scientific Acceptance

Antonella Guzzon and Luca Bucchini

Abstract Plant Food Supplements (PFSs) may present both risks and benefits. Methodologies for Risk-Benefit Assessment (RBA) of foods have been developed in the past two decades. As for risks, EFSA has published a guidance document on the safety assessment of botanicals and botanical preparations used in food supplements (EFSA J 7:1249, 2009a). On the other hand, beneficial health effects of PFSs are often controversial. Regulation (EC) No. 1924/2006 on nutritional and health claims currently applies to the health effects claimed for PFS. So far, the few claims for botanicals examined by the EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA) have not received a positive evaluation due to absence or poor quality data available; as traditional use is not accepted as an evaluation criterion, such assessments have been put on hold. Moreover, health benefits of botanicals are mostly not related to disease risk reduction, and therefore to standard measures of risks and benefits. Given the uncertainty in benefit assessment, an agreed procedure to perform an integrated risk-benefit assessment (RBA) of botanicals used in PFSs has not been put forward or applied. Based on a review of approaches applied to foods, and to herbal medicines, potential approaches are presented, taking into account the outcome of an expert workshop organized in the context of the PlantLIBRA project.

Keywords Risk and Benefits • Safety • Risk and Benefit Assessment • Botanicals • Plant Food Supplements (PFS)

14.1 Introduction

Plants, and also plant preparations, such as extracts, in liquid or powder form, have been part of the human diet for a long time. Water extracts of *Camellia sinensis* leaves or of *Coffea arabica* powdered beans are currently part of the diet of a large proportion of the world's population (Weinberg and Bealer 2001). For at least 3500 years, humans have been aware of the benefits of plants as a source of

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phytochemicals, but also of the toxicity of some plant species (Porter 2006). In modern times, depending on specific regulations of different countries, some plants may be permitted for use in concentrated form in food (or dietary) supplements, a special category which falls under the scope of food legislation (as opposed to medicine legislation). Such products are targeted to healthy individuals who wish to obtain specific physiological benefits (e.g., maintaining normal cholesterol levels, relaxation, etc). Pre-market authorisation is not required in the US (Dietary Supplement Health and Education Act of 1994) and in the European Union (European Parliament and Council 2002).

In line with requirements for foods, Plant Food Supplements (PFSs) must be safe, like their plant constituents (botanicals). Consumption of foods and nutrients is not totally devoid of risks. Vitamins or minerals are indispensable for life, and yet pose demonstrable risks at high dietary exposure.

In the food sector, in the past two decades, there has been a growing demand for the concurrent assessment and management of risks and benefits of foods. In the late '90s, for example, it has been highlighted that an exclusive focus on the risks of mercury and other contaminants in fish, when translated in policy, could lead to an increased consumption of red meat, with a resulting negative health impact; the concept of countervailing risks emerged (Graham and Wiener 1997). Research projects (among others, the EC-funded BRAFO project (Hoekstra et al. 2012) and regulatory opinions (US FDA 2009; EFSA 2008) have started to look at the net balance between the risks and benefits of foods.

PFS are special foods. However, given that they do not provide nutrients and are not regulated as medicines (for which a regulatory framework encompassing risks and benefits is in place), it is not clear whether benefits and risks of PFSs should be directly compared, or whether they should be assessed independently.

14.2 Risk-Benefit Assessment of Food

14.2.1 EFSA Guidance

In 2010, the European Food Safety Authority (EFSA) published a guidance document on human health risk-benefit assessment of foods (EFSA 2010a), outlining the authority's approach to Risk-Benefits Assessment (RBA) and summarizing the state-of-the art in the field.

According to EFSA, the purpose of a food RBA is to allow a risk-benefit manager to weigh the probability of a health risk against the probability of a health benefit, by a qualitative and/or quantitative approach. Three parts in the assessment are identified: risk assessment, benefit assessment and risk-benefit assessment.

The structure for the benefit assessment (positive health effect identification, positive health effect characterisation, exposure assessment and benefit characterisation) mirrors the risk assessment paradigm (hazard identification, hazard characterisation, exposure assessment, and risk characterisation). The final stage of the RBA is the weighing of results of the risk assessment and of the benefit assessment, using a common scale.

EFSA devotes attention to situations that may require a RBA, many of which could theoretically be applicable to botanicals and PFSs. They include situations where a compound or food constituent has both positive and negative health effects occurring in the same population (zinc, vitamin A, iron, etc.) or in different populations (e.g. folic acid fortified food for prevention of neural tube defects in unborn child and potential hazards as masking vitamin B_{12} deficiency in elder people). In other cases, positive and negative health effects result from different components in the same food (e.g. fatty fish, where positive effects from omega-3 fatty acids should be weighed against negative effects due to dioxins or PCBs). Other situations are related to levels of dietary exposures, or chemicals used to reduce microbial contamination.

EFSA also makes an important distinction: current approaches to RBA of foods restrict the risk-benefit assessment to net health effects without taking into consideration other factors such as social, economic and legal ones, which may legitimately influence risk-benefit decisions.

EFSA's approach has three steps http://onlinelibrary.wiley.com/doi/10.2903/j.efsa.2010.1673/epdf:

Step 1: *initial assessment*,
Step 2: *refined assessment,*
Step 3: *assessment using a composite metric*.

In the initial assessment risks and benefits are estimated separately and their health effects are compared to assess whether risks far outweigh the benefits or *vice versa*, via two scenarios. In the "benefit scenario", if benefits at low dietary exposure are much larger than risks at high exposure, the analysis will focus on benefits only, as risks may be minimal (e.g., if the ADI is not exceeded at all exposure levels). In the opposite "risk scenario", if risks at low dietary exposure are much larger than benefits at high exposure, the analysis will focus on risks only, as it is clearly the key concern (e.g., if all exposure levels are below the effective dose). If risks and benefits do not clearly outweigh each other, then the assessment should be refined, as in step 2, with a new formulation of the problem (endpoints and populations to be considered, refined exposure assessment and potential for quantification). If after the refinement of the assessment there is still no sufficient evidence for a net risk or a net benefit, additional refinement might be necessary (step 3).

Step 3 involves the comparison of risks and benefits using composite metrics ("common currencies" in this chapter), a single measure reflecting a number of dimensions of health (increases or decreases in morbidity, mortality, disease burden, quality of life). The outcome is therefore a single net health impact value.

14.2.2 The BRAFO Approach

The EU-funded BRAFO research project has proposed another RBA framework for net benefit comparisons (Hoekstra et al. 2012), and applied it to natural foods (Watzl et al. 2012). It is also a stepwise process and consists of four tiers https://www.ncbi.nlm.nih.gov/pubmed/20546818:

Tier 1: *individual assessment of risks and benefits,*
Tier 2: *qualitative integration of risks and benefits,*
Tier 3: *deterministic computation of common health metric,*
Tier 4: *probabilistic computation*.

The difference existing in the four tiers lies in the extent of integration of risks and benefits. In Tier 1 risk assessment and benefit assessment are performed independently while from Tier 2 to 4 risks and benefits are increasingly integrated with incrementally sophisticated approaches. Uncertainty in the net health effects (no clear dominance of risks or benefits) is the trigger for transition from Tier 1 to 2.

In Tier 2 the comparison between risks and benefits is qualitative. It takes into consideration only the results of the positive health and hazard identification steps of Tier 1, using four health impact dimensions, i.e. incidence, severity of the health effect, duration and additional mortality caused by the effects. If Tier 2 provides an outcome with unacceptable uncertainty, then risks and benefits have to be integrated quantitatively in a common metric by a deterministic (Tier 3) or probabilistic approach (Tier 4). From Tier 3 composite metrics such as QALYs and DALYs are recommended.

This approach has been applied to farmed salmon and to soy protein (Watzl et al. 2012). In the case of salmon, the current low fish consumption level in Europe is compared with a scenario of higher intake. Initially, positive health effects (those due to ω−3 long chain polyunsaturated fatty acids, selenium, iodine and vitamin D) and hazards (methyl mercury, PCBs and dioxin) were identified. Tier 1 was inconclusive, but a comparison of intake and reference doses for benefits and risks at Tier 2 permitted to conclude that increased fish consumption would decrease the incidence of cardiovascular heart disease, outweighing risks. For soy protein, a scenario of 25 g/day of soy protein intake was weighed against no intake. Potential benefits of soy proteins are related to risk reduction for breast and prostate cancer, for cardiovascular disease and improved bone health while potential risks are related to disruption of sex hormone levels and changes in reproductive function, impaired thyroid function and cognitive functions, increased breast cancer risk. The RBA was concluded at Tier 2.

A common feature of the two methods (Hoekstra et al. 2012; EFSA 2010a) is their stepwise nature: it is foreseen that the risk-benefit assessor, after completing each step, would discuss with the risk-benefit manager on whether the net health impact is beneficial or negative, taking into account the uncertainties, and decide whether to continue to refine the assessment, or not.

A more complex approach was applied to the net health impact of mandatory fortification of bread with folic acid in The Netherlands (against a scenario of no

bread fortification) (Hoekstra et al. 2008). The methodology tested by the authors consisted of:

1. hazard and benefit identification (selection of adverse and positive health effects, selection of population)
2. hazard and benefit characterisation through dose-response function.
3. exposure assessment (selection of scenarios, computation of intake distributions)
4. risk-benefit integration (calculation of prevented and/or additional incidence of diseases, expressing change in disease incidence in a common metric).

The beneficial effects identified were the prevention of neural tube defects in women of childbearing age and of megaloblastic anemia in all the population, while the hazard was the masking of vitamin B_{12} deficiency in the elderly. Possible effects of folate in carcinogenesis were also identified: a protective role of folate from colorectal cancer at high intake but increased risk of cancer at very high intake levels. Four scenarios of levels of fortification were chosen (70, 140, 280, 420 μg folic acid/100 g bread) and compared against the reference scenario. As a common metric, DALY was used. The risk-benefit assessment showed that fortification of 140 μg folic acid/100 g bread caused the maximum change in DALY, resulting in the most reasonable scenario for improving public health.

14.2.3 The use of Common Currencies

For quantitative assessments, an overview of the metrics used for risk-benefit assessment is provided by EFSA (EFSA 2010a). DALYs (Disability Adjusted Life Years) and QALYs (Quality Adjusted Life Years) are the composite metrics commonly used to express risks and benefits in the food sector. DALY is a time-based measure of the overall burden of disease calculated as the sum of years of life lost due to premature mortality in the population and the years lost to disability weighed for the severity of disease. QALY is a metric for burden of disease adjusted for quality of life. To express a health adverse or beneficial effect using DALYs and QALYs four parameters are important; incidence, severity, duration of disease, the number of years of life lost for DALYs (and the number of healthy years for QALYs). The DALYs or QALYs of the population considered in the assessment are calculated by summing the DALYs/QALYs of all individuals.

14.2.4 Uncertainty in Food-Sector RBA

In RBA uncertainties are present and affect the estimated net health impact. Hoekstra et al. (2012) report a list of common sources of uncertainties that are:

– Uncertainties affecting problem formulation.
– Uncertainties affecting hazard and benefit identification.

– Uncertainties affecting intake assessment.
– Uncertainties affecting dose/relationships estimated from animal data.
– Uncertainties affecting dose/response relationships estimated from epidemiological studies.
– Uncertainties affecting the conversion to common health metrics.

Evaluation of uncertainties can be qualitative or quantitative. In many cases a qualitative description of uncertainties might provide an adequate basis for decision-making by risk-benefit managers.

