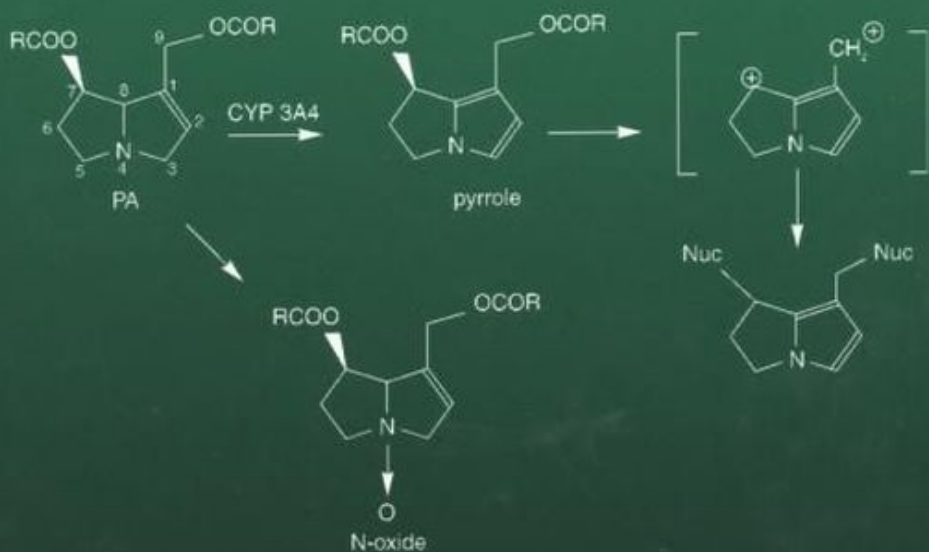
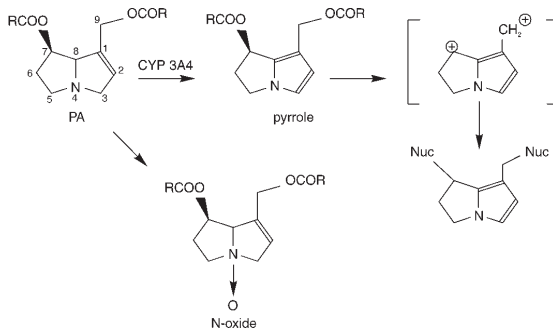


Food Toxicology

Edited by
William Helferich
and
Carl K. Winter



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Preface

At the turn of the 20th century, the average life expectancy was about 40 years of age. The main causes of death were infections due to microbial pathogens. As we enter the 21st century, the leading causes of death are now heart disease and cancer. Diet plays a significant role in both of these diseases, as evidenced by estimates that environment including diet may be related to 70% of all human cancers. By the middle of the 21st century, approximately one third of the U.S. population will be comprised of persons 65 years or older. While microbial safety will continue as a major health concern, the contribution that chronic consumption of bioactive chemicals from our diet plays in the development and progression of several diseases is becoming extremely important. New data frequently indicate that antioxidants may contribute to reductions in cancer risks and that chronic consumption of low levels of chemical carcinogens in our diet may contribute to an increased risk for developing specific types of cancers.

This book provides a comprehensive look at contemporary food toxicology issues. Its nine chapters all concisely address critical subjects and are authored by leading U.S. academic experts. The highlights of each chapter are summarized below:

- Food allergies and intolerance is a topic of increasing concern to consumers and food manufacturers alike. Allergic individuals must alter their lifestyles to severely limit their consumption of allergenic foods while food manufacturers must address potential cross-contamination in the manufacturing environment and provide appropriate labeling of potential allergenic components in food.
- Food contains a variety of chemicals that may exist as either dietary estrogens or dietary antiestrogens. The occurrence of these chemicals in our diet is discussed as well as their potential beneficial and detrimental health effects.
- Bioactive chemicals in our foods also include nonnutritive antioxidants. The mechanisms of how such antioxidants interact with dietary toxicants are discussed as are their ramifications in assuring safety of complex mixtures in our diet.
- The food industry is the largest user of biotechnology today. While various biotechnology methods have been used in food production for several hundred years, the use of modern genetic engineering techniques is receiving considerable attention from the public, industry, and regulatory sectors. Regulatory aspects of genetically

modified organisms are provided and comparisons between traditional breeding and genetic engineering to select desirable traits are made. The methods used in the genetic modification of foods also are discussed.

- Microbial toxins that are commonly found in contaminated foods may pose acute and chronic health risks. The economic significance and widespread distribution of susceptible seafoods and the etiology of toxin production and subsequent accumulation is discussed.
- Several naturally occurring plant compounds have been shown to be toxic and/or carcinogenic in animals and in humans. A comprehensive list of bioactive compounds (detrimental and beneficial) produced by plants is provided. The toxic effects of such chemicals are discussed as well their potential chemopreventative mechanisms.
- Pesticide residues in food have generated considerable public, scientific, and regulatory interest in the past 2 decades. Pesticide regulations, use patterns, and monitoring programs are discussed and the risks of dietary exposure to pesticides are evaluated.
- Food additives play a major role in many food processing practices. Their history of use, classification, and regulatory systems are discussed. Specific case studies of the toxicological assessment of several food additives are provided.
- Central to the understanding of risks posed by various types of chemicals in food that are subject to toxicological scrutiny is our ability to qualitatively and quantitatively analyze food for such chemicals. The methods commonly used in the trace analysis of chemical toxins in food are provided as well as discussions of pitfalls and limitations related to the sample collection, preparation, resolution, and detection practices.

It is clear that food toxicology is a diverse topic and we believe that we have assembled a comprehensive book discussing the most critical and timely chemical food safety issues. Many of the topic areas reside on both the cutting edge of technology and in the headlines of newspapers.

We wish to express our gratitude to the chapter authors for their cooperation and contributions and to Dr. Elisabeth Garcia and Judy Howard for their outstanding editorial and clerical contributions. We also are grateful to Lourdes Franco of CRC Press for her assistance and patience.

**William G. Helferich
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Editors

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His research activities focus upon examining the influence of diet and dietary compounds on the growth of estrogen-dependent breast cancer. His work is widely published in the leading toxicology and nutrition journals.

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His research and outreach work focus on chemical contamination of foods. His most recent work has concerned scientific and public policy aspects of dietary pesticide risk assessment, the influence of pesticides upon production of naturally occurring toxins, and studies of the biosynthetic pathways for the production of fumonisin mycotoxins from *Fusarium moniliforme*. He has authored more than 70 publications in the scientific and lay literature, gives more than 60 news media interviews annually, and has frequently been invited to provide testimony for the U.S. House of Representatives on pesticide/food safety matters.

Most recently, Dr. Winter has expanded his outreach efforts to include musical approaches for food safety education. Thousands of each of his two food safety musical CDs — “Stayin’ Alive: A Hearty Helping of Food Follies and Science Serenades” and “Sanitized for Your Consumption: A Menu for Musical Morsels” — have been distributed throughout the world and he frequently tours the country giving live performances.

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Food Allergies and Sensitivities

Steve L. Taylor, Susan L. Hefle, and Barbara J. Gauger

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*“Ut quod ali cibus est aliis fuit acre venenum”
“What is food to one is bitter poison to another”*

Lucretius, ca. 96 B.C.–55 B.C.
De Rerum Natura, Book IV, line 637

Introduction

Eating is necessary to sustain life. For most people, given the variety and abundance of food available to them, eating is an enjoyable experience. For individuals with food allergies and sensitivities, however, consuming certain foods can be a debilitating and possibly even life-threatening experience. For such people, the joy of eating is diminished by the ever-present concern that they might consume a food or food component that will cause an adverse reaction. The standard treatment for food allergies and sensitivities is the removal of the offending food from the diet. For such consumers, food selection often becomes a tedious task requiring meticulous reading of ingredient lists on labels, dependence on food manufacturers to maintain accurate labels, and a continual search for more knowledge about food composition. Food preparation for them requires, in many cases, careful attention to detail, cooking “from scratch,” and seeking alternative recipes for many dishes. These consumers live in constant fear that trace amounts of the offending food, sufficient to elicit an adverse reaction, might still exist in the foods that they consume.

Food allergies and sensitivities can be collectively referred to as “*individualistic* adverse reactions” to foods. These food-related illnesses are individualistic because they affect only a few people in the population. Often, these diseases are grouped together under the general designation of “food allergies,” but it must be recognized that this term covers a host of different diseases. In fact, true food allergies represent only some of the individualistic adverse reactions to foods. [Table 1.1](#) provides a classification scheme for the various illnesses that are known to occur as individualistic adverse reactions to foods. Knowing the difference between *immunological* food allergies and *nonimmunological* food intolerances is critical. Intolerances are often controlled by limiting the amount of food eaten; with allergies, *total avoidance* is essential.

Food allergy is an abnormal immunological response to a food or food component (almost always a protein).¹ Examples are allergic reactions to common foods such as peanuts and milk. Within this category are immediate hypersensitivity reactions (IgE-mediated allergies) and delayed hypersensitivity reactions (cell-mediated allergies).

Immediate hypersensitivities are IgE-mediated and occur within a few minutes to several hours after consumption of the offending food. Exercise-induced food allergies are a subset of food allergies that involve immediate reactions that occur only when the specific food is ingested just before or after

TABLE 1.1
A Classification Scheme for Food Allergies and Sensitivities

Food Sensitivity
<i>Primary Food Sensitivity</i>
Immunological (food allergies)
IgE-mediated
Typical food allergies
Immediate allergic reactions
Delayed allergic reactions
Exercise induced
Non-IgE-mediated
Celiac disease (not proven)
Nonimmunological
Allergy-like intoxications
Anaphylactoid reactions
Metabolic reactions
Food idiosyncrasies
<i>Secondary Food Sensitivity</i>
Secondary to another event like illness or drug therapy

exercise,² although many cases of exercise-induced allergies are not related to foods.³ Delayed hypersensitivities are cell-mediated involving the response of sensitized cells, usually lymphocytes, to the specific foreign substance that triggers the reaction. The ultimate result is tissue inflammation often restricted to certain sites in the body. Symptoms appear from 6 to 24 h after consumption of the offending food.

Nonimmunological food reactions or food intolerances, in contrast to true food allergies, and as the name implies, do not involve abnormal responses of the immune system.¹ Anaphylactoid reactions are a non-IgE-mediated release of the chemical mediators (mostly histamine) of allergic reactions in the body. Foods such as strawberries, shellfish, and chocolate can allegedly induce such reactions, but proof for this type of food intolerance does not exist. Metabolic food disorders are genetically determined metabolic deficiencies that result in adverse reactions to a food component. An example would be lactose intolerance, which is due to a deficiency of the intestinal enzyme, lactase, that is essential for the metabolism of the lactose in milk.⁴ Food idiosyncrasies are adverse reactions to foods or a food component that occurs through unknown mechanisms. Psychosomatic illnesses are included in this category and, frequently, the cause-and-effect relationship between the food or food component and the particular adverse reaction remains to be well proven. Examples include sulfite-induced asthma⁵, tartrazine-induced asthma⁶, food-associated migraine headache, and a variety of other illnesses. An allergy-like food intoxication is not an individualistic adverse reaction as everyone in the population is probably susceptible. However, such illnesses are often misdiagnosed as a food allergy. This reaction occurs

as a result of the ingestion of chemical mediators of allergic disease. The only example is histamine poisoning (also known as scombroid fish poisoning) which is commonly associated with the ingestion of spoiled tuna, mackerel, mahi-mahi, and other fish and also occasionally with cheese.⁷

Avoidance diets are the only reliable means of prevention for food allergies.⁸ Pharmacologic and other therapeutic methods of prevention of food allergies do not exist, although certain drugs, such as epinephrine (adrenaline) and antihistamine, can be used to treat the symptoms that develop during an allergic reaction. Thus, food-allergic individuals are forced to become avid label readers in an attempt to avoid offending foods and certain ingredients derived from these foods. Their efforts are fraught with difficulty because individuals with true, immunologically mediated food allergies, can react to mere traces of the offending food in their diet.^{8,9}

Immunological Food Hypersensitivities (True Food Allergies)

The main function of the gastrointestinal tract is to process ingested food into a form that can be absorbed and used by the body for energy and cell growth. The “gut associated lymphoid tissue” must remain unresponsive to a wide variety of nutrient materials, and yet stand ready to mount a rapid and potent response against pathogenic viruses, bacteria, parasites, and other foreign substances. The human immune system is very effective in reacting with unwanted and potentially harmful foreign substances in our bodies, often by mounting humoral (antibody-based) or cellular immune responses against specific proteins present in the foreign material. But, at the same time, the immune system must develop tolerance to the hundreds of thousands of different proteins that are ingested with the typical human diet, lest we become sensitized to many foods. The small portion of the population (approximately 5% of infants and 2 to 2.5% of adults) with true food allergies has a genetically based predisposition to develop abnormal immunological responses to substances, usually naturally occurring proteins, in their environment. These responses may take the form of environmental allergies to pollens, mold spores, animal danders, bee venom, etc. or they may take the form of allergic responses to specific foods. As noted earlier, a food allergy is defined as an abnormal immunological reaction in which the body’s immune system overreacts to ordinarily harmless substances in foods.

Mechanisms

Allergic reactions (or hypersensitivity reactions) are based on four different immunological mechanisms (Type I, II, III, IV) as first classified by Coombs

and Gell.¹⁰ These same mechanisms apply for food allergies and for allergic reactions to pollens, mold spores, animal danders, insect venoms, and drugs. The Type I mechanism also is called “immediate hypersensitivity,” and involves the formation of IgE. IgE-mediated reactions are the most important type of food allergy. Type II reactions are not associated with food hypersensitivities. Type III, or immune complex responses, may be involved in food allergies but evidence is rather limited.¹¹ Type IV reactions, also known as cell-mediated reactions or delayed hypersensitivities, probably play an important, although as yet undefined, role in food hypersensitivity.^{12,13} Celiac disease, which will be discussed later, may be a form of cell-mediated delayed hypersensitivity.¹⁴

IgE-Mediated Allergic Reaction (Immediate Hypersensitivity)

Hippocrates was the first to document the occurrence of food allergies. The beginnings of allergy as a clinical science may be traced to the experiments of Prausnitz and Kustner¹⁵ who subcutaneously injected a nonallergic individual with a fish extract and noted no adverse reaction. However, when the normal individual was first inoculated under the skin with serum from a fish-allergic person and then injected with the fish extract, there was an inflammatory skin reaction at the sensitized site. This experiment provided the first evidence that the blood contained some substance that sensitized the allergic individual to the fish. In 1966, Ishizaka et al.^{16,17} demonstrated that this reaginic activity was associated with a unique immunoglobulin and tentatively called this protein E. The protein was officially named immunoglobulin E or IgE by the World Health Organization (WHO) in 1968. Identification of IgE as a reaginic antibody provided immunochemical approaches to analyze the mechanisms involved in hypersensitivity reactions.¹⁸ Immunoglobulin E (IgE) is one of five classes of antibody that are present in the human immune system.

In IgE-mediated food allergies, the allergen-specific antibodies are produced in response to stimulus of the antibody-forming B cells by a food allergen, usually a naturally occurring protein present in the food. The IgE antibodies bind to the surfaces of mast cells in the tissues or basophils in the blood. When the same food allergen is encountered on a subsequent occasion, the allergen associates with the mast cell- or basophil-bound IgE, and cross-links two of the IgE molecules. This precipitates a cascade of biochemical events which causes cell membrane disruption and the release of a variety of mediators contained within granules existing in the mast cells and basophils. The granules in mast cells and basophils contain most of the important mediators of the allergic reaction.¹ While more than 60 substances have been identified as chemical mediators emanating from mast cell and basophils, histamine is responsible for most of the immediate effects of allergic reactions. The histamine-related effects include inflammation, pruritis, and contraction of the smooth muscles in the blood vessels, gastrointestinal tract, and respiratory tract.¹ Other important mediators include a variety of

prostaglandins and leukotrienes; these particular mediators are associated with some of the slower-developing responses observed in some cases of food allergy (e.g., late-phase asthmatic reactions).

A nonallergic individual will not respond to an exposure of a food protein with the production of an allergen-specific IgE. Even among individuals predisposed to allergies, exposure to food proteins does not usually result in formation of allergen-specific IgE. In normal individuals, exposure to a food protein results in oral tolerance through the formation of protein-specific IgG, IgM, or IgA antibodies.¹⁹ The true prevalence of food allergies is unknown, although it has been estimated that approximately 5% of infants and perhaps 1% of adults have food allergies.²⁰ Heredity and other physiological factors are significant in predisposing individuals to the development of allergies, including food allergies.²¹ Approximately 65% of patients with clinically documented allergy have first-degree relatives with allergic disease.²¹ Conditions that increase the permeability of the intestine to macromolecules such as viral gastroenteritis, premature birth, and cystic fibrosis, may increase the risk of development of food allergy. Although food allergies also may involve other types of immunological mechanisms, the IgE-mediated mechanism is, by far, the most well documented and understood.

Allergic reactions involve numerous symptoms ranging from mild to life-threatening (Table 1.2). The symptoms experienced by individuals with food allergies are quite varied, and no one likely suffers from all of the symptoms mentioned in Table 1.2. The nature and severity of the symptoms also may vary from one occasion to another in the same individual as a result of the amount of the offending food ingested and the length of time since the last previous exposure.

Among the many symptoms involved in food allergies, systemic anaphylaxis is the most severe manifestation. Systemic anaphylaxis, sometimes referred to as anaphylactic shock, involves many organ systems and numerous symptoms. Symptoms may include tongue swelling and itching, palatal itching, throat itching and tightness, nausea, abdominal pain, vomiting, diarrhea, dyspnea, wheezing, cyanosis, chest pain, urticaria, angioedema, hypotension, and shock.²² Anaphylactic shock is the most common cause of death in the occasional fatalities associated with true food allergies.^{23,24}

TABLE 1.2
Symptoms of IgE-Mediated Food Allergies

Gastrointestinal
Nausea, vomiting, diarrhea, abdominal cramps
Respiratory
Asthma, wheezing, rhinitis, bronchospasm
Cutaneous
Urticaria (hives), eczema or atopic dermatitis, pruritis, rash, angioedema
Other
Anaphylactic shock (systemic shock), headache, hypotension, palatal itching, swelling including tongue and larynx

Exercise-Induced Allergic Reactions

Little is known about the natural history of exercise-induced anaphylaxis (EIA). A syndrome characterized by exertion-related development of allergy-like symptoms was first described in 1936.²⁵ Increasingly, there has been recognition that in certain individuals experiencing EIA, the exercise must be preceded or followed by the ingestion of specific foods in order to elicit an allergic reaction. Shellfish,²⁶ peach,²⁷ wheat,²⁸ and celery²⁹ are among the foods that have been incriminated in food-dependent EIA. While the mechanism for food-dependent, exercise-induced anaphylaxis is unknown, enhanced mast cell responsiveness to physical stimuli may be involved.³ The symptoms in this type of food allergy are individualistic and similar to those involved in other food allergies. With awareness of the existence of this syndrome, and the recent national emphasis on physical activity, reports of this condition may continue to increase.

Cell-Mediated Reactions (Delayed Hypersensitivity)

As noted earlier, cell-mediated allergic reactions also are known as delayed hypersensitivity or Type IV reactions because the symptoms of these reactions usually begin to appear 6 to 24 h after ingestion of the offending food. These reactions develop slowly, reaching a peak at approximately 48 h and subsiding after 72 to 96 h. Cell-mediated food allergies involve interaction between specific antigens or allergens from the food and sensitized T lymphocytes. The stimulation of lymphocytes, which release cytokines and lymphokines, produces a localized inflammatory response.³⁰ In contrast to the Type I mechanism, these reactions occur without the involvement of allergen-specific antibodies.

T lymphocytes are a major component of the gut-associated lymphoid tissue.³¹ Evidence for the involvement of cell-mediated immune reactions in food allergies is sparse with the possible exception of celiac disease (discussed below). However, some reasonably compelling information exists on the possible role of cell-mediated reactions in some cases of cows' milk allergy. Both immediate and delayed reactions have been observed in cows' milk-allergic infants.³² Increased numbers of intestinal intraepithelial lymphocytes have been observed in cows' milk allergy.³³ These reactions may be involved in the development of enteropathy in some cows' milk allergic individuals, but further evidence is needed. No estimates of the prevalence of cell-mediated food allergies have been made.

Nature and Chemistry of Food Allergens

Allergens are almost always naturally occurring proteins found in food.³⁴ Any food that contains protein has the theoretical potential to elicit allergic sensitization and, upon subsequent exposure, to cause an allergic reaction in the sensitive individual. However, only a few foods are most commonly

TABLE 1.3**The Most Common Allergenic Foods or Food Groups**

Cows' milk
Crustacea (shrimp, crab, lobster)
Eggs
Fish
Peanuts
Soybeans
Tree nuts (almonds, walnuts, etc.)
Wheat

associated with food allergy (Table 1.3).³⁵ These eight foods or food groups are thought to be responsible for at least 90% of all food allergies.⁹ Foods frequently and falsely implicated by consumers as causes of food allergies such as chocolate, strawberries, and citrus fruits do not give positive results in double-blind food challenges in children with atopic dermatitis.³⁶ In infants and young children, cows' milk allergy is the most common food allergy but it is usually short-lived.^{36,37} Other common food allergies in this age group are allergies to peanuts, eggs, and soybeans.^{36,37} These foods are commonly allergenic in infants and young children in part because they are very frequently consumed foods for this age group. In contrast, peanuts and crustacea are likely to be the most common allergenic foods among adults in the U.S.¹ While frequency of exposure may have something to do with why these foods are among those most commonly associated with IgE-mediated food allergy, the inherent immunogenicity of the protein also must play an important role. Some commonly eaten, proteinaceous foods such as beef, pork, and chicken are rarely implicated in true food allergies.¹ Because of differences in the diet in other countries, the prevalence of true food allergies to other foods may be higher. For example, soybeans in Japan, codfish in Scandinavian countries, and buckwheat in South Korea are commonly allergenic and comparatively popular foods in those countries.

Relatively few food allergens have been purified and characterized (Table 1.4).³⁴ Some commonly allergenic foods contain multiple allergenic proteins including cows' milk, eggs, and peanuts. Foods may contain both major and minor allergens. Major allergens are defined as allergens that bind to serum IgE antibodies from more than 50% of patients with that specific food allergy.

Cows' milk contains several major allergens. The major proteins in cows' milk — casein, β -lactoglobulin, and α -lactalbumin — are major allergens.^{38,39} Several other cows' milk proteins are minor allergens that affect only a small percentage of cows' milk-allergic individuals.⁴⁰ The major cow's milk allergens retain their allergenicity even when subjected to severe heat treatments.^{41,43} Cows' milk appears to retain its allergenicity after such common heat-processing treatments as pasteurization, condensation, evaporation, and drying.⁴²

In most published studies of egg allergies, the egg white has been shown to be more allergenic than the egg yolk.³⁴ The major allergens have been

TABLE 1.4

Some Food Allergens That Have Been Purified and Characterized

Food	Allergenic Proteins
Cows' milk	Casein, β -lactoglobulin, α -lactalbumin
Egg white	<i>Gal d 1</i> (ovomucoid), <i>Gal d 2</i> (ovalbumin), <i>Gal d 3</i> (ovotransferrin), <i>Gal d 4</i> (lysozyme)
Shrimp	
<i>Penaeus aztecus</i>	<i>Pen a 1</i> (tropomyosin)
<i>Penaeus indicus</i>	<i>Pen i 1</i> (tropomyosin)
<i>Metapenaeus enis</i>	<i>Met e 1</i> (tropomyosin)
Codfish	<i>Gad c 1</i> (parvalbumin)
Peanut	<i>Ara h 1</i> , <i>Ara h 2</i> , <i>Ara h 3</i> (seed storage proteins)
Soybean	<i>Gly m 1</i> (oleosin)
Brazil nut	<i>Ber e 1</i> (seed storage protein)
Mustard	<i>Sin a 1</i> , <i>Bra j 1</i> (seed storage protein)

identified as *Gal d 1* (ovomucoid), *Gal d 2* (ovalbumin), and *Gal d 3* (conalbumin).³⁴ It should be noted, however, that IgE antibodies also can be directed to egg yolk proteins,⁴⁴ and cross-reactivity may exist between egg yolk and egg white proteins, and between eggs of various birds.⁴⁵ Bernhisel-Broadbent et al.⁴⁶ determined that ovomucoid is the major antigenic and allergenic egg white protein for humans. The allergenicity of ovalbumin was due primarily to the presence of small amounts of ovomucoid as a contaminant of commercial ovalbumin.⁴⁶ Ovomucoid or *Gal d 1* has a molecular weight of 28 kDa, comprises 11% of protein in egg white, is noncoagulable by heat, and is not denatured by 8 M urea.⁴⁷ Ovalbumin or *Gal d 2* has a molecular weight of 45 kDa, comprises 54% of egg white protein, and is easily denatured by urea and guanidinium salts. Some egg allergens, ovomucoid in particular, are considerably heat stable. Allergic individuals may react to foods containing cooked eggs as well as raw eggs.⁴¹

The most extensively characterized food allergen is *Gal c 1* (allergen M), a parvalbumin from codfish.³⁴ *Gal c 1* contains 113 amino acid residues and 1 glucose moiety, has a molecular weight of 12,328 and an isoelectric point (pI) of 4.75.⁴⁸ The three-dimensional structure is known, and *Gal c 1* apparently contains several IgE-binding sites.^{49,50} Synthetic polypeptides of the sequence of the domains of the *Gal c 1* molecule have the ability to bind IgE from the sera of cod-allergic individuals.^{49,50} *Gal c 1* is extremely resistant to physical destruction^{41,51} and would, therefore, be expected to retain its allergenic activity through most processing and cooking treatments.

The major shrimp allergen has been shown to be tropomyosin.⁵²⁻⁵⁴ This protein contains approximately 300 amino residues with a pI range of 4.8 to 5.4.⁵² Extensive cross-reactivity between different members of crustacea among crustacea-allergic individuals may be due to homology between tropomyosin from these sources.⁵²⁻⁵⁵ The allergenic activity of tropomyosin is heat-stable, and this shrimp allergen has been isolated from shrimp cooking water.^{56,57}

Multiple IgE-binding proteins have been identified in peanuts.³⁴ Barnett et al.⁵⁸ identified 16 IgE-binding protein bands in raw peanuts and 7 IgE-binding protein bands in roasted peanuts. While many of these peanut allergens remain to be purified and characterized, several of the major peanut allergens are relatively well defined. Barnett and Howden⁵⁹ purified a 65 kDa concanavalin A-reactive glycoprotein that they documented as a major allergen. Burks et al.⁶⁰ purified a major peanut allergen, *Ara h 1*, with a molecular weight of 63.5 kDa and a pI of 4.55. Although it appears as though *Ara h 1* and the concanavalin A-reactive glycoprotein may be the same based upon the similarity in molecular weight, *Ara h 1* does not bind to concanavalin A.⁶⁰ These same investigators also identified and characterized a second peanut allergen, *Ara h 2*, with a molecular weight of 17 kDa and pI of 5.2.⁶¹ More recently, yet a third major peanut allergen, *Ara h 3*, has been purified and characterized.⁶² The IgE-binding capabilities of a crude peanut extract and two of the major peanut allergens, *Ara h 1* and *Ara h 2*, were unaffected by heating at 37°C for 60 min, 56°C for 60 min, 100°C for 5 min, 100°C for 20 min, or 100°C for 60 min.⁶³ Processed peanut products containing detectable peanut proteins appear to retain their allergenicity through typical processing practices.⁶⁴

Soybeans also seem to contain multiple allergens.³⁴ Soybeans have two major protein fractions, the globulin and the whey. The major globulins are glycinin or the 11S fraction and β -conglycinin or the 7S fraction. A minor fraction, the 2S fraction, contains several trypsin inhibitors. Allergenic activity has been found in the 2S, 7S, and 11S fractions by radioallergosorbent test (RAST), RAST inhibition, and Western blotting.^{65,66} The soybean allergenic protein, *Gly m 1*, which is most strongly and frequently recognized by the IgE antibodies in sera of soybean-sensitive patients, has been identified as an oleosin or oil body-associated protein with a molecular weight of 34 kDa.⁶⁷ Certain components of the glycinin fraction also appear to be significant soybean allergens.⁶⁸ As with peanuts, the soybean allergens are remarkably heat stable.⁶³ Processed soybean products containing detectable and nonhydrolyzed soybean proteins possess allergenic activity.⁶⁹

The allergens in green peas are localized in the albumin fraction.^{70,71} These pea allergens are also heat stable. However, the allergens from green pea were not completely purified, identified, or characterized.

Comparatively less information is available regarding the allergens in tree nuts. A study by Bargman et al.⁷² determined that almond may have two major IgE-binding proteins of 20 kDa and 40 to 50 kDa. The immunoreactivity of the larger one was reduced by heat processing, while the smaller one was stable and maintained IgE binding after roasting and blanching.⁷² The Brazil nut is a common cause of allergic reactions in tree-nut sensitive individuals.⁷³ Studies have shown that several proteins with potent antigenic properties are found in Brazil nuts, the most prominent being a methionine-rich 2S protein.⁷⁴ The 2S protein found in Brazil nuts contains 18% methionine residues making it an excellent candidate to supplement sulfur amino acid-poor crops, such as soybeans. A recent study by Nordlee et al.⁷⁴ demonstrated

that a chimeric gene encoding the 2S Brazil nut protein transferred to soybeans resulted in the protein being expressed in the transgenic seed. This protein was then found to bind human IgE, making it a probable allergen. More recently, the major allergen has been isolated from walnuts and also is a small molecular-weight storage protein.⁷⁵

Matsuda et al.⁷⁶ have demonstrated that the proteins responsible for rice allergy are major components of the rice albumin proteins. These proteins have molecular masses of about 14 to 16 kDa and a pI of about 6 to 8. The ability to bind IgE was decreased when the fractions were heated.