14.3 Risk-Benefit Assessment of Vitamins

Renwick et al. (2004) have significantly advanced approaches to RBA of micronutrients, using Recommended Dietary Allowances (RDA) and Upper Levels (UL) as reference values. Their approach relies on the coefficient of variation and its ability to substitute a genuine dose-response curve for vitamins and minerals. The approach, however, may not work for plant compounds. On the other hand, the authors propose a useful probability-based presentation of the assessment's results to risk benefit managers.

14.4 Systematic Reviews and Meta-Analyses as Risk-Benefit Assessments

In recent years, systematic reviews and meta-analyses have become standard practice, not only in the field of medicine but also in that of food supplements (most famously and controversially, for antioxidant vitamins (Bjelakovic et al. 2007). To avoid pitfalls, efforts have been made to monitor and encourage use of standardized methodologies (Chung et al. 2009).

While meta-analyses and systematic reviews are conceptually distinct from risk assessments or risk benefit assessments, it is often claimed by their authors, also in the case of botanicals, that they allow conclusions about risks (Bent et al. 2006; Jepson and Craig 2008; Geng et al. 2010) and benefits (Bent et al. 2006; Schoop et al. 2006; Reinhart et al. 2009; Fernández-San-Martín et al. 2010; Geng et al. 2010; Wei et al. 2009) (Table 14.1); in fact, they even include qualitative assessments of uncertainty (Fernández-San-Martín et al. 2010; Geng et al. 2010). EFSA, in its assessment of health claims, has given significant weight and scrutiny to meta-analyses which, when positive, with relevant studies and without significant publication bias, have generally resulted in positive opinions (EFSA 2010b, c, d, 2011). If meta-analyses or pooled analyses are accepted on the benefit side, they should also probably be considered on the risk arm of an RBA.

Table 14.1 Systematic reviews of botanicals used in some PFS which provide information of risks and benefits (see text for references)

Botanical	Beneficial effect	Risk assessment	Benefit assessment
Cranberry	Urinary tract infection in women	"*Side effects were common in all studies, and dropouts/ withdrawals in several of the studies were high*"	"*There is some evidence that cranberry juice may decrease the number of symptomatic UTIs* [urinary tract infections] *over a 12 month period, particularly for women with recurrent UTIs*"
Echinacea spp.	Reduction of risk of common cold	–	"*Standardized extracts of Echinacea were effective in the prevention of symptoms of the common cold after clinical inoculation*"
Garlic	Blood cholesterol reduction	–	"*Garlic reduces TC to a modest extent*"
Ginseng	Cognitive function	"*No serious adverse events associated with ginseng were found*"	"*Results of the analysis suggested improvement of some aspects of cognitive function, behavior and quality of life*" "*Currently, there is a lack of convincing evidence to show a cognitive enhancing effect of Panax ginseng in healthy participants*"
Psyllium	Blood cholesterol reduction	–	"*psyllium could produce dose- and time-dependent serum cholesterol-lowering effect in mild and moderate hypercholesterolemic patients*"
Valerian	Reduction of sleep onset latency	–	"*valerian would be effective for a subjective improvement of insomnia, although its effectiveness has not been demonstrated with quantitative or objective measurements*"
Valerian	Reduction of sleep onset latency	"*without producing side effects*"	"*the available evidence suggests that valerian might improve sleep quality*"

In the future, such reviews, if properly standardized and conducted with an appropriate mandate, could substitute, or at least complement, current forms of assessments also in the regulatory domain. Meta-analyses have a powerful way to analyze data from studies, which often provide information on both benefits and risks, and offer quantitative estimates. Moreover, they are particularly useful in defining the requirements of further research, can easily be transposed into conditions of use (i.e. those in the studies), and may need fewer assumptions than model-based risk-benefit assessments. Other sources of information, such as animal data or adverse effects, could be integrated into the conclusions of a meta-analysis, either qualitatively or quantitatively.

14.5 Risk-Benefit Assessment of Herbal Traditional Medicinal Products

Risk-benefit assessment is a common concept in the field of medicines. The methodology, however, is not standardized (EMA 2010a). As a consequence, the European Medicines Agency (EMA), which is responsible also for assessing herbal medicines in the EU (the regulatory framework of herbal medicines is discussed in Paper 1 of this supplement), has begun a process to update the application of risk-benefit assessment to medicines, including herbal medicines (which, like PFSs, are made of botanicals).

With a report which was concluded in 2007 (EMEA/CHMP 2007), EMA concluded that RBA models could provide a series of important elements such as:

- RBA driven by the identification of the most important benefits and medically serious risks.
- Explicit weights assigned to individual benefits and risks depending on their importance.
- Strength of evidence and uncertainty identified and quantified.

The EMA report also suggested to:

- Use a structured and mainly qualitative approach.
- Describe explicitly the importance of benefits and risks in the specific therapeutic context.
- Describe uncertainties and their impact on the benefit-risk assessment.

EMA has also concluded that a quantitative approach (Tiers 3 and 4 of BRAFO) is probably not useful for medicines, a conclusion which may be relevant for PFSs as well.

More recently, EMA has summarized possible methodological approaches, including some which are used in the food sector (probabilistic simulations, DALYs, QALYs) (EMA 2010b).

Even if methodological research is ongoing, in the EMA assessments of botanicals (herbal substances) and botanical (herbal) preparations, references to RBA are common, and the conclusions of the assessments describe risks and benefits. In this respect, EMA has declared that, even for traditional products for which no proof of efficacy is necessary (the concept of plausibility is used instead), any risk has to be balanced against the plausible efficacy/potential benefit for the consumer (EMEA/HMPC 2005). Since the same botanicals are used in herbal medicines and PFSs, it is of interest to examine the methodology used by EMA in this context.

In comparing the approaches to RBA of EMA, as applied to herbal medicines, to the food-related RBA guidance of EFSA or BRAFO, it should be preliminarily noted that the separation of risk assessment and risk management does not seem a priority for herbal medicines as it is for food risk-benefit assessors. The EMA committee that assesses the risks of an herbal substance also makes a judgement of whether the risk is

acceptable, through an explicint recommendation to include a herbal preparation in the list of permitted herbal preparations and defining the conditions of its inclusion.

In terms of methodology, the EMA assessments are characterized by a qualitative approach; uncertainties are mentioned, but are not translated in figures or left to the managers to evaluate, in contrast for example with the proposal of Renwick et al. (2004). Quantitative estimates are rare, or absent, from the conclusions; the substance is safe, or not, and is efficacious, or not. Thanks to limiting, in a series of steps, the scope of the assessment and of the decision, to reduce uncertainty, the process always ends at EFSA's step 1 or BRAFO's Tier 1 (see above). This is similar to EFSA's compendium-building process (EFSA 2009c), where plants with toxic compounds are placed in an annex of plants with toxic, addictive, psychotropic or other substances that may be of concern, based on the presence of a hazard (if botanicals not on this list, an interpretation that EFSA discourages, were to be considered safe, a hazard-only approach would have been applied). It should be recognized, however, that EMA assessments do sometimes address exposure and dose-response.

Exposure to herbal medicines and PFSs can be conceptualized in two key arms, composition and intake. Variability of composition is related to the exposure to toxic, and beneficial, compounds. EMA provides an interesting approach to the assessment of composition. Like EFSA's ESCO PFS case studies (EFSA 2009b), segmentation is the primary tool used by EMA to address such variability**.** For example, in the case of leaves of *Peumus boldus* Molina (EMEA/HMPC 2007a), the herbal preparations are divided in two groups, with one with unacceptable risk (ethanolic extract) and the other with acceptable risk (leaf water extract), due to different levels of ascaridole.

As a rule, again in terms of composition, EMA assessments do not include a quantitative estimate or specification of expected levels of compounds or toxicants. For example, in the case of the leaves of *Rosmarinus officinalis* L. (EMEA/HMPC 2009a), carnosol, carnosic acids and camphor are discussed in detail in the assessment, while the expected values in the final products are not established. However, where beneficial well-established use is accepted (a higher standard of evidence, as opposed to traditional use), standardization is required in the specifications (EMEA/HMPC 2007b, 2008a); also, well-established use status is not granted based on insufficient data on preparation and extract composition (EMEA/HMPC 2008b). Use in pregnancy is not recommended if the species is not clarified in the reference study (EMEA/HMPC 2008b).

Assessment of compounds present or added to foods is generally mediated by the establishment of a reference dose (an Acceptably Daily Intake, ADI, or a Tolerable Daily Intake, TDI, derived from a No Adverse Event Level, NOAEL, with uncertainty factors); other approaches are proposed within this supplement (Chapter 7). The first step in both EFSA's and BRAFO's methodologies usually relies on these reference doses, including an effective dose for the benefit side of the assessment.

In this sense, EMA has not required an *a priori* establishment of a reference value for assessing toxicity; the necessity of a reference dose, and margins when compared to actual exposure, is sometimes excluded because of history of use. When a reference toxic dose can be estimated for a compound, it is used to confirm safety, and

considered not relevant when in conflict with the history of safe use (EMA/HMPC 2009b); even when toxicity studies are reported, they are not used to derive a safe dose (EMA/HMPC 2009b, c). when reference values are used for individual compounds, the calculated margins can be considered pivotal (EMA/HMPC 2008a), or of no relevance (EMA/HMPC 2009b) based on expert judgment; in some cases, the calculations are detailed (EMA/HMPC 2008b). Another relevant factor in exposure, duration, is often controlled via specifications, to reduce risks and uncertainty.

After segmentation of the variability in exposure, EMA makes a judgement of whether the toxicological profile is acceptable or not, a step which is similar to the hazard identification phase in food risk assessment. It is noteworthy (Table 14.2) that lack of data, or the highest level of uncertainty, does not preclude a positive final evaluation if clinical data or history of use do not reveal serious adverse events. Save exceptions, only uncertainty on mutagenicity is overriding all other considerations.

In terms of human variability, subpopulations are addressed separately. For children, adolescents, and pregnant women, EMA has a precautionary default; in case of lack or uncertain evidence use is not recommended. When clinical safety data are available for children, they are used down to the age in which serious adverse effects are reported (EMEA/HMPC 2008c). Pregnant women are included as potential users only when a clinical trial has been conducted (EMEA/HMPC 2008a).

For other subpopulations, use is allowed in the absence of convincing evidence of risk, though precaution is exercised in some cases. For example, the possible interaction with anticoagulants is ruled out after examination of case-reports (EMA/HMPC 2010a). At the same time, in the case of centaury (*Centaurium erythraea*) (EMEA/HMPC 2008d), textbook evidence (or evidence from compounds with similar action (EMEA/HMPC 2007b; EMEA/HMPC 2008b) is used to exclude patient groups; in the case of dandelion leaves (*Taraxacum officinale*) (EMA/HMPC 2008c), the theoretical possibility of hyperkalemia is used to advise against the use in patients with renal failure and/or diabetes, and/or heart failure.

More generally, the absence of adverse events related to the proposed preparations, reported by surveillance systems or in the literature, is used as sufficient information to conclude that a preparation is safe (EMA/HMPC 2009b), even in the absence of clinical studies. Adverse events related to combination products may not be considered relevant (EMEA/HMPC 2008d). When information is available from studies, mild adverse effects are not considered relevant for questioning the recommended use (EMEA/HMPC 2007b, 2008a).

One of the key differences between food sector assessments and those of EMA is in relation to uncertainty, lack of data, and precaution. In case of uncertainty, with the exception of mutagenicity and some vulnerable subpopulations, EMA generally accepts lack of robust evidence as an indication of absence of risk (EMA/HMPC 2010b): in the single study cited, mild gastrointestinal symptoms were reported for 62% of the 15 participants, and one more serious side effect required hospitalization. In the case of maté leaves (*Ilex paraguariensis* St. Hilaire*)* (EMA/HMPC 2008d), a favourable opinion is given even if aqueous extracts appear mutagenic in vitro, and there is epidemiological evidence that at least hot matè is carcinogenic to humans: the uncertainty in the information is used to argue that the data are not relevant.