Adverse reactions to buckwheat have been reported and, though rare, can be rather severe in some cases.^{77,78} Immunoblotting with the sera of one patient who had multiple episodes of buckwheat-associated anaphylaxis revealed four IgE-binding bands in the molecular weight range of 9 to 40 kDa.⁷⁹ Other investigators also using sera from buckwheat-allergic patients identified three proteins, including one trypsin inhibitor, in the molecular weight range of 8 to 9 kDa that bound to IgE.⁸⁰

Sesame seed is a food of increasing allergenic significance,⁸¹ and sesame seed allergy occurs comparatively commonly in some countries.⁸² Recently, the major allergens from sesame seed have been identified.⁸³

Adverse reactions to mustard have been documented by several studies.^{84,85} Mustard is made from the seed flour of mustard plants, namely *Brassica nigra* (black mustard), *Brassica alba* (white mustard), *Sinapis alba* L. (yellow mustard), and *Brassica juncea* L. (oriental mustard). Table mustard is usually made from yellow mustard and oriental mustard. The relative amount in the commercial product may be different depending on the manufacturer, with yellow most common in Europe, and oriental being most abundant in mustard extracts in the U.S. and Japan. Menendez-Arias et al.⁸⁶ describe the major allergen from yellow mustard seeds as a 2S albumin, designated *Sin a 1*. This protein is composed of two disulfide-linked polypeptide chains of 39 and 88 amino acids. The *Sin a 1* allergen is found to be related to other low-molecular-mass albumins, such as those isolated from rapeseed, castor bean, and Brazil nut. Gonzalez de la Pena et al.⁸⁷ isolated and characterized a 2S albumin from oriental mustard seeds. This protein, *Bra j 1*, was found to be closely related to *Sin a 1*.

Avoidance of True Food Allergies

The only treatment for all food allergies and sensitivities is the specific avoidance diet. Individuals with peanut allergy must avoid peanuts, for example. While such diets can be quite successful, adherence can be quite challenging. The construction of safe and effective avoidance diets and the difficulties faced by consumers who must adhere to such diets have been extensively reviewed elsewhere.^{9,88}

Patients with true food allergies are faced with three serious issues as they attempt to implement a safe and effective avoidance diet:

1. Will trace levels of the food elicit reactions or increase sensitization?
2. Do all foods and food ingredients made from the offending food contain the allergens?
3. Are cross-reactions likely to occur between closely related species?

First, trace levels of the offending food can elicit adverse reactions. Many experiences have been anecdotally related, such as reactions from touching utensils or bottles contaminated with the offending food, kissing the lips of someone who has recently eaten the offending food, opening packages of the offending food, inhalation of vapors from cooking the offending food, and the transfer of food allergens from lactating mothers to breast-feeding infants.¹ In such situations, the amount of the offending allergen that is ingested must be rather low. However, several episodes have been well investigated and lend credibility to the anecdotal reports.^{23,89,90} While, for all practical purposes, complete avoidance must be maintained, threshold doses do exist below which allergic individuals will not experience adverse reactions. The threshold doses are likely to be very low and variable from one allergic individual to another. In a recent clinical study of threshold doses for peanuts, the most sensitive peanut-allergic individual among a group of 12 began to experience subjective symptoms when exposed to 100 µg of peanut protein and experienced objective symptoms when exposed to 2 mg of peanut protein.⁹¹ However, four other peanut-allergic individuals in this study with equally impressive histories of allergic reactions to peanuts had no reaction when exposed to the highest dose used in the trial, 50 mg of peanut protein.⁹¹

Foods may become contaminated with trace amounts of other foods through various means. For food processors, the major concerns are the use of rework and the use of shared equipment.⁹ Contamination of food products with trace, unlabeled residues of allergenic foods is especially important to individuals who are exquisitely sensitive to the offending food and who experience life-threatening symptoms. No avoidance diet provides absolute safety, but careful adherence to an effective avoidance diet will minimize the chance of a reaction.

When considering foods derived from an allergenic food source, the presence of the allergenic protein is important. In a study by Nordlee et al.,⁶⁴ the allergenicity of peanut products was determined by RAST-inhibition using blood sera from peanut-allergic individuals. Most processed peanut products retained their ability to bind specific IgE from the sera indicating that peanut allergens are highly heat stable and survive typical food processes, such as roasting. The allergic reactivity of soybean products was determined, also using RAST inhibition, by Herian et al.⁶⁹ Some soy products, such as soy oil and soy lecithin, which do not normally contain soy protein may be safe for consumption by soy-allergic individuals. Soy products, such as hydrolyzed vegetable protein (HVP) and soy sauce, which are subjected to considerable proteolysis during processing and which, therefore, may not contain intact

allergenic proteins, remain unsafe for soy-allergic consumers in many cases as the samples evaluated in this study were able to bind serum IgE. Edible oils, if processed by the typical hot-solvent extraction process, do not contain sufficient levels of protein to elicit allergic reactions in sensitive individuals. Extremely low levels of protein (<1.0 ppm) can be detected in these oils. However, double-blind challenge tests have been conducted with allergic individuals using peanut, soybean, and sunflower seed oils and all have been documented to be safe for ingestion.⁹²⁻⁹⁴ If foods derived from allergenic sources contain detectable protein residues, the safety of these foods must be established by clinical trials in sensitive individuals. Alternatively, the foods should be labeled to declare the source of the ingredient.

No ubiquitous statement can be made about cross-reactions between closely related foods because only limited studies have been conducted. Cross-reactivity to closely related foods seem to occur among some food-allergic patients, but not others. For example, individuals with a shrimp allergy may be told to avoid all seafood including both crustacean and molluscan shellfish and fish. Considering the distant taxonomic relationships between edible seafood, it is unlikely that shrimp would cross-react with fish or molluscan shellfish.⁹⁵ However, patients with shrimp allergy will usually experience adverse reactions after ingestion of other crustacean species such as lobster, prawn, crab, and crayfish,⁹⁶ suggesting appreciable similarity in the IgE-binding epitopes of the offending allergens from these sources.⁵² A study by Bernhisel-Broadbent et al.⁹⁷ indicates that patients allergic to one or more fish species can often consume other fish species without adverse reactions. Some peanut-allergic individuals are allergic to other legumes, such as soybeans,⁶⁶ although this is not a frequent occurrence. Clinical hypersensitivity to one legume, such as peanuts or soybeans, does not warrant dietary elimination of the entire legume food family unless allergy to each legume is individually confirmed by double-blind, placebo-controlled food challenges (DBPCFC).⁹⁸ In contrast, cross-reactions are known to commonly occur between different species of avian eggs⁴⁵ and between cows' milk and goats' milk.⁹⁹

Cross-reactions also are known to occur between some types of pollens and certain foods. These include ragweed pollen and melons (watermelon, cantaloupe, honeydew); mugwort pollen and celery; mugwort pollen and hazelnuts; and birch pollen and various foods such as carrots, apples, hazelnuts, and potatoes.³⁴ Another allergic cross-reaction is that between latex and fruit, particularly banana, chestnut, and avocado.^{34,100} Patients with a history of allergic reactions to latex should be aware of the potential for allergic reactions to certain fruits.

Clinical observation of cross-reacting IgE antibodies are occasionally unexpected and confusing, but they don't always imply the existence of an allergy to each food. For the interpretation of IgE antibody assays, it is important to appreciate that finding IgE antibodies to an allergen does not imply that the patient has ever been exposed to that allergen or that they will react after ingestion of that food.¹⁰¹

TABLE 1.5**Steps to Minimize Cross-Contamination and Mislabeling**

1. Never substitute for listed ingredients without changing label accordingly.
 2. Package label should accurately reflect product contents.
 3. Check supplies to ensure all necessary precautions have been taken in-house.
 4. Inspect incoming ingredients upon receipt.
 5. Throw away unused supplies of outdated labels.
 6. Ensure that limited opportunities exist for cross-contaminants to transfer from adjacent lines and that the production process and equipment are designed for avoidance of cross-contamination.
 7. Inspect cleanup of equipment used for allergen-containing ingredients.
 8. If reworked product is included in the formulation, adopt a “like-into-like” policy.
 9. Schedule known allergenic foods and ingredients near the end of a run and prior to a full cleanup, shutdown, and inspection.
 10. Institute an in-house education program in food allergies and anaphylaxis.
-

Allergen Cross-Contact and Its Control

The issue of cross-contact of allergenic foods with other foods from the use of shared facilities and equipment came to the attention of manufacturers in the early 1990s as more incidents occurred where allergic individuals became ill from the consumption of products containing low levels of undeclared allergenic foods.⁹ To help prevent costly recalls and serious reactions in consumers, there are steps the food industry can take to minimize the opportunity for cross-contact or mislabeling (Table 1.5). Manufacturers need to be aware that inadvertent transfer of allergenic food residues from one product to another and accidental mislabeling may result in serious, life-threatening reactions among allergic consumers. The amount of allergen that can set off a serious allergic response is extremely small and well below visual detection, although visual detection is sometimes the only method for inspection. Processors should approach all of the commonly allergenic foods listed in Table 1.3 with extreme caution with respect to cross-contact. Since these allergenic foods comprise 90% or more of the problem, the focus should be placed primarily on these particular commonly allergenic foods or food groups. It appears that the potential for peanut and tree nut allergies to elicit more severe manifestations is greater than those for most other food allergies. There is also an increasingly widespread use of peanuts in the food sector. This is a risk especially with the use of peanuts in products where it would not be expected and where they might not be visible or apparent (e.g., peanut shavings garnishing a lemon meringue pie). It appears that there are no adverse reactions to fully refined peanut oils.⁹² However, sometimes a “cold-pressed” peanut oil is utilized in gourmet and ethnic food production which could contain allergenic protein residues.¹⁰² Also, some ethnic food products containing peanuts are fried in peanut oil; in such cases, cross-contamination of the oil is probable. So although refined peanut oils are unlikely to trigger adverse reactions, it would be appropriate for allergic individuals to be cautious.

Accurate labeling is the best approach to the prevention of severe allergic reactions to packaged food products. However, in most of the reported cases, the victim had no access to the label (e.g., food consumed from food service outlets). Restaurants, catering businesses, and similar establishments should consider including allergen training in their food safety training courses and placement of nonprescription antihistamine drugs in their first aid kits.

In Canada, with the cooperation of government agencies and industry, the Allergy Beware Program was launched in 1993. The program includes a videotape, instructor's manual, employee summary, and cross-contact audit checklists. Through the program, manufacturers can learn to be aware of the program, how to be more accurate in food labeling, how to avoid the problem, and action to take if, despite all efforts, an allergen goes undetected.¹⁰³ Programs like this one protect the consumer, the customer, the branch franchises, and the company.

Celiac Disease (Gluten-Sensitive Enteropathy)

Celiac disease, also known as celiac sprue or gluten-sensitive enteropathy, is a malabsorption syndrome occurring in sensitive individuals following the ingestion of wheat, rye, barley, and, in some instances, oats.^{104,105} Following the ingestion of these grains, the absorptive epithelial cells in the small intestine are damaged resulting in a decreased number of epithelial cells that are critical for digestion and absorption. The activity of the mucosal enzymes necessary for digestion and absorption also are decreased in the damaged cells. This damage to the absorptive function of the small intestine results in a severe malabsorption syndrome characterized by diarrhea, bloating, weight loss, anemia, bone pain, chronic fatigue, weakness, muscle cramps, and in children, failure to gain weight and growth retardation.^{106,107}

While the mechanism for producing this damage is not known, several theories have been promulgated:

1. Sensitive individuals lack some enzyme necessary for the digestion of the wheat protein fraction, gliadin.
2. Gliadin acts like a lectin and binds to abnormal glycoprotein receptors on the surfaces of the epithelial cells of sensitive individuals and this interaction results in a cytotoxic effect.
3. Sensitive individuals mount an abnormal immunologic response to a fraction of the gliadin protein.¹⁰⁸

Strober¹⁴ suggested that the mechanism of celiac disease might be a Type IV allergic mechanism, an immunocytotoxic reaction mediated by intestinal lymphocytes. Researchers have found that immunoglobulins synthesized by celiac mucosa have antigliuten specificity,¹⁰⁶ but this response may occur secondary to the intestinal damage.

Celiac disease is an inherited trait; however, its inheritance is complex and poorly understood. Celiac disease occurs in about 1 of every 3000 individuals in the U.S.^{104,108} The disease occurs with differing frequencies in other parts of the world. The highest incidence of celiac disease is in County Galway, Ireland, affecting 1 in every 300 individuals.¹⁰⁸ Celiac disease occurs more frequently among Europeans than among Americans of European descent for unexplained reasons. Celiac disease rarely, if ever, occurs in those of Chinese or African descent.¹⁰⁵

The intestinal damage that occurs in celiac disease is associated with the abnormal immunological response to the prolamin protein fractions of wheat, rye, barley, and perhaps oats. Specifically, in wheat, this fraction is called gliadin, but related prolamin proteins also occur in barley, rye, and oats.¹⁰⁶ It is likely that the cross-reactivity is due to the conservation of reactive peptide sequences in these complex protein fractions among these closely related grains. The role of oats in celiac remains somewhat uncertain. A recent Finnish study documented that celiac sufferers can safely ingest oats.¹⁰⁹ However, in commerce in much of the world, oats would often be contaminated with wheat so the avoidance of oats may still be a wise idea for those with severe celiac sensitivity.

The treatment of celiac disease typically involves the total avoidance of wheat, rye, barley, and probably oats and their products.¹⁰⁴ While the tolerance for wheat, rye, and barley protein in celiac sufferers is not precisely known, the symptoms of celiac disease can be triggered by ingestion of rather small quantities of these grains. Treatment with a gluten-free diet results in resolution of the damage to the intestinal mucosa and its absorptive function. Since a safe tolerance level cannot yet be estimated, complete avoidance is usually practiced by celiac sufferers. However, adherence to strict avoidance diets can be quite difficult since wheat and wheat products are so commonly used in food formulations. Questions remain about the necessity of excluding ingredients prepared from wheat, rye, barley, and oats if the ingredients contain no intact proteins. Examples might include wheat starch, rye whiskey, malt extract, and hydrolyzed vegetable protein. In the absence of data demonstrating the safety of these ingredients for celiac patients, most celiac sufferers will likely continue to avoid these products.

Nonimmunological Food Sensitivities

In contrast to true food allergies, many of the individualistic adverse reactions to food do not involve the immune system. The prevalence of these reactions is unknown. Most of the nonimmunological food sensitivities are associated with foodborne substances other than proteins. While true food allergies can be attributed to proteins, nonimmunological food sensitivities are associated with both naturally occurring and additive substances (Table 1.6).

TABLE 1.6

Substances Associated with Nonimmunological Food Sensitivities

Type of Reaction	Specific Illness	Known, Suspected, or Alleged Causative Substance
<i>Naturally Occurring Substances</i>		
Anaphylactoid	—	Unknown substances in strawberries, shellfish
Metabolic food disorder	Lactose intolerance	Lactose
	Favism	Vincine and convicine
Idiosyncratic	Migraine headache	Chocolate, cheese
<i>Additive Substances</i>		
Anaphylactoid	—	None known or suspected
Metabolic food disorder	—	None known or suspected
Idiosyncratic	Asthma	Sulfites, tartrazine, MSG, BHA, BHT, benzoates, sunset yellow
	Chronic urticaria	Tartrazine, BHA, BHT, benzoates, parabens, sunset yellow, aspartame
	Migraine headache	Aspartame
	Behavioral disorders	Food colorants, sugar
	MSG symptom complex	MSG

Anaphylactoid reactions result from substances in food that cause mast cells and basophils to spontaneously release histamine and other mediators of allergic reactions. However, unlike true food allergies, there appears to be no involvement of IgE or other immunoglobulins, and prior exposure is not a prerequisite.^{1,107}

Metabolic food disorders are adverse reactions to a food or food additive that occur through some effect of the substance on the metabolism of the individual.^{1,107} Two of the most common examples of metabolic food disorders are lactose intolerance and favism.

Idiosyncratic reaction is the term used to describe a variety of individual food sensitivities thought to occur through nonimmunological, but unknown, mechanisms.^{1,107} Many of the reported adverse reactions to food additives are placed in this category because of the lack of understanding of their modes of action. The cause-and-effect relationship between the food additives allegedly involved in idiosyncratic reactions and the adverse reactions is not yet clearly established. In a few cases, the cause-and-effect relationship is very well established. For example, the involvement of sulfites in asthma is well documented.^{110,111} However, the extent to which chocolate causes migraine headaches;¹¹² food coloring agents and sugar cause hyperkinesia;^{6,113,114} tartrazine (FD&C yellow #5), butylated hydroxyanisole (BHA), and butylated hydroxytoluene (BHT) cause hives;⁶ and monosodium glutamate causes the headache, facial flush, and chest pain termed “monosodiumglutamate (MSG) symptom complex” in sensitive individuals¹¹⁵ is unproven.

Anaphylactoid Reactions

In true food allergies, the release of histamine and other mediators of the allergic response from the mast cells and basophils is mediated by IgE, as discussed earlier. In contrast, anaphylactoid reactions are caused by substances that bring about the nonimmunologic release of these same mediators from mast cells without the involvement of IgE.^{1,107}

Occasionally, histamine poisoning (also known as scombroid fish poisoning) is included as an example of an anaphylactoid reaction.¹ However, histamine poisoning is actually a foodborne intoxication associated with the ingestion of foods containing unusually high levels of histamine.¹¹⁶ The histamine is formed during bacterial spoilage.¹¹⁷ All consumers are susceptible to histamine poisoning, so it does not truly fit into the category of food allergies and sensitivities, the so-called individualistic adverse reactions to foods. Histamine poisoning is clearly an illness that is distinct from food allergies as it involves the ingestion of exogenous histamine rather than the release of histamine from mast cells and basophils *in vivo*. It is frequently included because the symptoms resemble those encountered with food allergies. Histamine poisoning has been described in several reviews.^{116,118}

In anaphylactoid reactions, some substance in the implicated food is presumed to destabilize the mast cell membranes allowing the spontaneous release of the histamine and other mediators. Actually, no such histamine-releasing substances has ever been isolated or identified in foods. However, this mechanism is well-established with certain drugs. Therefore, the best evidence for the existence of anaphylactoid reactions is actually the lack of evidence for an IgE-mediated mechanism in a few types of food allergy, such as strawberry allergy. Strawberries are known to cause adverse reactions (frequently urticaria) in some individuals. Yet, strawberries contain little protein, and no evidence of a strawberry allergen has ever been found. Additionally, no evidence has been obtained for the existence of strawberry-specific IgE in the sera of strawberry-sensitive individuals. The symptoms of strawberry "allergy" are very similar to those occurring in IgE-mediated food allergy, so *in vivo* release of histamine and other mediators is a possible mechanism. Also, histamine poisoning is not a likely explanation since strawberries also contain only traces of histamine. Thus, by a process of elimination, non-immunological release of the mast cell and basophil mediators seems a plausible, if unproven, explanation.

Metabolic Food Disorders

Metabolic food disorders involve genetically determined deficiencies that either (1) affect the host's ability to metabolize a food component or (2) enhance the sensitivity of the host to some foodborne chemical via an altered metabolic pattern.¹ Lactose intolerance is an example of an illness that occurs when a genetic deficiency affects the host's ability to metabolize a food component. In lactose intolerance, a deficiency in the enzyme, β -galactosidase,

leads to an impaired ability to digest lactose. Favism is an example of a genetic deficiency that enhances the sensitivity to a foodborne chemical. In favism, a genetic deficiency in erythrocyte glucose-6-phosphate dehydrogenase causes an increased sensitivity to several hemolytic factors in fava beans. These two metabolic food disorders are certainly the most common and best understood within this category of food sensitivities.

Lactose Intolerance

Lactose, a disaccharide and the principal naturally occurring sugar in milk, is hydrolyzed into its constituent monosaccharides, galactose and glucose, in the intestinal mucosa in normal digestive processes. The galactose and glucose can then be absorbed and used metabolically as energy sources. In lactose intolerance, the activity levels of the key intestinal hydrolytic enzyme, known as β -galactosidase or lactase, are diminished.^{4,119}

Lactose cannot be absorbed in the small intestine unless it is hydrolyzed to galactose and glucose. In case of the lactose intolerance, the undigested lactose passes into the colon where it encounters large numbers of bacteria. The bacteria present in the colon metabolize the lactose to CO_2 , H_2 , and H_2O .¹ The symptoms of lactose intolerance (abdominal cramping, flatulence, and frothy diarrhea)¹²⁰ are the result of this bacterial action. The symptoms vary in intensity depending upon the individual's level of intestinal β -galactosidase activity and the amount of lactose ingested.

Lactose intolerance affects many people on a worldwide basis. While only about 6 to 12% of Caucasians are affected,⁴ lactose intolerance is much more prevalent in other ethnic groups and races, affecting as many as 60 to 90% of Greeks, Arabs, Jews, African-Americans, Hispanics, Japanese, and other Asians.^{4,119} Although lactose intolerance tends to worsen with advancing age and is often more common and more severe among the elderly, it can have its onset at any age, occurring as early as the age of three.^{119,121} The level of intestinal β -galactosidase activity is usually sufficient at birth to allow the digestion of lactose in mother's milk.¹ However, individuals born with an inherited deficiency of intestinal β -galactosidase suffer a decline in the activity of the enzyme as they age. At some point, symptoms may begin to develop following the consumption of dairy products containing lactose at levels that exceed the saturation point of the enzyme activity.

Lactose intolerance also can occur secondary to another intestinal illness or infection, such as a bout with viral gastroenteritis.¹²² Secondary lactose intolerance is often a short-term illness because enzymatic activity levels can recover after the original illness subsides.¹

The lactose tolerance test (LTT) is usually used as the basis for a clinical diagnosis of lactose intolerance.^{1,4} The LTT involves the oral administration of 50 g of lactose to a fasting individual with monitoring for blood glucose or breath hydrogen levels after challenge to determine whether lactose is being absorbed. Gastrointestinal symptoms are also monitored. While the LTT definitely establishes lactose intolerance, the dose is sufficiently high in the LTT

that the test does not establish the degree of lactose intolerance. Few individuals would ever ingest 50 g of lactose in a single meal. Newcomer¹²³ concluded that only 19% of lactase-deficient individuals were intolerant to ingestion of 8 oz of milk containing 12 g of lactose. As a result of such concerns, some physicians have advocated the use of lower doses of lactose in the diagnosis of lactose intolerance.¹ The use of sequentially increasing doses of lactose, while perhaps a tedious diagnostic procedure, would help to clarify the degree of intolerance to various doses of lactose and the extent to which lactose intolerance worsens with age in affected individuals.

Careful differential diagnosis is important in the assessment of possible cases of milk intolerance. Lactose may not be responsible for all cases of milk intolerance. True cows' milk allergy is another possibility that has already been discussed. Additionally, investigators have identified individuals with normal capacities for lactose ingestion, as indicated by the LTT, who experience the same symptoms as lactose-intolerant individuals when challenged with 8 to 12 oz of milk.¹²⁴ These investigators speculated that substances other than lactose in milk may be responsible for some cases of milk intolerance. Milk protein intolerance, an illness distinct with IgE-mediated cows' milk allergy, might be one possibility, although the symptoms often display a delayed onset of several hours in this form of milk intolerance.^{32,125}

Individuals with lactose intolerance are able to control their symptoms through the avoidance of dairy products containing lactose. However, the extent to which lactose avoidance must be practiced depends upon the lactose-intolerant individuals and their individual degree of tolerance for lactose. As noted above, some lactose-intolerant individuals may be able to tolerate some dairy products and some lactose. Individuals identified as intolerant to 50 g oral challenges in the LTT may, in some cases, be able to tolerate the lower amounts of lactose present in most dairy products. Dietary alternatives also exist for individuals with greater degrees of lactose intolerance who would experience symptoms from ingestion of typical amounts of many dairy products. Some of these individuals will be able to tolerate small, divided doses of milk. Lactose-hydrolyzed milk is also available in the marketplace.¹²⁶ This product is effective, but its sweet taste limits acceptance. The addition of β -galactosidase to milk just before consumption also seems to be effective.¹²⁷ Presumably, the enzyme retains its activity and hydrolyzes the ingested lactose in the gut. Martini and Savaiano have demonstrated that the tolerance for lactose increases when the lactose is consumed with a meal.¹²⁸ Because yogurt and acidophilus milk contain active cultures of bacteria with β -galactosidase activity, lactose-intolerant individuals appear to be more susceptible to these dairy products than others.^{129,130} The level of lactase activity varies from one brand of yogurt to another, so some brands are more easily tolerated than others.¹³¹ Since dairy products are excellent sources of calcium and also have other important nutritional attributes, the incorporation of maximal, tolerated levels of dairy products into the diets of lactose-intolerant individuals is important.¹ Birge et al. suggested that osteoporosis may result from the inadequate calcium intakes

associated with dairy product-avoidance diets among lactose-intolerant individuals.¹³² The main objective should be to determine the tolerance level for each sensitive individual and construct an avoidance diet that allows the maximum benefit and enjoyment of dairy products.

Favism

Favism occurs as the result of an intolerance to the consumption of fava beans or the inhalation of pollen from the *Vicia faba* plant.¹³³ As a result, the individual suffers from acute hemolytic anemia with symptoms including pallor, fatigue, dyspea, nausea, abdominal and/or back pain, fever, and chills. In rare and severe cases, hemoglobinuria, jaundice, and renal failure can occur. The onset time ranges from 5 to 24 h after ingestion. The disease is usually self-limited with symptoms resolving promptly and spontaneously following avoidance of any further exposure. Favism is most prevalent when the *V. faba* plant is blooming, causing elevated levels of airborne pollen.

Individuals with an inherited deficiency of the enzyme, glucose-6-phosphate dehydrogenase (G6PDH), in their red blood cells are susceptible to favism. G6PDH is a critical enzyme in erythrocytes because it helps maintain adequate levels of the reduced form of glutathione (GSH) and nicotinamide adenine dinucleotide phosphate (NADPH). GSH and NADPH help prevent oxidative damage to erythrocytes.

Fava beans contain naturally occurring oxidants, including vicine and convicine (Figure 1.1), that are able to damage the red blood cell membranes of G6PDH-deficient individuals causing hemolysis of the erythrocytes and the symptoms of hemolytic anemia.¹³³ While both lactose intolerance and favism are metabolic food disorders, the mechanism of favism is quite distinct from that of lactose intolerance. With favism, the genetic deficiency causes an increased susceptibility to the oxidative toxins present in fava beans.

G6PDH deficiency occurs very frequently affecting about 100 million people.¹³³ Prevalence is highest among Asian Jewish communities in Israel, among Sardinians, Cypriot Greeks, African-Americans, and certain African populations. G6PDH deficiency is virtually absent among Caucasians and Native Americans. Despite the high prevalence of G6PDH deficiency, the incidence of favism is low. Favism occurs primarily in the Mediterranean area, the Middle East, China, and Bulgaria where G6PDH deficiency is fairly

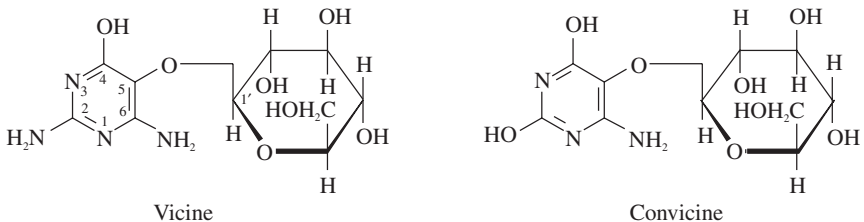


FIGURE 1.1
Structures of vicine and convicine.

common and where fava beans are grown and are frequently consumed. The treatment for favism is the avoidance of fava beans both in the diet and from the inhalation of the plant pollen.

Idiosyncratic Reactions

The mechanisms are unknown for some adverse reactions to foods experienced by certain individuals in the population.¹ Conceivably, a large number of different mechanisms could be involved in these idiosyncratic reactions.¹ As expected, the symptoms associated with this wide variety of illnesses range from the trivial to severe life-threatening reactions.¹

Some foodborne idiosyncratic reactions are rather well documented and the relationship with specific foods and/or food ingredients is firmly established. Sulfite-induced asthma would be a good example.¹¹¹ For many other idiosyncratic reactions to foods, the association with specific foods and/or food ingredients has not been clinically established. Examples would include the role of chocolate or aspartame in migraine headache; the roles of BHA, BHT, or tartrazine in chronic urticaria; the role of tartrazine in asthma; the role of MSG in asthma or MSG symptom complex; and the role of sugar in aggressive behavior.^{1,6} The role of psychological disorders in perceived reactions to foods has been the subject of several notable studies.^{134,135} In some cases, the symptoms are so subjective that the confirmation of the responses is difficult. In a few cases, the role of specific foods or food ingredients in idiosyncratic reactions has been disproven by careful clinical investigations. However, consumers may persist in the belief that these relationships are real. The outstanding example of such a reaction is the role of artificial food colors in hyperkinetic behavior in children. Food colorants were first implicated as causative factors in hyperkinesis by Dr. Benjamin Feingold on the basis of poorly controlled trials and anecdotal experiences.¹³⁶ The Feingold hypothesis received considerable publicity, and many consumers became convinced of the relationship between ingestion of artificial food colors and hyperkinetic behavior in children. Subsequently, several well-controlled, double-blind challenge trials revealed that few, if any, hyperkinetic children were adversely affected by ingestion of these food colorants.^{137,138} Despite this evidence, some consumers continue to believe that artificial food colorants are involved in hyperkinetic behavior in children. [Table 1.7](#) contains a partial list of food-related idiosyncrasies in each of these categories (proven, unproven, and disproven).