Table 14.2 Sample EMA assessment of botanicals; effect claimed; availability of data on clinical efficacy (Eff), traditional use/biochemical and animal evidence (Trad Use), clinical safety (ClSaf), toxicology (Tox); RBA outcome (RBA) [Explanations below]

Botanical (herbal substance)	Beneficial effect	Eff	Trad Use	ClSaf	Tox	RBA
Root of *Althaea officinalis* L.	Symptomatic treatment of oral or pharyngeal mucosa irritation and associated dry cough					MR, RU
Root of *Althaea officinalis* L.	Symptomatic relief of mild gastrointestinal discomfort					MR, RU
Seeds of *Aesculus hippocastanum* L.	Chronic venous insufficiency					RU
Aerial part of *Centaurium erythraea* Rafn s. L.	Dyspeptic/gastrointestinal disorders and in temporary loss of appetite					MR, RU
Root of *Echinacea pallida* (Nutt.) Nutt.	Supportive treatment of common cold					MR, RU
Root of *Echinacea purpurea* (L.) Moench,	Preventive or supportive treatment of common cold					RU
Seeds of *Trigonella foenum-graecum* L., semen	Lack of appetite					MR, RU
Leaves of *Ilex paraguariensis* St. Hilaire, folium	Increase the amount of urine as an adjuvant in minor urinary complaints					RU
Leaves of *Ilex paraguariensis* St. Hilaire	Fatigue and sensation of weakness					RU
Aerial part *Leonurus cardiaca* L.	Nervous tension					MR, RU
Leaves of *Urtica dioica* L., *Urtica urens* L.	Relief of minor articular pain					MR, RU
Leaves of *Urtica dioica* L., *Urtica urens* L.	Adjuvant in minor urinary complaints					MR,RU
Leaves of *Ribes nigrum* L.	Inflammatory conditions					MR,RU
Leaves of and essential oil of *Rosmarinus officinalis* L.	Symptomatic relief of dyspepsia and mild spasmodic of the gastrointestinal tract					RU
Bark of *Salix alba* L.	Symptomatic treatment of fever and pain					RU
Bark of *Salix alba* L.	Low back pain					RU
Leaves of *Taraxacum officinale* Weber ex Wigg.	Diuresis stimulation					RU
Essential oil of *Thymus vulgaris* L.	Expectorant in cough associated with cold					MR, RU

Colors indicate authors' estimate of uncertainty level (*dark brown*: no data; *light brown*: very limited data; *dark orange*: limited data; *light pink*: sufficient data) and the outcome of the assessment (*green*: benefits clearly outweigh risks). All the assessments appeared concluded at tier 1 (BRAFO). *MR* mutagenicity data required to proceed with assessment; *RU* restrictions in use apply

As for benefits, EMA relies on biochemical (related to individual compounds), or animal evidence to validate traditional use. In some cases, animal and *in vitro* evidence is available (EMEA/HMPC 2008b). In general, correspondence of preparations and clinical endpoints between assessed preparations and studies is not required. For example, in the case of *Rosmarinus officinalis* L. (EMA/HMPC 2009a), limited *in vitro* results, and not related to the preparations of interest, are used to validate a mild anti-spasmodic effect.

When well-established use, which requires stronger evidence, is accepted, meta-analyses often play a prominent role (EMEA/HMPC 2008a). Though well-established use is normally based on RCT studies, sometimes indications resulting from open studies have been accepted (EMEA/HMPC 2007b).

In summary, what EMA terms "benefit-risk assessment" for herbal products is really a an expert statement in which, according to the assessment, there is no risk for the subpopulations for which use is allowed and, generally, in which traditional use and *in vitro* evidence is used to argue that a benefit exists. In only one case (EMA/HMPC 2009c), a comparison is made with existing drugs (the leaves of *Ribes nigrum* L. are considered safer than other medicines, because of lack of reported side effects). EMA assessments are not actual comparisons of risks and benefits, but rather a useful and necessary qualitative assessment of safety and of possible benefits.

On the other hand, an actual RBA for a plant substance, with a structured comparison of the net health impact, has been carried out for ephedrine, a compound from plants in the genus *Ephedra*, by the US FDA (Food and Drug Administration) in 2007 (US FDA US Food and Drug Administration 2007).

In this case the standard applied was a relative weighing of "known and reasonably likely risks against its known and reasonably likely benefits". The US FDA considered evidence from pharmacology of ephedrine alkaloids, peer-reviewed scientific literature on the effects of ephedrine alkaloids, and adverse events.

Since ephedrine alkaloids raise blood pressure and augment heart rate, increasing the risk of serious adverse events such as stroke, heart attack, and death, the US FDA concluded that dietary supplements containing ephedrine alkaloids pose short-term and long-term risks. At the same time, according to the US FDA, on the benefit side, only a modest short-term weight loss is supported by the data, which is not sufficient to have a positive effect on cardiovascular risk factors or other health conditions associated with overweight or obesity. Other possible benefits were considered trivial in comparison to the health risk. The US FDA also concluded that labelling could not mitigate the risks.

14.6 Rationale and Approaches for Risk Benefit Assessment

Preparations of single or multiple botanicals, commercialized in supplement form, are popular with consumers, with apparent opposition to limitations or bans on their use. Thanks to their relatively long history of use, contrary to new or novel food-impacting technologies (GMOs, cloned animals, nanotechnology, etc), there does

not seem to be a demand, from the public, for a strongly precautionary approach. From a public health point of view, convincing evidence that all PFSs generally pose unreasonable health risks is not available. If misleading consumers with scientifically unsubstantiated claims is of concern, then the EU legislative framework for herbal medicines—which should prevent or cure disease, and not maintain health—does not provide a rationale for regulating botanicals exclusively as medicines, since for herbal medicines the standard of evidence is mainly tradition. Therefore, since an outright ban on all PFSs, would be unjustified, methodologies to assess risks and benefits, and to combine the assessments, are required (EFSA has proposed a methodology to assess safety of botanicals (EFSA 2009c)).

In fact, regulators and their scientific advisers across the world need to allow, or not to allow, or continue to allow, the use of a specific plant species—in concentrated form—in food supplements. They, and scientists in industry, are also faced with the task of assessing benefits but also individual multi-ingredient supplements. While management and policy decisions may take into account a number of other factors (economic, legal, etc.), it is generally expected that these assessment should be restricted to health benefits and risks only, as outlined by EFSA (EFSA 2010a).

EFSA (EFSA 2009b) has tested its assessment guidance of botanicals in a series of real case studies. These case studies show that, for the botanicals considered (hydroalcoholic extract of dried peel of *Citrus aurantium* L. ssp. *aurantium* L., dried green tea extract of *Camellia sinensis* (L.) Kuntze; dried leaves extract of *Ocimum tenuiflorum* L., dried fruits water extract of *Foeniculum vulgare* Mill. *ssp. vulgare* var. *vulgare*, dried ripe seeds of *Linum usitatissimum* L., and wheat bran from *Triticum aestivum* L), which are well known and widely used plants (with the exception of *O. tenuiflorum*), the safety assessments required in all cases additional unavailable data, could not be completed, and the botanicals could not be declared safe.

This situation is not unique to PFSs. For other foods as well, datasets are incomplete, and the doses at which benefits are claimed, if not proved, overlap with those at which risks cannot be ruled out. The risk manager is not left with a risk-only, low uncertainty scenario, and has to evaluate a risk-benefit and uncertain situation. A RBA methodology of botanicals could help combine, qualitatively and perhaps quantitatively, risks and benefits, leaving to risk assessors individual assessments, to managers the role to decide if there is an acceptable balance of benefits and risks, and to society at large to instruct managers on what an acceptable net health effect is.

14.6.1 Botanicals as Agents in the RBA

When dealing with PFSs, risk managers may have different questions, which define the agent in a RBA. Defining the question with clarity has been stressed by EFSA (EFSA 2010a).

In the case of PFSs, several EU countries (e.g. Italy, Belgium) have safety-based positive and negative lists of plant species, sometimes with the indication of the parts that can or cannot be used. Therefore, the obvious agent for the RBA are plants at the species level, with specification of the plant part used. However, the assessment of such plants often involves the assessment of individual phytochemicals, and the setting of maximum levels for the compounds of concern.

In other cases, compounds or groups of compounds (e.g. polyphenols, flavones, etc) can be the target of an assessment, regardless of the plant species. In this case, exposures from the diet (i.e., excluding PFSs) to the compound of interest would need to be combined with that from different PFSs.

14.6.2 Potential Features of PFSs RBA

EFSA and BRAFO's approaches to RBA include tiers, or steps, of increasing complexity; transition from a tier to the next is triggered by uncertainty in the net health effect. This seems a reasonable approach for assessing risks and benefits of PFSs as well.

Higher tiers of food-sector RBA involve quantification. On the contrary, RBA of medicines, including herbal medicines, remains mainly qualitative, while EMA stresses the importance of uncertainties, and focus on key hazards and health benefits (EMEA/CHMP 2007); consistency, transparency, and ease of audit are other important features. These features (uncertainty assessments, consistency, etc) should apply to PFSs as well; paucity of data, at least at species level, limits the scope for quantitative assessments, and for probabilistic modelling.

At the same time, in the field of herbal medicines, the separation between assessment and management is not emphasized. Given that this separation is the result of a long experience in the food sector, RBA of PFSs should be separated from management, as emphasized by EFSA's guidance for RBA (EFSA 2010a).

EMA assessments (EMEA/HMPC 2007b, 2008a, b, c, d; EMA/HMPC 2008a, b, 2009a, b, c, 2010a) and the EFSA compendium (EFSA 2009c) focus mainly or exclusively on hazard and positive health effect assessment. In the case of EMA, risk management measures are introduced (limiting the subpopulations of users, the duration of use, etc.) that not only control the risks but also reduce uncertainty in the assessments. This approach is applicable to PFSs as well; at the same time, a hazard-only approach does not appear in line with the current knowledge of health risks and methodologies for their assessment.

Chemical characterisation, and specifications, of the botanicals and their preparations, including attending variability, may significantly contribute to uncertainty in the assessment. Separate evaluation of different preparations is useful to reduce it. As experienced by EMA, it is likely that full characterisation would not be possible in all cases, and extrapolations across different preparations would be required, even if a rigorous approach to extrapolation could be recommended. Extrapolation from compounds, to botanicals, and vice versa, could be made with a

conservative default (in absence of specific data, the assessment would rely on the substance or preparation which appears most toxic).

Establishing a reference dose (an ADI, an effective dose, etc.) to assess risks and benefits is not required for herbal medicines. The alternative is reliance on history of safe use. Safety data from human studies should play a prominent role, especially when conditions of use can be controlled.

EMA does provide an interest example in setting defaults to ensure precaution when there is uncertainty. Adequate data are required to permit use in children and other vulnerable groups. A requirement for data on mutagenicity is not negotiable. Outside these boundaries, the EMA approach does not apply precaution consistently.

In terms of benefit assessment, EMA does not apply a consistent standard, but places emphasis on tradition, and non-human evidence, particularly biochemical data. Rigorously applied, extending the approach with a grading evidence system would leave to the policy-makers to decide what strength of evidence they deem appropriate, and potentially prescribe time-limited claim authorizations to enhance research.

Contrary to EMA's reports, the FDA ephedrine assessment is an actual RBA study in which risks and benefits are compared, and corresponds to EFSA's step 2 or BRAFO's Tier 2. It relates however to a compound (and not to a plant species) for which a considerable data set is available.