As noted above, the role of specific foods or food ingredients in many of these idiosyncratic reactions remains to be established. The cause-and-effect relationships can only be established through carefully controlled DBPCFC (double-blind, placebo controlled food challenges).¹ A positive DBPCFC confirms that the specific food or food ingredient is involved in the particular adverse reactions. Conversely, a negative DBPCFC may indicate either that

TABLE 1.7

Partial List of Food-Associated Idiosyncratic Reactions

Category	Reaction	Implicated Food or Ingredient
Proven	Asthma	Sulfites
	Urticaria	Aspartame
Unproven	Chronic urticaria	BHA, BHT, benzoates
	Asthma, urticaria	Tartrazine
	Migraine headache	Many foods, aspartame
	Aggressive behavior	Sugar
	MSG symptom complex	Monosodium glutamate
	Asthma	Monosodium glutamate
Disproven	Hyperkinesia	Food coloring agents

foods are not involved in the reaction or at least that the specific food or ingredient was wrongly incriminated.

A complete discussion of all of the many alleged food-associated idiosyncratic reactions is beyond the scope of this chapter. Instead, several idiosyncratic reactions will be highlighted as examples.

Sulfite-Induced Asthma

Sulfiting agents including sulfur dioxide (SO₂), potassium metabisulfite (K₂S₂O₅), potassium bisulfite (KHSO₃), sodium bisulfite (NaHSO₃), and sodium metabisulfite (Na₂S₂O₅) have been widely used in foods for many years.⁵ Sulfites are used as food additives for several important commercial purposes: to prevent enzymatic and nonenzymatic browning, as broad spectrum antimicrobial agents, as dough conditioning agents, to provide antioxidant protection, and as bleaching agents in the processing of maraschino cherries and hominy.⁵ As a result, residues of sulfite can occur in a variety of foods at levels ranging from a few ppm to >1000 ppm in dried fruits.⁵ Among the foods and beverages with the highest sulfite levels as consumed are dried fruits other than dark raisins or prunes, nonfrozen lemon and lime juices, wines, molasses, dehydrated potatoes, refrigerated or fresh hash brown potatoes, shrimp, white and pink grape juices, and sauerkraut juice. Sulfites also occur naturally in some foods, especially fermented foods, but the residues from naturally occurring sulfites are usually low.⁵

Sulfites added to foods can react with other food components such as reducing sugars, proteins, amino acids, aldehydes, and ketones.⁵ Consequently, very little free, unreacted sulfite remains in most foods. Instead, residual sulfites are typically bound to other organic constituents either reversibly or irreversibly. Sulfites also are oxidized to sulfate in some food systems. Sulfites also can be volatilized as SO₂, especially from acidic food and beverages. Thus, the residual sulfite levels in foods, measured as SO₂ equivalents, decreases with processing and storage in most food matrices.

Although sulfites were used for centuries with little evidence of harm to consumers, in recent years, sulfites have been implicated as triggers for asthmatic reactions in some sensitive individuals.^{110,111} The reactions usually occur within a few minutes after ingestion of a provoking dose of sulfite. The reactions can be quite severe on occasion, and deaths have been attributed to sulfite-induced asthma.¹¹¹

Asthma is the only well-documented symptom involved in sulfite sensitivity. The role of sulfites in asthma has been verified by numerous investigators through the use of DBPCFCs.^{110,111,139,140} Other symptoms have been reported as associated with sulfite sensitivity, but these reports are largely anecdotal and unverified by DBPCFC.¹³⁹ Double-blind challenges have been conducted with sulfite in capsules and in acidic beverages. Volatilization of SO₂ occurs in acidic beverages, and sulfite-sensitive asthmatics are more likely to respond to sulfited, acidic beverages than to capsules.¹⁴¹ In acidic beverage challenges, the increased sensitivity seems to be due to the inhalation of SO₂ vapors while swallowing.¹⁴¹

Sulfite sensitivity occurs rather infrequently among asthmatic individuals. From challenges conducted on over 200 asthmatics, Bush et al. concluded that severe asthmatics, defined as those requiring steroid-based drugs for control of their asthmatic conditions, are most likely to be sulfite-sensitive.¹⁴⁰ The prevalence among steroid-dependent asthmatics is estimated at 4 to 7%.¹⁴⁰ However, steroid-dependent asthmatics comprise only about 20% of the entire asthmatic population. Thus, the overall prevalence of sulfite sensitivity among asthmatics can be estimated at 1 to 1.5%. None of the mild asthmatics in the large clinical trial conducted by Bush et al. were confirmed to be sulfite sensitive.¹⁴⁰ Other investigators have estimated a higher prevalence of sulfite sensitivity among asthmatics,^{142,143} but these estimates may have been based mostly on challenges of steroid-dependent asthmatics rather than a representative cross section of the entire asthmatic population.¹³⁹

The mechanism involved in sulfite-induced asthma is now known. Hence, despite the well proven existence of sulfite sensitivity, it remains an idiosyncratic reaction. Multiple mechanisms have been proposed including IgE-mediated reactions, hyperreactivity to inhaled SO₂, and sulfite oxidase deficiency.⁶ The hyperreactivity to inhalation of SO₂ while swallowing seems to explain the sensitivity to ingestion of acidic beverages.¹⁴¹ However, this mechanism cannot explain adverse reactions to ingestion of sulfite in capsules.

Sulfite-sensitive asthmatics display thresholds for sulfites.¹¹¹ However, sulfite-sensitive asthmatics must avoid highly sulfited foods and beverages, as the reaction may be serious or even fatal. The threshold for sulfites varies among sulfite-sensitive asthmatics. In controlled challenges with capsules and/or acidic beverages, the threshold level of sulfite ranges from 3 to 130 mg of SO₂ equivalents.⁵ Sulfite-sensitive asthmatics are even more tolerant of sulfites in foods.¹⁴⁴ Perhaps the increased tolerance occurs because sulfite-sensitive asthmatics are more tolerant of bound sulfite than they are to free sulfite.¹⁴⁴ Sulfite-sensitive asthmatics are especially sensitive to sulfited

lettuce.^{144,145} Lettuce contains a preponderance of free, unbound sulfite¹⁴⁶ and may represent an especially hazardous food for sulfite-sensitive asthmatics.

As a result of growing concerns over reactions to sulfites among consumers, the U.S. Food and Drug Administration (FDA) has instituted several regulations for the protection of sulfite-sensitive asthmatics.¹¹¹ Since 1986, the FDA has banned the use of sulfiting agents on raw fruit and vegetables. This ban prohibits the use of sulfite on fresh lettuce and other vegetables and fruits in restaurant salad bars. This unlabeled use of sulfites was associated with many of the consumer reactions. Use of sulfites in shrimp has been limited to levels that will result in sulfite residues not exceeding 100 ppm total SO₂. Packaged food containing greater than 10 ppm of SO₂ equivalents must identify the presence of the specific sulfite on the ingredient declaration. Because of these public health interventions, the risk of sulfite reactions in sensitive asthmatics appears to be greatly reduced.

Role of MSG in Idiosyncratic Reactions

The involvement of monosodium glutamate (MSG) in idiosyncratic reactions remains to be proved. MSG has been linked to the so-called MSG symptom complex (headache, chest tightness, burning sensation along the back of the neck, nausea, and diaphoresis occurring within minutes after the ingestion of high levels of MSG in foods) and asthma. Recently, an extensive review of MSG reactions was conducted by a group of independent scientists under the auspices of the Federation of American Societies for Experimental Biology (FASEB).¹¹⁵ This review helped to put these safety concerns into perspective and reaffirmed the FDA's belief that MSG and related substances are safe ingredients for most people when eaten at customary levels. The FASEB review panel concluded that some evidence exists to suggest that certain people may develop short-term reactions (the MSG symptom complex) when they consume large doses (3 g or more) of MSG.¹¹⁵ No evidence was found linking the MSG symptom complex to consumption of lower levels (<3 g) of MSG.¹¹⁵ Few meals would contain more than 3 g of MSG. Also, the FASEB review panel failed to find convincing evidence for a role for MSG in more serious alleged reactions with the possible exception of asthma. The panel noted that there may be a small subgroup of people with severe asthma who may respond to ingestion of large doses of MSG (>3 g).¹¹⁵ However, scientific and clinical consensus on a role of MSG in the provocation of asthma has certainly not been achieved. Several clinical investigations have linked MSG exposure to asthma in a few severe asthmatics.¹⁴⁷⁻¹⁴⁹ However, some questions remain about the validity of the diagnosis in some of these cases because delayed (10 to 14 h) reactions occurred with some patients and very large doses of MSG (>3 g) were required in the majority of cases.¹⁰⁷ Moreover, other clinical investigators have failed to identify any MSG-sensitive asthmatics in clinical trials.¹⁵⁰⁻¹⁵³ However, the selection of patients in these trials may have diminished the likelihood of finding reactors, since mild

asthmatics were used for the most part. Thus, further clinical studies will be needed to confirm or refute the role of MSG in the provocation of asthma. However, it can certainly be concluded at this point that MSG-induced asthma, if it exists, is an extremely rare condition.

Tartrazine-Induced Asthma and Urticaria

Tartrazine, also known as FD&C Yellow #5, is a certified, artificial colorant used in foods, drugs, and cosmetics in the U.S. and other countries. In 1959, Lockey¹⁵⁴ presented the first anecdotal evidence of tartrazine-induced urticaria (hives) after the ingestion of yellow-colored drugs. Later, clinical evidence was presented that seemed to link asthma in a small percentage of aspirin-intolerant asthmatics with provocation by tartrazine as well.¹⁵⁵ Mounting evidence, mostly from anecdotal reports or non-blinded or open challenges with tartrazine, led the FDA to require the specific labeling of FD&C Yellow #5 on food products in 1979.¹⁵⁶ Today, the failure to properly declare FD&C Yellow #5 on food labels is one of the most frequent causes of food recalls in the U.S.

Since the FDA action in 1979, many additional clinical trials have been conducted on tartrazine-induced asthma and urticaria; these trials have recently been critically reviewed.⁶ Many of these trials were flawed in one respect or another, such as the failure to use double-blind, placebo-controlled trial designs or the withdrawal of key medications just before initiation of the trial.⁶ The trials that were conducted in double-blind, placebo-controlled fashion represent a strong test of the hypothesis that tartrazine is involved in the causation of asthma and urticaria. The results of the double-blind oral challenges with tartrazine have indicated that tartrazine plays virtually *no* role in either asthma or urticaria.^{6,157,158} With respect to asthma, the most carefully controlled double-blind, placebo-controlled trials with tartrazine have failed to identify any tartrazine-sensitive subjects even when the patient population was comprised of aspirin-intolerant asthmatics.^{157,158} The clinical studies that have implicated tartrazine in the causation of asthma have often been complicated by withholding bronchodilator drugs from patients with unstable, chronic airway disease.^{6,157,158} Stevenson et al.¹⁵⁷ concluded that tartrazine does not induce asthma and that the early reports were simply the exacerbations of asthma in patients with unstable airways who had been deprived of their bronchodilators.

With regard to urticaria, a very small number of tartrazine-sensitive individuals have been identified in double-blind, placebo-controlled trials.^{6,157} As was the case with the studies on the role of tartrazine in asthma, most of the clinical studies of tartrazine on urticarial patients are complicated by the failure to blind the challenge, a lack of placebo controls, and/or the withholding of antihistamines. The withholding of antihistamines is an especially significant clinical design element because such drugs are essential for the control of symptoms in patients with chronic urticaria.^{6,157}

Tartrazine is, at worst, a cause of urticaria in only a few of the many individuals with this symptom.^{1,6,157}

Other Food Additives in Chronic Urticaria and Asthma

Chronic urticaria is a disease with few known causes. Most chronic urticaria patients must take antihistamines on a daily basis to control the urticarial lesions. The clinical study of causative factors in chronic urticaria is complicated by the chronic and episodic nature of the illness. Since the hives appear on an episodic basis, careful placebo control of clinical studies is essential to document that any lesions are the result of the challenge material and not occurring on the basis of chance. As noted above in the discussion on tartrazine, the withdrawal of antihistamines can really complicate the interpretation of these clinical challenge studies. When a chronic medication such as the antihistamines are removed before challenge, any urticarial lesions could be the result of the challenge material or breakthrough urticaria from the withdrawal of the medication. However, if the patient is maintained on the antihistamines, it can be argued that a much higher dose of the challenge material would be needed to elicit urticarial lesions because the challenge material would have to overwhelm the antihistamine in the system. Few clinical trials conducted on food additives have succeeded in controlling these important design elements. Thus, the results of these trials can be questioned.⁶

In the search for causative agents in chronic urticaria, considerable attention has been focused on food additives: tartrazine, sunset yellow (FD&C Yellow #6), sodium benzoate, benzoic acid, and the parabens, and BHA, and BHT. Numerous clinicians have concluded that these additives play a causative role in chronic urticaria,⁶ but as noted above, the study designs have been flawed in most cases.

Asthma is also a chronic, episodic illness. Asthmatic individuals must take medications on a daily basis to control the illness and maintain good respiratory function. Several ingested substances, such as aspirin and many of the common allergic foods, are well documented to provoke asthmatic reactions in certain individuals within the overall asthmatic population. However, the role of food additives in asthma is far less clear. As noted above for tartrazine and MSG, many of the studies that have been conducted on food additives and their role in asthma did not employ proper placebo controls, were not done in double-blind fashion, and/or did not allow the patients to maintain critical medications. Because asthma is a chronic condition, withdrawal of medication could easily lead to false-positive results. Although asthma also has been linked to certain other food additives beyond tartrazine and MSG, the relationship of these additives to exacerbation of asthma is not well proved.⁶

The evidence implicating various food additives in chronic urticaria and asthma is suspect. Changes in the use and regulation of any of these food additives on the basis of this type of evidence are unwarranted.¹

Summary

Food allergies and intolerances are adverse reactions that plague a large number of people. Although the symptoms of these allergies and intolerances are manifested in only a small segment of the total population, the public can view such illnesses as a major health concern. The public and even some healthcare professionals fail to distinguish between the different types of illnesses that fall within this general category. Food allergies and intolerances are an increasingly important concern to consumers and food manufacturers alike. Allergic individuals must alter their lifestyles on a continuing basis to avoid the offending food, and the food industry must continue to be alert to the needs of these consumers by providing accurate and complete labeling. Manufacturers also must be aware that cross-contact between allergenic foods and other foods within the manufacturing environment may cause residues of the allergenic food to be present in the other food but not declared on the ingredient statement. Cross-contact, improper use of rework, and accidental mislabeling can result in serious, life-threatening allergic reactions among sensitive consumers.