A RBA for food supplements should include, where applicable, an assessment of background exposure, unless it can be shown that its magnitude makes it irrelevant for the assessment. This is not the standard practice for herbal medicines.

The evaluation of synergistic effect is particularly complex, but should be taken into account on both sides of the assessment.

An RBA methodology for botanicals does not need to propose alternatives to existing guidance on the evaluation of safety and efficacy of botanicals. As noted, the EFSA guidance (EFSA 2009a) is a useful reference for the risk component of the assessment. However, a RBA methodology should be capable of producing an outcome even with sparse data, with a proportional level of uncertainty, and within a reasonable and defined timeline (EFSA 2010a). In this case, it would be up to the risk manager to accept or reject the level of uncertainty associated to the outcome.

The methodology should also create incentives in favour of the incremental production of data and assessments. While it seems that existing EU regulation may have set a threshold for botanicals which is either too high, or too low (herbal medicines), and is therefore failing to produce an increase in research, EMA's requirement of testing for mutagenicity may result in further understanding of the risks.

The methodology needs to have discriminatory power, assuming that, at least for some botanicals, the net health benefit balance should be positive.

The ability to explore risk mitigation and benefit enhancement scenarios is also important for managers. Mandating minimum concentrations is, for example, a benefit enhancement measure. On the other hand, restrictions on use via label warnings may mitigate risks, and they should be explored in the assessments, much like EMA has done.

Finally, a complementary approach that cannot be ruled out is the extension, standardization of systematic reviews, which could provide quantitative insights even without requiring the complexities of a probabilistic RBA.

14.7 Appropriateness of a Direct Risk-Benefit Assessment for Botanicals

In the light of the different opinions on the RBA of PFSs, a scientific debate day among scientists from the PlantLIBRA consortium and experts in the field was organized (Norwich, May 23, 2013). The aim of the debate was to discuss critical issues in relation to the risk-benefit assessment of botanicals, with the overall objective to achieve a shared view among experts. The starting point for the discussion was the concept of direct risk-benefit assessment and the appropriateness of its applicability to botanical preparations in PFSs.

The debate day gathered the views of academic, public, private experts in the field of toxicology, nutrition, biochemistry, risk and risk/benefit assessment, consumer behavior, experts in the regulatory field and botanists. The experts addressed different aspects concerning the risk-benefit assessment of botanicals, including:

1. Ethical and scientific appropriateness of a direct risk-benefit approach to PFSs.
2. Regulatory framework in relation to the RBA of PFSs.
3. Consumer behavior in relation to PFSs;
4. Risk-benefit balancing: type of benefit and evidence needed for proving beneficial health effects;
5. Technical feasibility of a direct risk-benefit assessment and application of common currencies, including a comparison of qualitative versus quantitative approach.

The debate was organized in five different sessions, each one addressing one of the listed topics. One or more questions were formulated in each session and submitted to experts for comments. Experts discussed their different views and, at the end of each session, came to a shared view on the related topic.

14.7.1 Appropriateness of Direct Risk-Benefit Comparison for PFSs

Phytochemicals differ from vitamins; botanicals are not (taken) as a source of nutrients. In this context, is a direct risk-benefit approach ethically and scientifically appropriate for botanicals? If there is some evidence of risk, can we really weigh risks against benefits related to well-being (and not disease prevention)?

Experts agree that Currently RBA is common practice in many fields, not only nutrition. Thus, theoretically there would be no conceptual barriers in extending RBA application to the field of botanicals. In practice, when applying RBA methodology to botanicals some difficulties may arise, in view of the present regulatory framework and limitations in available data for performing the assessment, specifically for the benefit-side of the evaluation. While there might be adequate evidence for assessing risks, data limitations on the beneficial effects of botanicals and, additionally, interpretation of the available data, might represent major constraints. An option is to clearly and transparently explain dataset limitations for the beneficial effects, or for risks. Clearly highlighting any lack of data would represent a result of the assessment and, if this criterion is applied, botanicals might be assessed in a way similar to the RBA currently used for food.

Actually, the problem of insufficient available evidence for botanicals is generated by the fact that in the RBA framework of food evidence from randomized controlled trials is needed to prove health benefits. This requirement is too strict if applied to botanicals, because very few interventions studies have been conducted on botanicals addressing specific physiological effects in the general population. Most of the human studies have evaluated efficacy of botanical preparations in specific subgroups having a disease, and thus the result could not be extended to the general population. The simple application of the data requirement for food to botanicals would have as a result that no evidence would be available to demonstrate the efficacy of PFSs. Instead a decision should be made on the amount of information specifically required for proving health benefits of botanicals, in terms of number and type of studies (e.g. one in vitro or animal study or one human study or more than one).

The amount and quality of evidence depends on the level of confidence researchers have to achieve. Experts noted that setting the level of confidence is not responsibility of scientists but is indeed a societal question, in the sense that has to be based on societal values. Scientists have the role to assess the relative level of confidence obtained for a specific health impact given a certain amount of evidence, which in the end may be equal or not to the confidence level defined based on societal values. It is also necessary to develop a methodology to characterize the degree of certainty the evidence provides; an example is provided by EFSA that describes it in terms of probability (EFSA 2006).

The amount of evidence needed for proving benefits could also depend on the knowledge of risk, in the sense that a distinction in the approach to RBA between botanicals containing dangerous compounds and those which do not should be made. In case of botanicals containing toxic compounds, RBA is needed and the balance between risks and benefits with the related evidence should be well explained and, if there is a certain level of risk, then more evidence to prove the benefits would occur. An extreme case would be represented by a botanical for which there is evidence of risk, for example it contains a compound above TDI or a genotoxic carcinogen, and the benefit is not quantifiable; then the assessment should be stopped and the botanical or PFS not permitted. It is important to determine if, in case of a toxic compound in the botanical preparation, the compound giving the risk is the same providing the benefit. If so, then the risk and benefit assessment should

be performed. In case of compounds providing the risk and the benefit being two different molecules, a possible strategy to reduce the potential negative health impact is to avoid the toxic substances by regulating their levels. In addition, the whole preparation might be beneficial although there is one toxic compound. Some experts believe that the risk part of the assessment should not be based only on single compounds because experimental data for single compounds are obtained from studies in which the animal is treated with very high doses of the compound, which are not representative of concentrations in the preparations.

In case the product is substantially harmless because no compounds of concern are present, the amount of evidence required for the benefit would instead be lower.

14.7.2 Ethical Implications

The main ethical implication related to the RBA of botanical deals with the individual's freedom of choice between risks and benefits. There are also types of food or vitamins, that might have a risk, but they are marketed and the decision is left to the individual. In this context, consumers' perceptions of possible risks associated with PFSs consumption is an issue because they might be more focused on the putative benefit of the PFSs when buying the product while completing ignoring the risk. To make consumer able to take informed decisions, adequate information on both risks and benefits has to be provided.

14.7.3 Risk-Benefit Assessment in the Current Regulatory Framework for PFSs

Would a direct risk-benefit assessment for botanicals fit the current EU regulatory framework? Would it be more informative for decision-makers than separate risk and benefit assessments? It was remarked that at present there is no obligation in the European law to perform RBA of botanicals. It is possible but it cannot fall within the remit of the European Food Safety Authority (EFSA) because it is not in the mandatory framework of EFSA to perform risk benefit assessments. EFSA can perform it but only on self-tasking. If RBA has to be applied in the area of food law, it may within the European Commission rather than EsA. It would remain a scientific process but it would be more a risk-manager tool.

The general opinion was that direct RBA would be more informative for risk-managers than parallel assessment of risks and benefits. In a direct RBA, scientists are forced to make clear and transparent assumptions, which are then visible to people that can make judgments about the process undertaken and the uncertainties of the balance of risks and benefits. It is therefore more informative for the risk-manager, since there is an objective comparison of risks and benefits. In addition, leaving the decision up to risk manager might have negative consequences. In all the

cases in which there are no proven benefits and very minor risks, the risk manager might adopt the precautionary principle with possible drawbacks for the PFSs on the market.

14.7.4 Consumers' Perception of the Risk-Benefit Relationship in PFSs

Of relevance to any discussion on the application of RBA to botanicals is the attitude of consumers. Specifically, consumers may be aware that botanicals may carry benefits, risks, and be informed by both in their decisions.

While there is limited research on the behaviour of consumers in this area, if RBA is intended as a tool for risk-managers to make a decision which implies balancing risks and benefits and then communicating the result to the risk manager, then the consumers would not have a role in the RBA.

As for risks, it is not conceivable for foods of uncertain safety to be put on the market. As for the benefit arm, the utility of an evidence grading system directly available to consumers (up to 3–4 grades) for the evidence of beneficial health impacts is debatable. Such systems have been used in the US and in the UK (e.g. traffic light system used for food). Nevertheless, grading of evidence to the final consumer may be considered.

Some issues were indeed identified in relation to the grading of the evidence for risk and benefit. First it was considered to be an imperfect representation of the degree of uncertainty around the benefits or the risks. There might be studies belonging to the same category of evidence (e.g., in vitro, animal, clinical) but with different levels of uncertainty. In addition, grading of the evidence, even if perfect, would not be a solution because it might happen that the grade for risk and the grade for benefit are the same, thus not providing useful information The information needed concerns the levels of risks and benefits and the uncertainty around them.

There was agreement that was that more research is needed on the health behaviour of consumers regarding PFSs; there is not yet evidence to answer the question regarding consumers' role in RBA of PFSs. Actually, this approach is too simplistic because the question of leaving the RBA to consumers depends on how much information is provided. It has also to be remembered that consumers when deciding on a product do not simply make a RBA but they consider a multitude of reasons for choosing a product.

14.7.5 Evidence for Health Benefits

Most botanicals in use have a long history of use, and are likely to present negligible risks for consumers. Nevertheless, the evidence of proven health benefits is also missing in most cases, as few human trials have been conducted, and resources are unlikely to be available to investigate most botanicals in use through clinical

research in the near future. It should be noted that, in the European Union as well as in the United States of America, botanicals, when used in food (EU) or dietary (US) supplements, are required to be safe, but not effective as detemined in specific human studies. Risk benefit assessment has normally been used, in the context of foods, when risks and benefits are both believed to be significant.

In order to apply systematically RBA to botanicals, one would thus need to address a different scenario, in which most botanicals have very limited risk, and would thus be legally safe, and have no proven benefit. In this context, one may ask if freedom of choice and tradition could explicitly counted as a benefit. As noted above, EFSA (2010a, b, c, d) has recognized that other legitimate factors exist though they cannot be accounted for in a scientific risk assessment.

14.7.6 Feasibility of Direct Risk-Benefit Assessment for Botanical in Light of Limited Datasets

The datasets for many botanicals are limited. Often, the relevant plant, plant parts and preparations have not been characterized in terms of their natural composition. Data on biological effects may even be more sparse. As a consequence, an assessment of feasibility, given available data, should be performed on a case by case basis. In some cases, it may be necessary to conclude that an assessment is not possible. Several approaches exist to deal with paucity of data, such as expert elicitation.

14.7.7 The use of Common Currencies

Risks and benefits of foods are usually measured with different metrics. In the case of botanicals, risks are usually measured in relation to health guidance values, whereas benefits are measured in reference to results of human trials, or evaluated considering traditional uses. As in the case of risk benefit assessment of foods, the need for a common currency to compare risks and benefits arises. DALYs have been historically used for RBA of foods, more often than other common currencies.

However, DALYs require significant effort and large datasets to apply, and imply prevention of disease. The advantage of using DALYs as opposed to comparing qualitatively risk and benefits is that, in a qualitative assessment, the respective "weight" assigned to risks and benefits is not transparently stated. As weights for DALYs of several conditions associated with risks and benefits of botanicals, DALYs of similar conditions could be used. A major unresolved issue is how to

attribute a value to "quality of life". DALYS are used especially for disease; in the case of botanicals, the focus is often not on disease but on a certain health state; the weights of such states is more difficult to assign. For example, a botanical that contributes to maintaining normal cholesterol levels, a health benefit, may result in less cardiovascular events, and thus seems amenable to the use of DALYs; on the other hand, improved concentration is a beneficial physiological effect, but does not relate to prevention of a disease.

14.7.8 Qualitative Versus Quantitative RBA

From a theoretical standpoint, quantitative risk benefit assessment has the advantage of highlighting and quantifying uncertainty. Following this logic, it can be argued that quantitative assessments should always be performed.

In the current context, however, risk assessment for foods is normally performed qualitatively, unless large datasets and sufficient resources are available, and the question appears sufficiently uncertain. However, there is a trend towards applying more quantitative approaches to exposure assessment or dose-response data (e.g., benchmark dose as opposed to NOAEL). On the other hand, the approach to assessment of health benefits, for example in the case of health claims, is of a qualitative nature. Therefore, risk benefit assessments for botanicals, in combining a risk and a benefit assessment, are likely to be of a qualitative nature, and such qualitative assessments should be more readily acceptable to the other risk assessors, and risk managers.

14.8 Discussion and Conclusion

PFSs are among the situations for which EFSA's risk benefit guidance (EFSA 2010a) anticipates the need for a RBA (e.g., foods or compounds with both beneficial and negative health effects in the same population). The step-wise general approach proposed by EFSA (EFSA 2010a) seems suitable for PFS. However, existing assessments of botanicals, made under the legislative framework of medicines, or the case studies prepared by EFSA's working groups (EFSA 2009b) suggest that data will rarely allow to progress beyond step 1, or perhaps 2. Reference doses (ADI, etc.) may not be available or necessary, if a protective and scientifically satisfactory methodology to use tradition and biochemical evidence (benefits) and history of use and clinical safety (risks) is found and accepted.

While no limitation was perceived by experts to perform RBA of PFSs on a theoretical level, much more difficulties were highlighted in practical terms. The risk-side of the assessment was considered to be feasible, instead experts were more hesitant

when considering the benefits, due mainly to data limitations. As a consequence of limited data, the uncertainty in the final assessment might be unacceptable to the society or the decision-maker. The actual need is to decide the kind and amount of evidence needed to prove a benefit of a botanical. In addition, although current methodologies for RBA do take into consideration only the health impact in the evaluation, other factors may also have a role, especially on the benefit side. Some examples of factors that the assessor might include in the analysis are freedom of choice or tradition of use.

Looking instead at the risk-side of the assessment, the level of risk was considered by assembled experts as the trigger for performing the RBA. Various strategies may be applied in case of a botanical with significant risks, like banning the product in case the botanical contains a genotoxic compound, reducing the level of the toxic compound or look at the effect of the botanical preparation as a whole. It should be recognised that experts could not reach a consensus of the most suitable strategy to adopt; the decision would be better taken on a case by case basis. From an ethical point of view, in case of a very high risk product the suggestion would be that the decision-maker should take the decision of not permitting the product because PFSs on the market have to be safe. But when there is instead uncertainty on risks and benefits the decision may - from an ethical standpoint - be left to consumers.

From a regulatory point of view, it appeared clearly that there is no obligation to perform a RBA of botanicals; EFSA can perform RBA but under self-task. If the RBA should be performed, the process would be partially made scientifically but under the current regulatory framework it may be the Commission and not EFSA to perform such work. In this case, a direct RBA was considered to be more informative than parallel assessments or risks and benefits. The process would be much more transparent, because all the assumptions eventually made should be clearly stated by the risk-assessor. On the contrary, in the case of indepedent parallel assessments of risks and benefits, the decision would be taken by the decision-maker and he might make assumptions that would not be communicated because they are not part of the assessment. He may also tend to apply the precautionary principle in all cases of risks, with potential drawbacks for the PFSs market.

When considering communication to consumers in relation to the outcome of the RBA, experts did not come to an agreement. For some the outcome of the RBA should be communicated in the same way to the risk-manager and to consumers, while for others they should be differentiated, in the sense that only management measures, taken as a consequence of the assessment results, should be communicated to consumers. Again in the context of communication to consumers, grading the evidence and its indication on the label was proposed as an approach to provide consumers with more information. Some hesitation regarding the grading of evidence was anyway expressed, especially in relation to grading of the risk. Consumers assume that products on the market are safe and an indication of the level of risk on the label could impact their behaviour; actually more research would be needed on this aspect. Finally, in relation to consumers' role in the RBA, experts agreed on the fact that RBA should not be left to consumers.

In conclusion, experts shared the view that RBA is technically feasible. The degree of uncertainty related to the evidence for risks and benefits and to the outcome of the assessment should be communicated because it would be informative. Common currencies (e.g. DALYs and QALYs) may also be applied tentatively but still some thinking has to be done. The suggestion would be to perform initially a qualitative assessment to evaluate if there is clear dominance of risks or benefits and then to proceed quantitatively.

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Chapter 15
Consumers' Understanding of Plant Food Supplements: Benefits, Risks and Sources of Influence

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Abstract The popularity of plant food supplements continues to increase with research indicating that approximately 20% of the population consume such products. This use may reflect disaffection with traditional models of medical care and a shift in focus to maintaining overall health and wellbeing. Consumers of plant food supplements tend, in the main, to be older, female, well-educated and with a higher than average income. Evidence indicates that plant food supplements are used for a variety of reasons but their principal roles are in the prevention of certain health conditions or the treatment of specific problems. Consumers tend to perceive plant food supplements as natural substances and consequently are often unaware of the potential risks associated with consumption of these products. These risks may include adverse effects, potential interactions with prescription or over-the-counter drugs and product—related issues. Furthermore consumers are often reluctant to discuss the use of supplements with healthcare professionals and as a result are reliant on a range of other sources of information, including the internet, family, friends and the mass media. Given the complexity of the information environment consumers may struggle to distinguish between resources that are trustworthy, reliable and underpinned by evidence. Further research is needed to examine the influences on consumers' decision making and behaviours in relation to consumption of plant food supplements, with a view to informing policy makers and regulators.

Keywords Consumer • Health • CAM • Plant food supplement • Information sources • Regulation • Risks

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15.1 Introduction

In our increasingly health-literate society more and more people are turning to Complementary and Alternative Medicine (CAM) as a way of taking a more active role in looking after their health (Vos and Brennan 2010). These consumers are motivated by a desire to improve/maintain their health above and beyond disease prevention, with many users also engaging in healthy lifestyles (Schuster et al. 2004). Consumers are adopting a more holistic approach to their personal health and have moved from focusing on the absence of illness to perceiving health in terms of preventing illness and improving overall wellbeing. Associated with the increased usage of CAM in general, is a substantial increase in the use of dietary supplements (Ford 2001; Harnack et al. 2001; Kiely et al. 2001; Planta et al. 2000).

Industry sales data on supplements reveal that in the United States of America vitamins and dietary supplements continue to grow in popularity, reaching a value of 27.6 billion dollars in 2016 (Euromonitor 2016). Within that wider supplement market US herbal supplements sales reached 6.4 billion dollars in 2014 (Smith et al. 2015). The dietary supplement market in Western Europe was valued at 5.4 billion euros in 2015 and is expected to grow by 6.3% by 2020 (Statista 2015).

In considering consumers' use of supplements it is important at the outset to understand the terminology used. The term 'dietary supplement' is a broad one and encompasses a range of products, including vitamins, minerals, herbal and botanical substances, fish oils, glucosamine, creatine and essential fatty acids. The European Union (EU) Directive on Food Supplements (2002/46/EC) defines dietary supplements (which include plant food supplements, PFS) as:

"food stuffs the purpose of which is to supplement the normal diet and which are concentrated sources of nutrients or other substances with a nutritional or physiological effect, alone or in combination, marketed in dose form, namely forms such as capsules, pastilles, tablets, pills and other similar forms, sachets of powder, ampoules of liquids, drop dispensing bottles, and other similar forms of liquids and powders designed to be taken in measured small quantities". Examples of commonly used plant food supplements include echinacea, ginkgo biloba, ginseng, green tea extract, St John's Wort and valerian.

In the USA the Dietary Supplement Health Education Act (DSHEA) defines a dietary supplement as a "product other than tobacco that is intended to supplement the diet and bears or contains one or more of the following dietary ingredients: a vitamin, a mineral, an herb or other botanical, an amino acid, a dietary substance for use by man to supplement the diet by increasing the total daily intake, or a concentrate, metabolite, constituent, extract or combination of these ingredients". Furthermore a dietary supplement is intended for ingestion in pill, capsule, tablet or liquid form; is not represented for use as a conventional food or as the sole item of a meal or diet; and is labelled as a dietary supplement (DHSEA 1994).

Although available information about consumers' use of dietary supplements in general is fairly extensive (Gunther et al. 2004) information relating specifically to the use of plant food supplements is surprisingly limited with research findings

often reported only within the broader topic of CAM use. Another difficulty in identifying specific information about plant food supplements is the range of terminology used to refer to them, with PFS interchangeably being referred to as 'plant foods', plant extracts', botanicals', 'herbals' and/or 'herbs'. PFS in the USA often exist as a sub-division of supplements known as 'Non-Vitamin Non-Mineral (NVNM)' in which botanicals, proteins, amino acids and shark cartilage, are included. A further complication arises due to certain plants being used as herbal medicines and not specifically as dietary supplements, even though the plants are one and the same.

In this chapter we examine consumer usage of plant food supplements with a particular focus on understanding how they perceive the benefits and risks associated with them and how this may influence their decision to use such products.

15.2 Consumers' Use of Plant Food Supplements: Benefits and Risks

15.2.1 Who Uses Plant Food Supplements?

The limited data available on plant food supplement usage largely derives from either academic literature or business reports. The majority of academic studies addressing the use of dietary supplements come from the USA where data is available from a number of sources including the National Health and Nutrition Examination Surveys (NHANES), National Health Interview Surveys (NHIS) and Health and Diet Surveys.

Data from the 2007 NHIS Complementary and Alternative Medicine supplements provided a picture of the use of CAM more broadly among US adults and children (Barnes et al. 2008). The most common types of CAM therapy used by adults in the previous 12 months were nonvitamin, nonmineral, natural products (17.7%); for children this was 3.9%. The NVNM products most commonly used were fish oil, omega-3 or DHA (docosahexaenoic acid) (37.4%), glucosamine (19.9%), echinacea (19.8%) flaxseed oil or pills (15.9%) and ginseng (14.1%). Another study similarly used data from the National Health Interview Survey (NHIS), but across three time points- 2002, 2007 and 2012, to provide national estimates of the use of complementary health approaches among US adults (Clarke et al. 2015). Again the use of NVNM dietary supplements proved to be the most popular approach across the time period- 18.9% in 2002, 17.7% in 2007–2012.

The total usage of dietary supplements may however be higher than reported in some surveys because they capture usage only in the 30 days prior to the respondent's interview and do not allow for seasonal or occasional variation; a point highlighted by Dickinson et al. (2014) with respect to data from NHANES. They reported on five years of data (2007–2011) from a series of consumer surveys that addressed usage, the products used and the health habits of users. These online surveys were

undertaken annually by the Council for Responsible Nutrition (CRN) with 2000 respondents; a demographically representative sample of the US population. Nutritional or dietary supplements were defined as "vitamins, minerals, herbals, botanicals, sports nutrition or other speciality supplements".