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2

Dietary Estrogens and Antiestrogens

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Introduction

Over the past 50 years dietary estrogens have played an important role in human health and animal agriculture. In the late 1940s compounds present in subterranean clover were shown to alter reproduction in sheep. This reproductive failure had a severe economic impact on the sheep industry in Australia.¹ However the compound responsible for the reproductive failure was not identified for over a decade. In 1953, genistein, a compound present in legumes, was shown to enhance uterine weight in rodents and, thus, was classified as a phytoestrogen (plant estrogen). In the mid-1960s several phytoestrogens were discovered because of their importance in reducing fertility in sheep grazing on subterranean clover in Australia. Not only did these sheep have fertility problems, virgin ewes and wethers expressed milk, which also is indicative of consumption of potent estrogenic compounds. In the 1970s phytoestrogens were classified as a naturally occurring toxicant in food by the National Academy of Science.² It is interesting to note that in the 1990s many of the bioactive components in foods, which were previously listed as toxicants in food, are being promoted for their potential health

benefits. The phytoestrogens, specifically the soy estrogenic isoflavone genistein, has been one of the most studied phytochemicals in food over the past 5 years. It is important to note that estrogen-like compounds in food have the potential to prevent or alter many chronic diseases such as cardiovascular disease, cancer, and osteoporosis.

Estradiol, the female hormone, plays a critical role in function of the reproductive system. Additionally, estrogens are critical factors in such biological processes as cellular development, proliferation, and differentiation. Although estrogens are essential to reproductive function, it is believed that estrogens play a critical role in development of several human, hormone-dependent cancers, such as breast and uterine cancer.³ The biological actions of estrogen, as well as that of estrogen agonists, are mediated by a soluble protein, the estrogen receptor (ER), to which estrogen binds with high affinity.⁴⁻¹¹ Once the ligand binds to the ER, the liganded complex undergoes transformation and a chaperone protein (Heat Shock Protein 90) dissociates. This dissociation exposes the DNA-binding domain and allows the liganded ER to form a homodimer. These homodimers bind with high affinity to specific DNA sequences, known as estrogen responsive enhancers (ERE), which are upstream of estrogen responsive genes. The liganded receptor complex bound to the ERE initiates transcription of estrogen responsive genes. Estrogen-like compounds that bind to the ER and stimulate an estrogenic response are considered estrogen agonists. Those chemicals that bind to the ER and block the estrogenic response are considered estrogen antagonists. Another class are those compounds that do not bind to the ER, but inhibit an estrogenic response such as estrogen-dependent gene expression or cell growth. It is likely that these compounds mediate their effect post-receptor binding but pre-transcriptionally. Since these compounds do not mediate the effects by blocking estrogen binding to the ER, these compounds are considered antiestrogens. The emphasis of this chapter will be on the biological activities of phytoestrogens which are found in foods and feedstuffs consumed by humans, livestock, and wildlife. We will discuss phytoestrogens that can act as estrogen agonists and antiestrogens. Collectively these estrogen-like compounds will be referred to as dietary estrogens and antiestrogens.

Affinity of estradiol (E) for the ER is high, with K_D of ~ 0.1 nm. There are numerous dietary E ligands, all of which have chemical structures containing opposing hydroxys¹² on phenolic rings (Figure 2.1). These dietary ER ligands have affinity for the ER that are 100 to 1000 times lower than estradiol.^{13,14}

Humans and animals are exposed to environmental estrogens that give rise to varying biological effects depending on the dosage and the specific chemical. Sources of these estrogens include estrogenic drugs and industrial compounds, such as pesticides, nonionic surfactants, and chemical precursors used in the manufacturing of plastics.¹⁵⁻¹⁹ A subclass of the environmental estrogens are the dietary estrogens, which have been identified in several plants. These compounds are classified as phytoestrogens.^{13,20,21}

Dietary estrogens comprise a diverse group of compounds with varied chemical structure and biological activities. In this chapter we will focus on

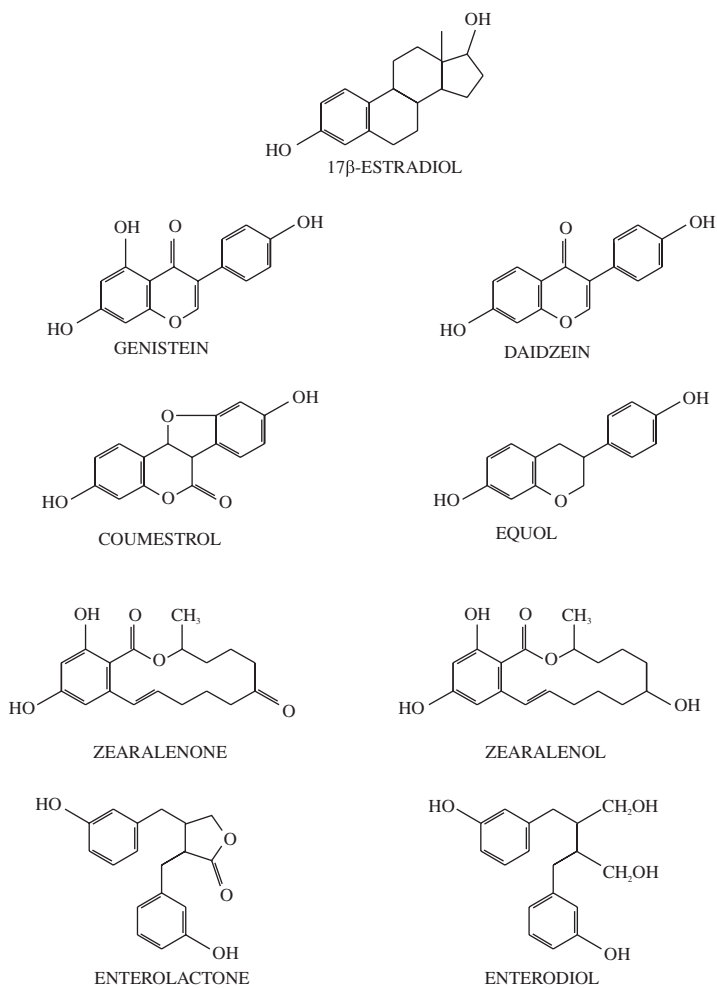


FIGURE 2.1
Structures of dietary estrogens.

several dietary estrogens and antiestrogens. These include lignans, zearalene, coumestans, isoflavones, and indole-3-carbinol.

Dietary Estrogens and Antiestrogens

Lignans

Lignans are found in many plant foods and make up a large portion of the known dietary phytoestrogens. Precursor compounds that form mammalian

lignans have been identified in grains, seeds, berries, and nuts.²² In the case of grains, these compounds are located in the outer fiber-containing region called the aleurone layer.²³ The first mammalian lignans were identified in vervet monkeys and human females as unknown, cyclically occurring compounds. Later, independent of each other, two groups identified these compounds as enterlactone and enterodiol.^{24,25} These two lignans have since become the most well known and researched compounds of their type.

The metabolism of mammalian lignans is thought to be dependent on the activity of the animal's gut microflora. Precursor compounds from plant sources enter the digestive system of the mammal where, in the lower gut, host microbes metabolize these precursor compounds into their respective lignans. Matairesinol and secoisolariciresinol are metabolized in the lower gut into enterlactone and enterodiol, respectively.²⁶ Setchell et al.²⁷ and Adlercreutz²³ proved the dependency of this metabolic pathway and microbe involvement by detecting a significant decrease in urine enterlactone and enterodiol concentration when they disrupted the normal gut flora with antibiotics. In many cases there was almost complete elimination of these compounds upon disruption of the gut flora.²¹

The biological effects lignans have in mammals are very similar to those of the other dietary estrogens. Also, as with the other compounds discussed in this chapter, lignans carry out their function by acting as weak estrogens. Sathyamoorthy et al.²⁸ demonstrated that enterolactone stimulated estrogen responsive, MCF-7 breast cancer cells to produce pS2. This result is a clear indication of the estrogenic activity of these compounds. Also, lignans are believed to have numerous other biological effects including: anticarcinogenic, antiviral, bacteriostatic, and fungistatic activities.^{29,30} The relationship of mammalian lignans to several different forms of cancer has been well researched in recent years. Hirano et al.³¹ demonstrated that lignans suppress mitogen-induced proliferation of human peripheral blood lymphocytes. It also has been suggested that lignans may play a role in decreasing the incidence of breast cancer by competing with estradiol for type II estrogen-binding sites and by affecting uptake and metabolism of sex hormones through regulation of synthesis of plasma sex hormone-binding globulin in the liver.²³

Zearalenone

Another class of dietary estrogens that occur in foodstuffs are derivatives of resorcylic acid lactones which are produced by numerous species of *Fusarium* fungi growing, under favorable conditions, on grains prior to and after harvest.³² The most prevalent of these compounds is zearalenone, [6-(10-hydroxy-6-oxo-trans-1-undecenyl)- β -resorcylic acid lactone], and its derivatives. This estrogen-like compound was first isolated from corn infected with *Fusarium*.³³ Since that time, over 300 derivatives of zearalenone have been isolated.³⁴ *Fusarium* fungi infect numerous agriculturally important crops, including cereal grains that make up a significant part of the human diet. The

grains shown to be most affected by this mycotoxin include corn, wheat, barley, sorghum, and hay.³⁴⁻³⁶ Numerous *Fusarium* fungi produce this mycotoxin including *Fusarium roseum* and *F. moniliforme* which invade kernels of corn and *F. saubinetii* that is known to infect barley.^{36,37} High concentrations of zearalenone are found in grain products most often as a result of an infected grain being stored, allowing the fungi to thrive. However, small amounts of zearalenone have been identified in fresh cut grains.³⁸

Zearalenone was first characterized as having estrogenic activity by Mirocha et al. when the compound was shown to increase uterine weight in rats.^{36,37} It was later discovered, that as is the case with many of the other dietary estrogens, zearalenone acts via the estrogen receptor. In 1978, Boyd and Wittliff³⁹ performed competitive binding assays proving that zearalenone does, in fact, bind to the estrogen receptor.³⁹ Since that time, binding assays have been performed with many of the derivatives of zearalenone⁴⁰ and one derivative (low melting point zearalenol) has been identified as having the highest affinity for the estrogen receptor of any of the known dietary estrogens. These *in vitro* studies also concluded that, like the other dietary estrogens examined, zearalenone caused growth of estrogen-dependent MCF-7 human breast cancer cells.¹³ More recent studies have proved that zearalenone has detrimental effects on the reproductive efficiency of swine and mink. In these studies, mink receiving zearalenone mated, but only 25% whelped.⁴¹ In swine, hyperestrogenism generally appears when corn is contaminated with zearalenone at 1 ppm, but it can occur at doses as low as 0.1 ppm.⁴² Zearalenone has proved to be a potent environmental estrogen and as a result it is used in beef cattle production as a growth-promoting supplement.

Coumestans

Another class of dietary estrogens is the coumestans. These phytoestrogens are found in many vegetables and forages including soybeans,⁴³⁻⁴⁶ alfalfa sprouts,^{44,47-48} large lima beans, mung bean sprouts, round split peas, red bean seeds, and clover sprouts.⁴⁷ Coumestrol is the most commonly studied coumestan and it is the predominant form found in alfalfa and other forages.⁴⁹ However, the highest concentration of coumestrol has been measured in soybeans.⁵⁰

As with the other dietary estrogens, coumestrol exhibits estrogenic activity through interaction with the estrogen receptor. Coumestrol has been shown to have other biological activities related to lipid and calcium metabolism. In studies performed by Dodge et al.,⁵¹ coumestrol, genistein, and zeranol were all shown to lower serum cholesterol in ovariectomized rats. Furthermore, coumestrol and zeranol prevented ovariectomy-induced bone loss.⁵¹ In a similar study, Draper et al.⁵² went on to demonstrate that coumestrol reduced urine calcium excretion and bone resorption markers pyridinoline and deoxypyridinoline after one week of treatment.

Genistein

The phytoestrogen genistein is an isoflavone with low affinity to ER, which is present in high concentrations (1 to 2 mg/g) in soybeans and soy products. Genistein is known to reduce reproductive performance of sheep grazing on subterranean clover, rabbits fed soybean hay, captive cheetahs fed diets containing soybean protein, and desert quail feeding on desert brush.^{53,54} All of these diets consumed by the various species contained substantial amounts of genistein. Additionally, a decrease in reproductive performance also was observed in female rats fed either a soy-based or a genistein-supplemented diet.⁵⁵ Estrogenic activity from components in these diets may prevent normal estrus in these animals and is a likely mechanism by which these diets alter reproduction. Human diets, containing 60 g/d of soy products (providing 45 mg/d of isoflavones) increased the length of menstrual cycles in women,⁵⁶ suggesting that dietary phytoestrogens also are capable of producing a biological response in humans.

Genistein and other isoflavones exist in plants as the glycoside conjugates. In fact, studies in the 1970s revealed that 99% of the isoflavonoid compounds in soy are present as glycosides.⁵⁷ It is generally accepted that these dietary glycosides must be hydrolyzed to aglycones by gut microflora before absorption can occur. Individuals consuming soy milk, in three different amounts, had a dose-dependent increase in plasma genistein concentration ranging from 0.74 to 2.15 μM .⁵⁸ In another study, humans weighing 61.9 kg consumed soy drinks that provided isoflavones at 30.9 $\mu\text{mol/kg}$ body weight. This is a very high dose that provides approximately 500 mg isoflavones per day. Blood concentrations of genistein and daidzein in the individuals consuming these large amounts of isoflavones were approximately 6 μM each.⁵⁹ Soy protein (60 g) containing 45 mg of isoflavones (20 mg genistein) given daily to women for 1 month significantly increased follicular phase length, delayed menstruation, or both.⁵⁶ These results indicate that dietary soy is estrogenic in adult women.

Genistein binds to the ER with an affinity 50 to 1000 times less than that of estradiol.²¹ We conducted competitive-binding experiments with rat uterine cytosol and confirmed that genistein binds to the ER with an affinity 1/50 to 1/100 that of estradiol.⁶⁰ As with several of the other dietary estrogens presented here, there are numerous reports that genistein can act as an agonist in ovariectomized animals as indicated by increases in uterine weight and mammary development.¹⁶ Maturation of the mammary gland was observed in pubertal Sprague-Dawley rats administered subcutaneous genistein at 500 $\mu\text{g/g}$ body weight.⁶¹ These authors hypothesized that an ER-mediated mechanism promoted mammary epithelial cell proliferation and enhanced mammary gland maturation. In studies using ovariectomized female rats, dietary genistein at 750 ppm can enhance mammary gland development.⁶⁰ Feeding genistein or estradiol to ovariectomized rats led to an increase in serum prolactin levels,⁶⁰ which also suggests estrogenic action of genistein on the hypo-

thalamus and pituitary gland *in vivo*. Dietary genistein induced expression of the estrogen-responsive gene *c-fos* in uterine RNA isolated from ovariectomized rats. Further, genistein can act as an estrogen agonist to stimulate growth of cultured human breast cancer (MCF-7) cells at concentrations as low as 200 nm.^{13, 62}

The estrogenic and antiproliferative activities of genistein present an apparent paradox. Epidemiological data suggest that diets rich in soy products, which contain high levels of phytoestrogens, are associated with a lower incidence of breast cancer.^{63,64} There also are numerous reports that genistein inhibits growth of cultured human cancer cells.⁶⁵⁻⁶⁷ We conducted experiments to resolve this apparent paradox by using both ER-negative (MDA-MB-231) and ER-positive (MCF-7) human breast cancer cells to evaluate the growth-inhibitory effect of genistein on cultured breast cancer cells. At concentrations above 20 μm , we observed a dose-dependent decrease in growth of both MDA-MB-231 and MCF-7 cells.⁶⁸ This inhibitory effect is independent of ER because it is observed in both ER-positive and ER-negative cells.