This study reported that the prevalence of supplement use varied from 64 to 69% from 2007 to 2011; the prevalence of regular use ranged from 48 to 53%. A more detailed analysis of the data from the 2011 survey revealed that vitamin or mineral supplements were used by 67% of all respondents, speciality supplements by 35%, herbals/botanicals by 23% and sport supplements by 17%. The finding of herbal or botanical supplement usage in the past year by 23% of respondents, was comparable to the NHANES 2003–2006 finding that about 20% of adults reported use of a botanical in the previous month (Bailey et al. 2011). The National Health Interview Survey similarly reported that 18% of adults said that they used herbal supplements in the 2007 alternative medicine supplement (Wu et al. 2011).

Data on the use of dietary supplements generally and plant food supplements specifically elsewhere in the world are more limited. A representative population survey was conducted on the use of complementary and alternative medicine in South Australia in 2004, involving 3015 respondents and addressing the use, cost, beliefs and quality of life of users of CAM (MacLennan et al. 2006). The products used included herbal medicines, traditional medicines, vitamin, mineral and nutritional supplements as well as homeopathic medicines and aromatherapy products; CAM practices included a diverse range of therapies.

Results from the survey indicated that CAMs were used by 52.2% of the population surveyed, in the past year. Vitamins were used most (39.2%) followed by herbal medicines (20.6%) and mineral supplements (13.6%). The greatest use was in women aged 25–44 years, with higher income and education levels. CAMs were used mostly to maintain general health with reasons for use varying with age, marital status and education; although CAM users had lower quality of life scores than non-users.

The increasing use of dietary supplements in Europe was reported in a number of studies (Knudsen et al. 2002; Messerer et al. 2004; Ocké et al. 2005) but with limited information on the prevalence and types of supplements used. Information on dietary supplement use emerged from the European Prospective Investigation into Cancer (EPIC) and Nutrition calibration study in which specific questions were asked on dietary supplement use as part of single 24-h dietary recalls (Skeie et al. 2009). The EPIC study included more than half a million participants in 10 European countries- Denmark, France, Germany, Greece, Italy, the Netherlands, Norway, Spain, Sweden, and the United Kingdom (Riboli et al. 2002) Across all countries the crude mean percentage of supplement users varied from 2 to 51.8% for men and from 6.7 to 65.8% for women. There was a clear north-south gradient in use with the highest consumption in Scandinavian and the lowest in Mediterranean countries. Participants mainly reported the use of vitamins, minerals or combinations of the two with herbs/plant-based supplements representing 8–17% of the products used.

More recently Garcia-Alvarez and colleagues reported on the usage of plant food supplements across six European countries in the PlantLIBRA Consumer Survey

(Garcia-Alvarez et al. 2014). Data on the usage of plant food supplements were collected in a cross-sectional, retrospective survey of PFS consumers in Finland, Germany, Italy, Romania, Spain and the United Kingdom. Overall an estimated 18.8% of screened survey respondents used at least one plant food supplement and across the countries 491 different botanicals were identified in the products used. Characteristics of supplement users included being older, well-educated, never having smoked, and self-reporting their health status as good.

Other studies of dietary supplement use focus on specific populations, including pre- and post-menopausal women (Pakzad et al. 2007), individuals with chronic conditions (Mehta et al. 2008) and older adults (Marinac et al. 2007). The latter study involved a survey of the use of herbal products and dietary supplements among American adults aged 60 years and older. A face to face survey was conducted with 267 men and women in Kansas City metropolitan area with questions on usage, patterns of use, attitudes about and knowledge of herbal products and dietary supplements. Results revealed that 21% of respondents were currently taking at least one herbal product or dietary supplement with glucosamine, garlic, echinacea and *Gingko biloba* being the most used products. The most common reasons provided for use were to improve general wellness, to help manage arthritis, to help prevent or manage colds or to improve memory. White women with some college education were most likely to report usage but preservation of health was the most predicative factor for use.

Children are another of the specific populations studied. Data on the feeding of dietary botanical supplements and teas to infants in the United States were obtained from the Infant Feeding Practices Study II, a longitudinal survey of women studied from late pregnancy through their infant's first year, conducted by the US Food and Drug Administration and the Center for Disease Control, between 2005 and 2007 (Zhang et al. 2011). Less than one tenth of infants (9%) were given botanical supplements or teas in their first year, including infants as young as one month. Maternal herbal use, longer breastfeeding and being Hispanic were significantly associated with giving supplements. Sources of information included friends or family, health professionals and the media.

The prevalence and predictors of children's dietary supplement use in the US were also examined using data from the Complementary and Alternative medicine supplement of the NHIS 2007 in which information was provided by proxies for children less than 18 years old (Dwyer et al. 2013). These results indicated that 37% used dietary supplements; 31% using multivitamin multimineral supplements exclusively and 2% using non vitamin, nonmineral products either singly or in combination with other supplements. Users were more likely to be Asian, white or non-Hispanic; to belong to families with higher parental education, and income levels, to be in good, very good or excellent health and have private medical insurance. Herbs and herbal medicines were used by 1.26%, more than twice as much use as in the next highest category of NVNM products.

In general higher use of dietary supplements has been associated with being female, better educated, having a higher income, being white and being older (Bailey et al. 2011; Nayga and Reed 1999; Timbo et al. 2006). The study by

Dickinson and colleagues, for example, reported that supplement use increased with age and was higher in women than in men (74% vs. 64%); higher in older age groups, (78% in those aged 55 and older, 69% in those aged 35–54 and 60% in those aged 18–34) and more prevalent in people with higher household income (73% in households with incomes of $50,000 or more per year and 64% in households with lower incomes). Alkhateeb et al. (2006) examined what factors influence consumer spending on herbal products in a survey of a stratified random sample of 1300 consumers in the USA (18+). A total of 181 consumers reported using herbal products and the significant influences on spending were age, (older people spent more than younger people), over the counter (OTC) drug use, (positively associated with spending on herbals) and use of an herb professional as a source of information.

Other evidence shows that users of PFS are more likely than non-users to have better dietary patterns, exercise regularly, maintain a healthy weight and avoid tobacco products (Mackay and Dickinson 2014; Wiens et al. 2014).

15.2.2 Why Do Consumers Use Plant Food Supplements?

Numerous studies have addressed the question of why consumers use dietary supplements and what benefits they are seeking. The overall conclusion is that supplements are used primarily to improve or maintain overall health and wellbeing but motivations for use may also be condition –specific.

A study in a Southern US city explored the consumption patterns and behaviours of vitamin and herb users, employing both qualitative and quantitative methods (Peters et al. 2003). Interviews were conducted with consumers of dietary supplements and pharmacists and a survey distributed, via pharmacies, to 900 customers, who had consumed vitamins. Of the 225 survey respondents 76% reported taking vitamins on a daily basis and 27% taking herbs, flowers or roots. Consumers cited a wide range of reasons for using supplements, including curing an ailment, preventing chronic diseases, gaining “peace of mind”, supplementing a poor diet, saving money on medical care and achieving cosmetic benefits. For the majority of consumers treating a particular ailment was given as the primary reason for supplement use; several reported using vitamins and herbs to counteract the negative side effects of prescription drugs. Many participants expressed the opinion that taking supplements gave them an element of control over their health.

Similar findings have emerged from other studies conducted with both the general population and specific groups. In a review of the use of herbs as medicines Winslow and Kroll concluded that consumption of herbal products could be attributed to patients desiring greater autonomy in the management of their health, including the management and prevention of long –term conditions such as diabetes and arthritis (Winslow and Kroll 1998). In their analysis of the data from the Council for Responsible Nutrition survey of 2011 Dickinson and colleagues reported that the reasons most often cited for supplement use were overall health and wellness (58%) and to fill nutrient gaps in the diet (42%). Supplement users were significantly

more likely than non-users to say that they tried to eat a balanced diet, visit their doctor regularly, get a good night's sleep, exercise regularly and maintain a healthy weight (Dickinson and MacKay 2014).

In contrast other research has suggested that dietary supplement users may rely on supplements to compensate for the effects of poor lifestyle choices (Lonn 2012) or that supplement use may promote a licensing effect, allowing users to engage in risky or unhealthy behaviours (Chiou et al. 2011).

A survey of patients undergoing elective surgery was undertaken by Adusumilli et al. (2004), using a self-administered questionnaire that examined self-health perceptions, herbal medicine use, and communication of usage to surgical staff. Over half of respondents, (57%), had used herbal products at some point in their lives; 38% in the past two years. The most common products used were echinacea (48%), aloe vera (30%), ginseng (28%), garlic (27%), and *Ginkgo biloba* (22%). One in six patients continued to use these products during the month of surgery. Patients reported using herbal products for personal autonomy on health (26%), dissatisfaction with conventional health care (17%), ease of availability (14%), and for spiritual and religious beliefs (5%). Some reported using these products for chronic medical problems (38%); other reasons included weight problems, to improve concentration, energy, memory and general health, to resolve stress and sleeping problems, to prevent the ageing process and cancer.

Similar results emerged from a study undertaken with users and non-users of PFS in Italy, Romania and the United Kingdom within the PlantLIBRA European project (Authors unpublished results). Users viewed PFS as being 'natural' products, unlikely to cause significant harm and an alternative to conventional medicine. In addition PFS were perceived as compensating in part for demanding and/or unhealthy lifestyles and addressing dietary deficiencies.

The reasons underlying the consumption of supplements may be more complex as evidenced by a number of studies in the UK that examined the use of dietary supplements in a cohort of women (Conner et al. 2001, 2003). These studies employed the Theory of Planned Behaviour (Ajzen 1991) to investigate supplement use. Intentions emerged as a strong predictor of supplement use; health value and susceptibility to illness were also significant predictors. Users of supplements believed more strongly than non-users that taking dietary supplements would prevent them getting ill and help them to be healthy.

15.2.3 What Risks Are Associated with the Consumption of Plant Food Supplements?

The increased use of dietary supplements has led to concerns about the safety of these products and the potential health risks associated with their usage. Consumers perceive that dietary supplements can be helpful in maintaining good health, reducing health risk factors and preventing chronic diseases but problems may arise

for a variety of reasons including improper/inappropriate product use, adverse effects and product-related issues including poor quality, questionable composition, purity and strength.

Adverse effects have been reported for plant food supplements (Di Lorenzo et al. 2015; Lüde et al. 2016; Restani et al. 2016) and are described in detail in Chap. 5. Results from the US Health and Diet Survey of 2002 revealed that 73% of respondents had used a dietary supplement of which 4% had experienced an adverse event, that they believed might be related to the dietary supplement (Timbo et al. 2006). A higher proportion of supplement users with adverse events than users without adverse events were taking supplements and prescription medicines concurrently or were taking supplements instead of conventional medicines to treat or prevent a health condition.

There is evidence from numerous studies that consumers are unaware of the potential harmful interactions between medicines and dietary supplements (Eisenberg 2003;Tsen et al. 2000) and this is a cause for concern given the finding that 16% of prescription drug users take one or more dietary supplement (Kaufman et al. 2002). An analysis of data from the 2002 NHIS survey revealed that 21% of adult prescription medication users reported using a nonvitamin dietary (NVD) supplement in the prior 12 months with the most commonly used supplements including echinacea, ginseng, ginkgo, garlic and glucosamine chondroitin. Furthermore, of those using both prescription and NVDs 69% did not discuss their use of a combination of products with a conventional medical practitioner (Gardiner et al. 2006).