Blood concentrations of genistein reported in humans consuming soy-containing diets are relatively low. To determine whether lower levels of genistein could induce an estrogenic response *in vitro* in ER-positive cells, we⁶⁴ and others⁶⁹ conducted dose-response studies in MCF-7 cells in which the concentration of genistein (in charcoal-stripped media) ranged from 10 nm to 100 μm . Results from these studies support the dual threshold hypothesis: when genistein is administered at low concentrations, a dose-dependent increase in MCF-7 cell growth is observed, with maximal growth occurring at 1 μm , whereas concentrations above 20 μm lead to a dose-dependent inhibition of growth. Genistein concentrations of 1 μm and 10 μm were also evaluated in MCF-7 cells by determining changes in expression of *pS2* mRNA by Northern blot analysis.^{64,70,71} Expression of *pS2* is an established marker of estrogen-dependent gene expression.⁷¹

Indole-3-Carbinol and Metabolites as Antiestrogens

Indole-3-carbinol (I3C) is a secondary plant metabolite found in cruciferous vegetables, such as cabbage, Brussels sprouts, and broccoli. Consumption of these vegetables has been associated with decreased risk for cancer in humans.⁷² I3C has been evaluated in human clinical trials as a potential chemopreventive agent against breast and ovarian cancers.⁷³ It has been known that I3C suppresses the growth of both estrogen-dependent and estrogen-independent human breast cancer cell lines.⁷⁴ Dietary I3C has been reported as inhibiting spontaneous tumorigenesis and tumor induction by direct-acting carcinogens⁷⁴⁻⁷⁸ in various estrogen-responsive target organs, including mammary tissue,⁷⁹⁻⁸¹ liver,^{82,83} endometrium,⁸⁴ lung,⁸⁵⁻⁸⁸ and other target organs^{89,90} in various animal models. However, there are other studies that demonstrated stimulation of tumor promotion by I3C *in vivo*.^{91,92} When

I3C was administered orally to animals, it induced chemopreventive effects against a wide variety of carcinogens. Chemoprevention properties of dietary I3C in most of the models are evident when it is administered with the carcinogens or prior to initiation. There are reports that, when given after initiation (promotion-progression stage), I3C can enhance carcinogenesis.^{75,77,83} There also is some evidence that I3C may be mutagenic when administered in the diet along with nitrites.⁹⁵ Earlier studies^{77,83} documented the ability of I3C to promote aflatoxin B₁-initiated hepatocarcinogenesis at relatively high dietary levels (1000 ppm).

The chemopreventive properties of I3C are proposed to occur through several possible mechanisms, including the alteration of estrogen metabolism.^{81,96-99} I3C is known to inhibit glutathione S-transferase-mediated steroid binding activity,¹⁰⁰ act as a scavenger of free radicals,¹⁰¹ modulate the activity of multidrug resistance,¹⁰² and alter the expression of various phase I and II drug metabolizing enzymes^{99,103-106} contributing to detoxification of carcinogenic compounds. Dietary intake of I3C has antiestrogenic as well as estrogenic activities¹⁰⁷ and also binds to the arylhydrocarbon receptor (AhR).^{99,108,109} I3C is known to be an inducer of intestinal and hepatic xenobiotic metabolizing enzyme activities.^{105,110-112} Although I3C induces several phase II enzymes,¹¹² the indoles induce multiple families of cytochrome P450-dependent isozymes. I3C induces CYP1A family (e.g., TCDD), CYP2B family (e.g., phenobarbital), and CYP2A family (e.g., dexamethasone) isozymes.^{79,103,106} Grubbs et al.⁷⁹ report that after 15 weeks of exposure to I3C, the livers of Sprague-Dawley rats continue to have higher activities of both phase I and II enzymes. I3C acts as an antiinitiator as well as a promotor of carcinogenesis, and increases in activities of cytochrome P450-dependent monooxygenases and in phase II enzymes (conjugation).¹⁰⁹ Many of the aromatic hydrocarbon receptor (AhR) agonists are environmental toxicants.¹¹³ These researchers found that I3C was an AhR agonist with weak binding affinity and an inducer of monooxygenase activity *in vivo*. It has been reported that I3C binds to the same AhR site as a potent environmental pollutant, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and other arylhydrocarbons.¹⁰⁸ I3C exhibited antiestrogenic activities at concentrations that did not induce ethoxyresorufin *O*-deethylase activity (EROD). An additional mechanism for the chemopreventive effects of I3C in estrogen-responsive tissues is a modulation of cytochrome P450-dependent estradiol metabolism. Estradiol is metabolized via two competing pathways. Hydroxylation at C-2 yields 2-hydroxyestrone; hydroxylation at C-16 α yields 16 α -hydroxyestrone which is reduced to form estriol.¹¹⁴⁻¹¹⁶ 16 α -hydroxyestrone covalently binds to the estrogen receptor, decreases its degradation and has estrogenic effects. Increased estradiol-16 α -hydroxyestrone has been associated with increased risk for breast cancer in women¹¹⁴ and mice,¹¹⁶ and 16 α -hydroxyestrone has been reported to be genotoxic in mammary cells.¹¹⁷ Attempts to decrease estradiol 16-hydroxylation have not been successful. Thus, their studies focused on increasing

the alternate 2-hydroxylation pathway of estradiol.^{96,97} In rodents, I3C induces CYP1A1/CYP1A2-dependent estradiol 2-hydroxylase activity and the formation of 2-hydroxyestradiol/2-hydroxyestrone has been associated with protection from estrogen-induced mammary, endometrial, and other tumors development.^{81,84,116,118-121} Toshifuma et al.¹²¹ reported that I3C increased 2-hydroxylation in estrogen-dependent human breast cancer cells but has little effect on 16 α -hydroxylation. In human breast cancer cells, induction of estradiol 2-hydroxylase activity is a CYP1A1-dependent response, and several studies have reported induction of this activity by AhR agonists, I3C.^{81,122,123} However, I3C induced CYP1A1 in MCF-7 cells only at high concentrations (500 μ M),¹²² and induced CYP1A1 mRNA only at concentrations \geq 100 μ M.¹²³ In contrast, McDougal et al.¹²⁴ reported that after 48 h incubation of MCF-7 cells with 10 μ M, I3C resulted in a more than fourfold increase of estradiol 2-hydroxylase activity.¹²⁵ Therefore, induction of estradiol 2-hydroxylase by 10 μ M, I3C may be CYP1A1-independent or may involve *in vitro* activation of P450 isoenzymes in MCF-7 cells.

Many of these enzyme-inducing effects are due to the condensation products of I3C produced upon contact with gastric acid.^{104,109,126} Some of these oligomers have been shown to interact with the AhR. This may be involved in induction of the CYP1A family which is thought to be primarily responsible for the inactivation of estradiol in breast tumor cells and other drug metabolizing enzymes. Increased estrogen conjugation and excretion via induction of phase II enzymes could result in these effects.¹²⁷ A major condensation product, the dimer 3,3'-diinloylmethane is an effective inhibitor *in vitro* of cytochrome P450.^{109,128,129} Oligomers of I3C enhance estradiol 2-hydroxylation in the human through the CYP1A family.⁹⁷ Indolo[3,2-b]carbazole (ICZ) is one of the acid-condensation products of I3C that is produced *in vivo* and *in vitro*.¹⁰⁹ ICZ binds to both the estrogen receptor and AhR. ICZ decreases estrogen receptor levels in breast cancer cells in culture.¹⁰⁷ ICZ competitively binds to the AhR, induces P450 (CYP1A1/2) gene expression, and transforms the cytosolic AhR to a form that binds to a dioxin or xenobiotic responsive element.^{99,108,109,130,131} ICZ is the most potent AhR agonist among condensation products of I3C. Like I3C, ICZ is also similar to the TCDD. Both compounds exhibit antiestrogenic activities including inhibition of estrogen-dependent growth of cultured breast tumor cells.¹⁰⁷ And, both substances induce CYP1A1 activity *in vivo* and *in vitro*.¹⁰⁹ ICZ is not only an inducer of the CYP1A1 gene, but also a potent and selective inhibitor of CYP1A1 enzyme activity.¹³² ICZ inhibited estradiol-induced cell proliferation at concentrations above 10 nM.¹³² At lower dietary I3C levels (<1000 ppm), estrogenic activities of I3C acid derivatives promote hepatocarcinogenesis in rainbow trout. Much stronger promotion was induced at high dietary I3C levels (\geq 1000 ppm), at which levels of CYP1A also were induced.¹³³ I3C and related condensation products also have been characterized as AhR agonists and exhibit structure-dependent binding affinity for the AhR.^{108,109,131}

Summary

The dietary estrogens and antiestrogens discussed in this chapter are found in fairly high concentrations in many foods that are routinely consumed daily. For example, lignans are found in foods that are high in fiber. Genistein is found in soy foods. Zearalenone is found in moldy corn which has been contaminated with *Fusarium*. Many of the dietary estrogens are present in high concentrations; for example, genistein has been found in foods ranging from 0.8 to 1.2 mg/g of food.

It is important to note regarding the dietary estrogens that although these chemicals bind weakly to the ER, they are in high concentrations in foods. For example, genistein has a low affinity relative to estradiol for binding to the ER. However, genistein is in concentrations of 1 mg/g of soy food (dry matter). Thus, it is possible for an individual to easily consume 50 mg in one day. This dietary consumption of genistein would produce a circulating plasma concentration in excess of 200 nm (aglucone form). This plasma concentration is 200 times higher than the concentration of estradiol in a premenopausal woman. Even though genistein is a weak agonist, the concentration is high enough to elicit an estrogenic response. Regarding the dietary antiestrogens, many of the chemicals discussed are found in *Brassica* vegetables, such as cabbage, Brussel sprouts, and broccoli.

In the past decade, many of the estrogens and antiestrogens discussed in this chapter have been shown to be protective against several types of cancers. Because of the potential beneficial effects of these chemicals, extracts containing high concentrations of these bioactive chemicals are now available as dietary supplements or for use as food additives. Additionally, plant geneticists are now selecting cultivars which are high in some of these chemicals. For example, soybeans containing high amounts of isoflavones are currently under investigation. Additionally, broccoli cultivars with high concentration of glucobrassicins also are available. At some point, the content of these chemicals will become too high and become a chemical safety concern. Currently it is believed that consumption of levels of these bioactive chemicals is safe as long as the levels do not exceed that found in food. If we increase the content of these chemicals by selection or genetic engineering to five times the average levels, can we still assume this is safe? Another important point that must be made is regarding subpopulations. If we develop a food with high concentrations of isoflavones as a "natural" mechanism to consume estrogen-like chemicals for prevention of bone mineral loss, is this same product safe for another subpopulation with an estrogen-dependent cancer? The safety of consumption of high amounts of dietary estrogens and antiestrogens remains an important unanswered question.

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3

Nonnutrient Antitoxicants in Foods

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Introduction

In the U.S., recent assessments of human cancer risks due to exposure to environmental toxicants entering the food supply have shown clearly that industrial, agricultural, and other manufactured environmental contaminants constitute a nearly negligible risk, whereas naturally occurring toxicants in the food supply are of at least minor significance to human cancer risk.¹ What these assessments do not take into account is the coexistence in the food supply, alongside foodborne toxicants, of numerous naturally occurring food

components that may act as antitoxicants. A more realistic assessment of cancer and other health risks due to toxicants in the food supply must eventually factor in these health protective substances. But many, if not most of these potential antitoxicants, are incompletely characterized. Food composition is not well understood, partly because food has not been well recognized as a source of much more than the obligatory nutrients, and because the chemical analysis of lifeforms is highly complex and, therefore, extremely challenging. It is reasonably likely that many potentially antitoxic food components remain to be identified chemically, let alone characterized biologically. Furthermore, experimental models that may be most useful in predicting both short- and long-term human health effects of such substances are only in the embryonic stages of development. Application of such findings, which should revolutionize healthcare by focusing upon the prevention of diseases rather than their treatment, exist only as a “twinkle in the eye” of imaginative scientists, at present.

A more fundamental scientific problem that must be addressed along with developing appropriate models, is the characterization of mechanisms of action of potential antitoxicants. Most of the food components studied to date as antitoxicants seem to have multiple mechanisms of action: scientists are like the blind men and the elephant — each scientist has a different perspective on what mechanism to study and a different set of assays with which to study the “elephant” of foodborne antitoxicants. What results is a collage of biologically antitoxic effects against a wide variety of toxic substances, with an incomplete picture of the whole. Understanding the interrelation of effects of antitoxic food components, especially in model systems which can be well justified as relevant to human exposures to toxicants will be most important to human health. Understanding the mechanisms of action of antitoxicants also will produce an understanding of toxicant action and of basic life processes that may be applied to the sustenance not only of human life but of all other lifeforms.

Antitoxicants act by two major mechanisms. They either limit the access of the toxicant to its site of action or either directly or indirectly block the effects of the toxicant. Access of toxicants to their active sites may be limited by sequestering the toxicant, diluting it, and preventing its absorption into the body, or by enzymatically transforming the toxicant into a less reactive or nontoxic product that can be readily eliminated from the body. Toxicant effects can be blocked by inhibiting the production of key tissue-damaging substances that toxicants stimulate, such as reactive oxygen species or prostaglandins. Toxicant effects can be blocked by substances which act as analogs to either toxicants or toxicant-stimulated products, such as estrogens or other hormones. Toxicant effects also may be blocked by post-translational modification of toxicant-induced proteins, e.g., modification of ras protein anchorage in cell membranes, or inhibition of toxicant-induced enzymes. Foodborne antitoxicants can act by each of these mechanisms, in some cases, multifunctionally.

Antitoxicants and Chemical Carcinogenesis

Our understanding of the mechanisms of action of antitoxicants is proceeding from the outside in. The foundation of our knowledge comes from animal feeding studies of effects of foods on chemical toxicity, especially of chemical carcinogenesis. From foods or food extracts, many antitoxicants have been purified and their effects further studied in those same animal models, and also at cellular and molecular levels in many cases.

Chemical carcinogenesis studies may be criticized with respect to their relevance to human exposures to toxicants because many of the important model carcinogens tested are not commonly found in any significant quantity in the human diet or elsewhere in the human environment (e.g., acetamidofluorene or phorbol esters). But, what has been learned over more than 5 decades of intensive carcinogenesis research strongly supports the existence of fundamental mechanisms occurring in discernible stages of cancer development whether the cancer occurs in a laboratory animal or in the human population.

Cancer development is initiated by heritable genetic alterations, produced by the formation of DNA-carcinogen adducts in interaction with endogenous DNA repair mechanisms.² There are many carcinogens studied in experimental animals which cause similar damage in human tissues, either in tissue culture or in studies where human tissues are examined after natural exposures to such carcinogens (e.g., nitrosamines, benzo(a)pyrene, heterocyclic amines).³ Anti-initiating antitoxic effects have been demonstrated for several foods and food components. These antitoxic agents act to limit the access of initiating toxicants to their sites of action on DNA.