Studies with consumers reveal an unwillingness to report or discuss suspected adverse reactions with health care professionals or any official bodies. One such example is the study by Walji and colleagues that examined how consumers responded when they believed they had experienced Natural Health Product (NHP)-related adverse drug reactions (Walji et al. 2010). Qualitative, semi-structured interviews with 12 consumers who had self-identified NHP-related adverse drug reactions revealed that generally they were not comfortable discussing their suspected adverse reactions with their health care provider, preferring instead to do so with personnel from a health food store, friends or family and no one reported their reactions to a regulatory body.

A series of experimental studies were conducted by Lynch and Berry (2007) with convenience samples of the general population with a view to investigating their views about the efficacy and specific risks of herbal, over-the –counter (OTC), and prescribed medicines and their likelihood of taking a second, (herbal or OTC), product in addition to a prescribed medicine. Participants believed herbal remedies to be less effective but safer than OTC and prescribed medicines and they were perceived as being less likely to give rise to adverse effects, to interact with other medicines and to lead to dependency. Results indicated that participants would be more likely to take a herbal medicine than an OTC medicine in addition to a prescribed product and less likely to consult their doctor in advance.

In the study by MacLennan et al. (2006) in South Australia 49.7% of CAM users reported the use of conventional medicines on the same day and 57.2% did not

report the use of CAMs to their doctor. It emerged that about half of the respondents thought that CAMs were tested independently by a government agency- 74.8% believed they were tested for quality and safety, 21.8% for what they claimed and 17.9% for efficacy.

These findings lead to consideration of the regulations for dietary supplements, which are very different from those for prescription and over-the-counter (OTC) drugs. An extensive legal framework is in place for pharmaceutical products; in Europe the European Medicines Agency (EMA) has responsibility for the evaluation and monitoring of the safety and efficacy of pharmaceuticals and Directive 2001/83/EC requires that all medicines are registered before they are placed on the European market (EC Directive 2001; Eussen et al. 2011).

Food supplements, in contrast, are regulated under food safety legislation by the Food Supplements Directive (2002/46/EC). Herbs and botanicals may occur as ingredients in certain dietary supplements i.e. in combination with vitamins and minerals but also constitute a distinct sub-group of supplements (EC Directive 2002). A product containing herbs or botanicals may be considered a medicine, depending on the purpose of its use i.e. if presented as having properties for treating or preventing disease or when it is used to restore, correct or modify physiological functions by exerting a pharmacological, immunological or metabolic action (EC Directive 2004). This may lead to confusion on the part of the consumer as a product with the same active ingredient(s) and in the same dosage may be marketed and sold as a medicine in some European countries but as a dietary supplement in others. The regulation of plant food supplements is dealt with in detail in Chap. 2.

In 1994 the introduction in the United States of the Dietary Supplement Health and Education Act (DSHEA) created a new structure for regulating dietary supplements. Under the DSHEA it became the manufacturer's responsibility to ensure the safety of a dietary supplement before it came to market and generally it was not necessary to register or gain approval from the Food and Drugs Administration, (FDA), before introducing products for sale. This allowed botanicals to be marketed with few regulatory controls, provided that no claims were made regarding disease prevention, cure or detection; instead so-called structure-function claims could relate to enhancing or maintaining normal physiological functions of the body. The introduction of the DSHEA resulted in a dramatic increase in the use of plant food supplements.

Subsequently a number of studies have examined how information about the regulation of dietary supplements can affect consumers' beliefs about the safety and effectiveness of the supplements. In a study by Ashar et al. (2007) 335 physicians were tested on their knowledge of the FDA's role in regulating dietary supplements - only 59% of questions were answered correctly. In a study by Dodge and Kaufman (2007) with a sample of college students (n = 262) results showed that individuals were not very knowledgeable about the FDA's role in regulating dietary supplements. Making participants explicitly aware that the FDA did not approve a dietary supplement lowered the safety ratings of the product but had no effect on effectiveness ratings. The addition of a structure- function disclaimer (which specifically states that that the product is not intended to treat, cure, diagnose or

prevent a disease) had the opposite effect i.e. it lowered the effectiveness ratings but did not affect safety ratings.

Peters et al. (2003) addressed the issue of over-consumption in their study of vitamin and herb consumption. Their interviews with consumers revealed that they could be segmented into two groups i.e. those who may be over-consuming dietary supplements because of confusion as to what constitutes an appropriate daily dosage or those who make a conscious decision to take more than the recommended daily dosage on the package.

The former group of consumers expressed some concern about their consumption and indicated that this was due to a lack of knowledge about supplements and appropriate dosages. They were often confused by the range of dosages available and the instruction to take one pill per day. The latter group of consumers expressed the opinion that supplements were not harmful and perceived that high dosages help to prevent and treat diseases.

Toxicity associated with PFS may arise also from problems with the production of products, such as the misidentification or mislabeling of the botanical used in the product, use of incorrect parts of plants, contamination or adulteration with pharmaceutical agents or contamination with pesticides, herbicides, heavy metals or microbes (Van Breemen et al. 2008). To counter these problems there is a need for efficient and rigorous systems of quality control at all stages of product production.

15.3 What Influences Consumers' Use of Plant Food Supplements

In striving to understand why consumers use plant food supplements it is important to recognise and examine what factors may affect their decision to use supplements. Indications from the literature are that a broad swathe of influences may have a role in informing consumer choice and affecting consumer behaviour, including health professionals, family, friends and the mass media (Peters et al. 2003; Ritho et al. 2002). Given the broad range of factors, at different levels, that may be influencing consumers, it is useful to utilise a social ecological model as a framework for the discussion An ecological framework recognises that behaviour is affected by multiple levels of influence, often including intrapersonal (biological, psychological), interpersonal (social, cultural), organisational, community, physical environmental, and policy (Sallis et al. 2006).

Considering each of these levels we can examine the socioecological influences that may affect consumers' decisions to use plant food supplements (Fig. 15.1).

The factors at individual level include knowledge, attitudes and beliefs and these have been dealt with to a large extent in Sect. 15.3.2 as have the social determinants of PFS consumption. The next level of influence represents an individual's interactions within their social networks such as family and friends. Several studies

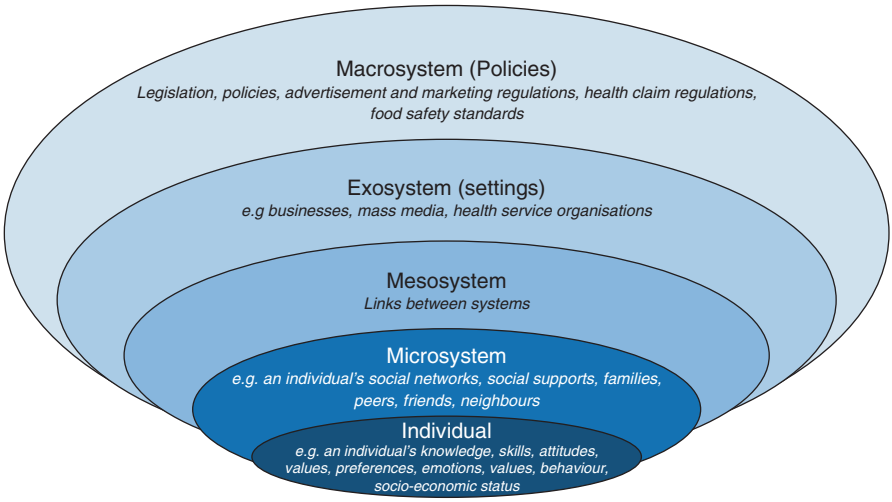

Fig. 15.1 A socio-ecological model for understanding plant food supplement consumption. Adapted from McLeroy et al. (1988)

with consumers of dietary supplements have cited family and friends as a source of recommendation, for example a study of US adults, 18 years and older revealed that family, friends and written materials were the leading sources of information (Harnack et al. 2001). Ritho and colleagues reported that friends were by far the most common source of information about herbs, used by 67.4% of participants in their survey of what influences consumer use of herbal therapies (Ritho et al. 2002). Family and friends were also mentioned in the study by Peters and colleagues and regarding family some consumers mentioned taking supplements on the advice of a family member and rarely questioned that advice (Peters et al. 2003).

The exosystem represents the wider community environment that influences access to and the consumption of plant food supplements. This is the society that the individual inhabits and we can identify some potentially key influences that consumers may interact with and seek advice from i.e. manufacturers and retailers, the mass media and healthcare professionals.

15.3.1 Manufacturers and Retailers

The retail environment in which plant food supplements are purchased represents a significant influence on consumers. In the study by Ritho and colleagues grocery stores and health food stores were the most prevalent places where herbs were purchased (44.9% and 44.2% respectively), followed by pharmacies (33%). Consumers refer in some instances to store displays as a source of information (Marinac et al. 2007) and in a study of consumers in an urban health food store 41% relied on retail

staff for dietary supplement information (Archer and Boyle 2008). The availability of dietary supplements in supermarkets and other retail outlets has become more widespread, increasing access to these products for consumers but providing little in the way of advice on the appropriate use of these substances.

In a dynamic, competitive environment marketing of supplements needs to use an information strategy that is very clear and considers the segmentation of the market and the target groups, from medically skilled professionals to the mass consumer market (Mark-Herbert 2003). The development and marketing of dietary supplements is directed by the needs of the new conscious consumer, careful to follow healthy lifestyles, who wants correct information to facilitate choosing products in a competent way (Ethan et al. 2015).

Given the constraints of the regulatory environment manufacturers and retailers need to rely on how they promote their products and what information is presented on product packaging. Packaging is a vital tool in marketing and fulfils two functions- to contain the product and to serve as the interface with the consumer (Sara 1990). Package design provides product category information and can 'position' a product within a category (Ampuero and Vila 2006) as well as communicating brand identity and values (Schoormans et al. 2010). There is ample evidence that consumers' product attitudes and purchase decisions are influenced by their preferences relating to package design (Creusen and Schoormans 2005) and that package graphics have the potential to influence consumers' product related behaviours (Westerman et al. 2013). However there has been little research to date on the effect of the different elements of packaging on consumers' perceptions of plant food supplements.

Packaging was identified in the Plant LIBRA consumer survey as one of the principal sources of information for consumers. A number of different studies within the PlantLIBRA project examined consumer responses to the packaging used for plant food supplements. In the previously mentioned focus groups, with users and non-users of PFS, in Italy, Romania and the UK it emerged that users had a strong preference for packaging that was simple, clear and informative (authors' unpublished results). A further study examined the use of packaging cues to determine the perceived risks and benefits of a particular plant food supplement, in this instance *Ginkgo biloba*. A sample of users and non-users of PFS, again in Italy, Romania and the UK, were presented with a series of product variants whereby certain product attributes, (product name, image used, claim type and warrant type) were varied and participants were asked to rate the anticipated benefit and risk for each product. The results indicated that 82% of participants' judgements of benefits were influenced by packaging and 69% of their judgements of risks were also influenced by packaging. Furthermore participants attended to different elements of the packaging to make judgements of benefits and risks (Authors, unpublished results).

In addition to packaging marketing of supplements may rely on the use of scientific jargon to persuade consumers to purchase supplements and may claim to promote health or prevent disease in ways that confuse the consumer (Bolton et al. 2008). Health claims have the potential to provide consumers with important information regarding supplements; health claims are those that state, suggest or imply a

relationship between a food or food category and health, with a requirement that the claim is scientifically proven (EC Regulation 2006). In the EU medical claims, i.e. claims for the prevention, treatment or cure of human diseases are reserved for medical products whereas food supplements are subject to the Health Claims Regulation (EC) 1924/2006. It is within this regulatory environment that PFS manufacturers and retailers have to communicate the benefits of their products.

Several studies have demonstrated that consumers do not always understand nutrition and health claims as they are intended (Bech-Larsen and Grunert 2003; Verhagen et al. 2010). In the United States structure/function claims were authorized under the DSHEA (1994) to describe the effect of a dietary supplement on the structure or function of the body. Such claims do not require FDA approval before being used on labels and must be accompanied by the following disclaimer: "This product is not intended to diagnose, treat, cure or prevent any disease". In a US study the authors examined consumer beliefs derived from structure function claims and disease claims including how such claims were interpreted when accompanied by disclaimers (Russo France and Fitzgerald Bone 2005). In this study consumers made no distinction between structure -function claims and disease claims on a supplement label although consumers existing beliefs systematically biased product- specific judgements regarding efficacy and scientific certainty. General beliefs about the supplement industry affected product specific efficacy judgements and consumers for whom disease prevention was important were more likely to place faith in both types of claims. Relatively little is known about the factors that may enhance the persuasiveness of the information used to promote dietary supplements. A study by Haard and colleagues (Haard et al. 2004) examined the effect of the use of scientific jargon and attributed versus unattributed citations on message persuasiveness. Results indicated that the use of scientific jargon to create the impression of a sound scientific basis for claims increased message persuasiveness status. In addition no effect was found for attributed versus unattributed citations.

15.3.2 The Mass Media

The mass media represents an important means of communication with consumers and for the majority of consumers will be the prime source of information on plant food supplements, (Weeks and Strudsholm 2008). With the rise in the use of dietary supplements over recent decades coverage in media sources has increased and contributed to the growing awareness of plant food supplements, in part by providing reports on the effects of botanicals (Chang 2000). A 2007 survey of Americans, aged 60 and over, revealed that 73% of participants reported television as the most common source of information, followed distantly by magazines and radio, newspapers, friends and store displays (Marinac et al. 2007). These findings reflected those from a study with women in an urban health clinic which revealed the most common sources of dietary supplement information to be radio and television, followed by family, newspapers and magazines and friends. In the study

by Peters et al. (2003) the media influences reported by participants included advertisements, books, magazine articles, newspapers, television stories and the internet. Results from their survey indicated that the more products a consumer uses the more influence sources other than the doctor have on consumer‘s supplement use and that the media is particularly important.

Consumers of multiple supplements in this particular study emphasised the importance of self-education as medical staff were seen often to be lacking knowledge about these products. Access to various media sources was seen as a means to facilitate this self-education with respondents confident that they only used valid, reliable sources. The authors questioned the ability of the public to distinguish between truly independent research on dietary supplements from, for example, advertising which may appear scientific in its content and presentation.

Dietary supplements, including plant food supplements, are widely marketed and readily available for purchase online via internet retailers. In addition, a plethora of non-retail websites provides information about these dietary supplements. The internet has contributed to the increased interest in and use of herbal supplements. Various studies have indicated that 36–55% of all internet users have accessed medical information, with one US study revealing that 48% of those seeking information on CAM had used the internet (Baker et al. 2003).

The extremely high number of adults using the internet to look for health-related information (approximately 83% of all American adults, Zickuhr 2010) has led to increased concerns with respect to the potential for inadequate labelling of dietary supplements, including exaggerated reports of efficacy and minimal safety warnings. In fact, for many of these products there is little evidence of their use and information available online is often lacking, insufficient, or incorrect.

In 2014 researchers at Idaho State University published the results of a study that focused on the state of online information available for top selling herbal products according to the most recent US nationwide survey (include cranberry, Echinacea, flaxseed oil, ginseng, ginkgo biloba, garlic, green tea, grape seed extract, saw palmetto, and soy) (Owens et al. 2014). Researchers conducted two different searches (general and shopping) to analyse Web site content that would likely be encountered by the typical consumer going online to learn about or purchase an herbal product. The general search was used to evaluate information on nonretail sites and the shopping search to evaluate information on retail sites.

For the retail search, the researchers noted whether the site included the correct plant species name, statements regarding product standardization, the Food and Drug Administration (FDA) disclaimer, a list of potential adverse effects, medical contraindications, safety of use in certain circumstances (pregnancy, lactation, pediatrics), known interactions, a recommendation to consult with a healthcare professional before use, and also the presence of references or citations of medical or scientific literature. For the nonretail search, the safety information assessed was similar, but they also noted whether a mechanism of therapeutic action was included, as well as recommended dosage, and whether any indication of efficacy was mentioned.

The study revealed that, of the 1179 websites examined, less than 8% provided information regarding adverse effects, drug interactions, or other safety information. Only 10.5% of the sites recommended discussing the use of the herb with a healthcare professional. Sites that sold products were less likely to recommend consulting with a healthcare professional compared to sites that only provided information. Less than 3% of websites cited any scientific literature to back their claims. Fourteen percent of the retail websites included information that violated FDA criteria by making claims about diagnosing, treating, preventing, or curing a disease. These claims were more common for sites selling soy, black cohosh, and green tea extract with 20 to 38% of claims violating FDA criteria.

The authors thus concluded that retail sites continue to make illegal disease claims, but do so less commonly than 10 years ago, since just more than 13% of retail sites make such claims compared with 55% of sites in a previous analysis by Morris and Avorn (2003).

Nevertheless, not all herbal information on the internet is misleading; nonretail sites are frequently found when conducting an online general search for information on herbal products. A review of the most popular hits on a general search will include information from the NCCAM (National Centre for Complementary and Alternative Medicine), the Mayo Clinic, and other medical or educational institution supported sources that more often provide balanced information with respect to efficacy and safety. There is often a recommendation on these websites sites for individuals to consult their healthcare providers before using a supplement.

However, it should be noted that there also are many sites that will be found in a general search that are administered by amateur herbalists and enthusiastic proponents of these products. Such sites may appear to be or are portrayed as "medical information" but in fact are maintained by individuals whose credentials are questionable or absent. Taken together, however, the information contained on many nonretail websites is more often referenced and at a minimum contains the warnings and safety precautions for special populations (pregnant women, children) which afford some consumer protection, when compared with information on retail sites. Many of these sites contain authoritative information that can help consumers make more informed decisions about the use of these products.

Among people using the internet for health-related information there are not only lay consumers but also physicians and other healthcare professionals; besides traditional sources of information, such as scientific publications and medical sales representatives, they are increasingly using newsletters, company and nonretail websites and social media to remain constantly updated on this issue.

Another very important influence at this level are the interactions that consumers have with healthcare providers, be it a medical doctor or one of a range of other health professionals. Results from in depth interviews with dietary supplement users in the US indicated that a primary source of influence is their physician and other health professionals may also have a role, including dentists, nutritionists, pharmacists and personal trainers and several respondents valued their opinions more highly than those of their physician (Peters et al. 2003).

15.3.3 Health Professionals

Given their potential role in advising consumers several studies have been conducted among healthcare professionals addressing their attitudes, beliefs and knowledge regarding dietary supplements (Dickinson et al. 2009; Lederman et al. 2009; Ten Hove 2011). The majority of studies have focused on dieticians who are regarded as experts on healthy diet and lifestyle and thus may have a significant influence on consumers' decisions regarding plant food supplements.

A study in Oregon examined the perceived knowledge, attitudes and practices of licensed dietitians, (n = 202), regarding the effectiveness and safety of functional foods, nutrient supplements and herbs as complementary medicine (Lee et al. 2000). More than 80% were confident of the effectiveness of functional foods and nutrient supplements for the prevention of illness and treatment of chronic illness and 89% were confident of their safety for these uses. However fewer than 75% considered herbs to be safe and only 50% had confidence in the effectiveness of herbs. Only 10% of respondents considered themselves knowledgeable about herbs for prevention and treatment of illness.

Another study addressed the knowledge, attitudes, opinions, personal use and recommendations to clients about herbal supplements by Massachusetts registered dietitians (Cashman et al. 2003). Results from the study indicated that 73% of respondents had little or no knowledge of herbal supplements although 22% had recommended herbs to clients in the past year. In the European context a study by Ten Hoeve (2011) examined the beliefs and recommending practices of Dutch dieticians. Herbal –based supplements were recommended by 13%; green tea mainly for all purposes (prevention, treatment or enhancement), followed by St John's wort. The majority of respondents (94%) perceived dietary supplements to be at least moderately safe, 75% considered supplements moderately effective in prevention, 91% for treatment and 59% for performance enhancement.

In the broader context Kemper et al. (2006) conducted a cross-sectional survey of physicians, pharmacists, nurses, dietitians and trainees about Herbs and Dietary Supplements (HDS) in 2004–2005, questioning knowledge, confidence and communication practices. Results gave mean scores of 66% correct for knowledge; 53/95 on the confidence scale and 2.2 out of 10 on communication practices. On average scores were lowest for those who used fewer HDS, trainees and nurses compared to physicians, pharmacists and dietitians. A previous survey by the same authors (Kemper et al. 2003) had also identified the need for significant improvement in levels of knowledge and confidence about herbs and dietary supplements as well as communication practices.

The final level in the model is that of policies, which are adopted and implemented at the national or international level. The legislative and regulatory policies are detailed elsewhere in this book.

15.4 Conclusions and Implications for Future Policy and Research

The use of plant food supplements continues to be popular among consumers with studies indicating that approximately 20% of the population consume such products. This use is thought, in part, to reflect an increasing desire by individuals for greater autonomy over their health and a shift in focus from the absence of ill-health to the maintenance of both physical and mental wellbeing. The general profile of a consumer of PFS is older, female, well-educated and with a higher than average income.

Consumers use plant food supplements for a variety of reasons but in the main they appear to be seeking to prevent certain health conditions occurring or alternatively to treat specific problems. Traditionally pharmaceutical drugs were used to cure/treat disease but over time the use of medicines to reduce the risk of certain diseases has become normal practice. Concurrently there has been a rise in the use of dietary supplements and functional foods which occupy the middle ground on the continuum from foods to medicines. In contrast to functional foods dietary supplements are sold as pills, powders, capsules or liquids and are intended to supplement the diet but the evidence suggest that consumers do not view supplements, particularly plant food supplements in this way. Consumers tend to perceive PFS as 'natural' substances and hence safe, and as potentially effective for a number of health problems, which is a cause for concern for stakeholders. However the reasons for use may be more complex and research needs to shift from simple, descriptive studies to exploring the use of socio-behavioural models that provide a better understanding of the reasons for PFS consumption.

As a consequence of the perception that PFS are safe consumers are often unaware of the risks associated with consumption of these products, both in terms of adverse effects and the potential for interactions between PFS and prescription or over-the-counter drugs. There is ample evidence that consumers do not discuss the use of PFS with their primary healthcare provider and consequently there is no means of monitoring usage of these products. There is further confusion in relation to how much of a product should be consumed and for how long, leading to the risk of over-consumption. Furthermore it becomes evident that the status of PFS as food is unknown to many consumers, who assume, wrongly, that these products are tested and regulated in the same manner as pharmaceutical drugs.

Consumers often regard their medical practitioners as lacking in knowledge regarding supplements and tend to rely on other sources of information. In the main consumers tend to rely on the internet and on family and friends for information on PFS; those who use these sources also tend to trust them. The consumer information environment is multilayered and it may be difficult for consumers to distinguish between those sources which are reliable and underpinned by sound scientific evidence. This is a cause for concern for both stakeholders and some consumers who deplore the lack of qualified information and professionals' involvement and who highlight the need for accessible trustworthy information. The provision by regulatory bodies, for example, of reliable internet resources on PFS may be one solution.

It becomes apparent that consumers considering PFS are subject to a range of influences and policy makers may need to adopt a more holistic approach to policy development in this area, taking account of pre-existing beliefs and biases that affect consumers' decision making and behaviours. Future research will need to suggest a solution for the misalignment between policies and consumer perceptions (education, stricter regulation etc.)

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