Antitoxicants as Sequestrants and Diluents

Some anti-initiators such as dietary fibers sequester toxicants, physically and/or chemically, preventing their absorption from the gastrointestinal tract.^{4,5} Dietary fibers and fiber-associated components may decrease absorption of heterocyclic amines⁶ and nitrites.⁷ Wheat bran decreased absorption of aflatoxin B₁ in male rats by more than 20%, based upon urinary excretion data, and feeding 15% wheat bran, substituted for maize starch, from 3 to 16 weeks of age decreased signs of aflatoxin toxicity and carcinogenicity when evaluated at 109 weeks of age.⁸ Insoluble fibers can sequester hydrophobic substances, but soluble fibers may oppose this effect by solubilizing those substances,⁹ supporting the need for careful attention to dietary fiber composition in studies of antitoxic effects of dietary fibers. The fiber-associated polyphenols, quercetin and chlorogenic acid, decrease absorption of

benzo(a)pyrene by 20% in adult male Sprague-Dawley rats.¹⁰ Another fiber-associated component, phytic acid, may sequester carcinogenic and otherwise toxic minerals such as lead. Sodium phytate salts, but not magnesium phytate or phytic acid, increase N-butyl-N-(4-hydroxybutyl)nitrosamine-initiated urinary bladder cancer in male F344/N rats when fed at 2% of the diet.¹¹ This suggests that sodium phytate should not be introduced into the diet as a food additive, but that amounts of phytic acid as found in foods may exert some beneficial effects.

Not only are anti-initiating effects accomplished by dietary fibers and associated components, but general antitoxic effects of these food components also are seen. Weanling male Sprague-Dawley rats fed 10% psyllium seed or carrot powder show no toxicity from consuming 5% FD&C Yellow #6 for 14 days, whereas rats fed a basal diet and Yellow #6 show 70% lower body weights. Cellulose (2.5 to 10%), 10% wheat bran, or 10% alfalfa meal also significantly lessens toxic effects of the food color.¹² Alfalfa feeding at 5 to 20% of the diet of rats dosed with T-2 toxin, a tricothecene mycotoxin, protects the animals from feed refusal and lowered body weight gain, probably by limiting absorption of the toxicant.¹³ Dietary fiber effects on toxicants, in concert with effects of a high calcium, low fat diet also has been investigated in humans.¹⁴ For 5 days, men were fed a diet containing 148 g fat, 6 g dietary fiber, and 324 mg calcium, or a diet containing 22 g fat, 42 g dietary fiber, and 1900 mg calcium. Compared with an *ad libitum* diet, fecal water bile acid concentration was reduced by nearly 50%, and the ability of fecal water to lyse erythrocytes *in vitro* also was reduced by 50%. This study suggests an important role for dilution of toxic factors (e.g., bile acids) by dietary fiber. In summary, studies of dietary fiber and associated components generally support the ability of such components to limit access of toxicants to their sites of action.

Antitoxicants Alter Toxicant Biotransformation and Suppress the Initiation Phase of Carcinogenesis

Other antitoxicants induce biotransformation enzymes that divert initiating agents from their proximate carcinogenic forms. Anti-initiating biotransformation inducers may limit the ability of the initiator to act as an electrophile by forming a covalent bond with a conjugant (e.g., glutathione, glucuronic acid, or sulfate) at the electrophilic site, thus preventing DNA-carcinogen adduct formation. These conjugation reactions do not always create less electrophilic species. For example, safrole and acetamidofluorene actually have greater electrophilicity and are in their most reactive proximate carcinogenic forms as sulfate conjugates.¹⁵ Likewise, glutathione conjugation activates certain halogenated hydrocarbons.¹⁶ There are many initiators, such as aflatoxin B₁ and benzo(a)pyrene, that are clearly detoxified by conjugation reactions. Induction of enzymes catalyzing conjugation reactions suppresses initiation

by such agents. Induction of conjugation reactions also alters the initiator's solubility, which can shunt the transformed initiator toward the cytosol, the blood plasma and the urine, facilitating excretion of the initiator, limiting the ability of the initiator to cross membranes and, therefore, also limiting initiator access to DNA.

Identification of anti-initiating antioxidants may be done *in vivo* in animal models of carcinogenesis, examining the effect of coadministration of suspected antioxidants during the initiation phase on later cancer development. For example, rats fed cabbage containing 1 ppm aflatoxin B₁ (AFB₁) for 26 weeks had fewer than half the number of tumors per liver as did rats fed AFB₁ alone.¹⁷ Garlic powder fed to rats containing 7,12-dimethylbenz(a)anthracene (DMBA) for 24 weeks suppressed mammary tumorigenesis in rats.¹⁸

Compounds identified in these foods also inhibit initiation. Wattenberg has reviewed the ability of isothiocyanates from cruciferous vegetables to inhibit initiation by DMBA, N-nitrosodiethylamine (NDEA) and benzo(a)pyrene (BP). D-limonene from citrus inhibits NDEA initiation and indoles from cruciferous vegetables inhibit DMBA initiation. Organosulfur compounds from garlic and onion inhibit initiation by 1,2-dimethylhydrazine and NDEA.¹⁹ Garlic components, diallyl sulfide, allyl methyl sulfide, and diallyl disulfide, significantly suppress initiation of mammary tumorigenesis by DMBA, when these compounds were given 96, 48, and 24 h before DMBA.¹⁸ Another organosulfur compound, S-methyl cysteine sulfoxide derived from Brassica vegetables, also may suppress initiation. This compound and its metabolite, methyl methane thiosulfinate, when coadministered with benzo(a)pyrene, inhibit micronucleus formation in mouse bone marrow, but methyl methane thiosulfinate had only a tenfold margin of safety, suggesting that the use of such compounds in amounts exceeding their content in foods would be unwise.²⁰

In vitro screening, using mammalian or bacterial cell mutagenesis systems, such as the Ames assay, is a cost-saving method to identify potential anti-initiators. For example, tobacco smoke and product extract mutagenicity in *Salmonella typhimurium* strains TA 100 and TA 98 was decreased significantly by the walnut flavonoid, ellagic acid, and the porphyrins, bovine hemin and chlorophyllin.²¹ The mechanism of action of these compounds was not determined, but in many cases, the ability of such compounds to alter biotransformation enzyme activity can be used as a screen for potential anti-initiators, because induction or inhibition of such enzymes is predictive of anti-initiating effects *in vivo*.

Antioxidant Inducers of Biotransformation Enzymes

Wattenberg has termed initiator-detoxifying antioxidants as type A or type B.¹⁹ The type A anti-initiator, such as the organosulfur compound, diallyl sulfide, induces phase II enzymes, especially glutathione S-transferases, but also UDPglucuronosyltransferase (UDPGT), epoxide hydrolase and NAD(P)H-quinone reductase. Glutathione S-transferases (GSTs) are needed for the detoxification of initiators such as aflatoxin B₁,²² at least in some species.

Although salmon are much less sensitive to aflatoxin carcinogenesis than are trout, trout have threefold greater hepatic GST activity than salmon. Even when trout GST was induced by β -naphthoflavone, very little glutathione-aflatoxin conjugate was detected in bile, compared to undetectable glutathione-aflatoxin conjugate in salmon bile.²³ Thus, the basis of interspecies variation in susceptibility to initiation by aflatoxin cannot be attributed solely to GST. Aflatoxin detoxification may involve glucuronidation as well. BP is also detoxified by GSTs, as well as UDPGT. Piperine, the active component of black pepper, increased BP-DNA adduct formation in V-79 lung fibroblasts, while suppressing both GST and UDPGT activities.²⁴

Induction of GSTs by anticarcinogens varies by species and organ. For example, rat GST- α is induced in esophagus by flavone, α -angelicalactone and especially by coumarin. Pancreatic GST- μ is induced by ellagic acid. Esophageal GST- α is induced dramatically by coumarin, whereas GST- π in stomach is induced by α -angelicalactone as well as by coumarin, and pancreatic GST- π is induced by flavone.²⁵ The isothiocyanate, goitrin, an *in vivo* inhibitor of aflatoxin-DNA binding in rats, specifically induces GSTs 1b (GST- α) and 7(GST- π) in liver.²⁶ A 40% increase in plasma GST- α , the major GST isoform in humans, was found in men after 3 weeks of daily consumption of 300 g Brussels sprouts, a glucosinolate-rich vegetable.²⁷ The effects of induction of the different classes of GSTs with differing organ specificity on initiator detoxification and carcinogenesis remains a largely unanswered question.

Induction of other phase II enzymes, such as UDPGT and NAD(P)H:quinone reductase (DT diaphorase), also play roles in anticarcinogenesis. Green tea, which contains the anticarcinogenic (-)-epigallocatechin gallate, specifically induced UDPGT in rat liver.²⁸ A very large dose of turmeric (10% of the diet) increases rat hepatic UDPGT, and 5 to 10% turmeric also increases hepatic GST.²⁹ Butylated hydroxyanisole (BHA) increases by tenfold rat hepatic quinone reductase.³⁰ This anticarcinogen also increases GST activity. Dietary Brussels sprouts (25%) and indole-3-carbinol (250 ppm) also increase rat hepatic quinone reductase.³¹ The anticarcinogenic isothiocyanate, sulforaphane, derived from the glucosinolate, glucoraphanin, found in SAGA broccoli (*B. oleracea italica*) is the major inducer of GSTs and NAD(P)H:quinone reductase from this plant food.³² Isothiocyanate structure-activity relationships in altering Phase II enzymes show that among phenolic isothiocyanates, phenethyl isothiocyanate (PEITC) stimulated NAD(P)H:quinone reductase to the greatest extent compared with fewer or greater numbers of carbons in the bridge between the phenyl ring and N=C=S.³³

Type B compounds, such as indoles, induce both Phase I and Phase II enzymes, and as such, are more complex in effects. Among the carotenoids, canthaxanthin, but not β -carotene, increased rat hepatic cytochrome P-450, GST, and UDPGT activities after feeding 300 mg/kg for 15 days.³⁴ The ability of canthaxanthin to act as an antitoxicant is not known. Indole-3-carbinol is the best studied Type B inducer. As reviewed by Stoewsand,³⁵ indole-3-carbinol inhibits initiation but promotes carcinogenesis in animal models, but the induction of cytochrome P-450 by this compound alters estrogen

metabolism and reduces breast and ovarian cancer risk factors in women. Indole-3-carbinol's effect on cytochrome P-450 is probably largely due to the formation of polymeric metabolites under acidic conditions. The binding affinity of some of these metabolites for the aryl hydrocarbon-responsiveness (Ah) receptor, which induces CYP1A, is within two orders of magnitude of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, the most potent binder of the Ah receptor known.³⁶ Trout hepatic cytochrome P-4501A induction by indole-3-carbinol and its polymeric metabolites is transient, and these compounds also can irreversibly inactivate CYP1A.³⁷ It is likely that the anticarcinogen effects of indole-3-carbinol over time are mediated by several different mechanisms.

Biotransformation of toxicants also may be accomplished by nonnutrient food components that alter gut microfloral composition and/or enzymatic activities. Toxic dietary components may be metabolized by gut microflora if the absorption of the toxic components occurs primarily in the ileum, cecum, or colon. The production of nitrite, a necessary precursor of endogenous nitrosamine formation, also can be altered by dietary fiber.⁵ The effect of non-nutrients on the gut microfloral metabolism of toxicants is not always antitoxic and deserves further attention.

Antitoxicant Inhibitors of Toxicant Biotransformation

Limiting access of initiators to DNA also is thought to occur by inhibition of the formation of electrophilic proximate carcinogens by inhibition of the first step in their biotransformation, catalyzed by cytochromes P-450. The synthetic flavonoids, α - and β -naphthoflavone, competitively inhibit trout hepatic cytochrome P4501A activity and aflatoxin B₁-DNA binding *in vitro*.³⁸ Flavonoids and isoflavones may act as competitive cytochrome P-450 inhibitors to be anti-initiators, but their mechanism of action is not entirely clear because some of these compounds not only inhibit the *in vitro* mutagenicity of compounds that require metabolic activation but also can inhibit the mutagenicity of compounds that do not require metabolic activation. Apigenin inhibits the mutagenicity of benzo(a)pyrene as well as that of 2-nitrofluorene (an activation-independent mutagen) in *S. typhimurium* TA-98. As antimutagens against 2-aminoanthracene, flavonoid glycosides were inactive; isoflavones such as daidzein and biochanin A were moderately active and nontoxic to TA-98, with the exception of the inactive formononetin. The prenylated form of daidzein, neobavaisoflavone, and other prenylated flavonoids were highly antimutagenic but also toxic to TA-98.³⁹

Birt et al.⁴⁰ showed that apigenin was antimutagenic against benzo(a)pyrene and, apigenin and robinetin were antimutagenic against 2-aminoanthracene in TA-98, although Wall et al.³⁹ found apigenin to be inactive against 2-aminoanthracene. The reason for this contradiction is not known. Other compounds also inhibit cytochrome P-450 activity. Feeding 0.2% of a phenolic component of coffee and tea, caffeic acid or its glycoside, chlorogenic acid, inhibited mouse intestinal cytochrome P-450 activity by

25%, and by 55% when P-450 was induced by benzo(a)pyrene pretreatment.⁴¹ Although caffeic acid is antineoplastic,⁴² the mechanism(s) of these effects is not clear. It also is possible that wine phenolics account largely for the antineoplastic effects of white or red wines given to male C3H/HeJ mice for 41 weeks with ethyl carbamate because ethanol alone in similar concentrations had little antineoplastic effect.⁴³ Another class of compounds, including 4-phenylbutyl-, 6-phenylhexyl- and phenethyl- isothiocyanates, inhibit oxidation of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) in rat and mouse lung microsomes. These compounds are also antineoplastic against this carcinogen in mouse lung.³³ Most of the cytochrome P-450 inhibitors described above have other effects, such as the ability to induce Phase II enzymes or to act as antioxidants. The relative importance of the different potentially antitoxic mechanisms of such compounds with multiple effects is not yet understood.

Antitoxicants Against the Promotion Phase of Carcinogenesis

Limiting initiation is an important strategy in cancer prevention, but preventing the growth of initiated cells by limiting expression of growth-promoting genes or inhibiting promotion may be even more important. Because control of natural environmental exposure to initiators is a daunting proposition, efforts focusing upon the reversal of promotion offer a reasonable and practical defensive strategy because cancer development can be blocked at an early stage. Promotion mechanisms involve disruption of signal transduction, stimulating signals that selectively turn on the growth of the initiated cell, or suppressing signals that maintain cell stasis. Targets of chemical carcinogens acting at the promoting stage include activation of protein kinase C (PKC),⁴⁴ inhibition of protein phosphatases,⁴⁵ inhibition of sphingosine production,⁴⁶ which in turn may activate PKC because sphingosine is a negative regulator of PKC;⁴⁷ and stimulation of eicosanoid production.⁴⁸ Promoting agents generally increase oxidative stress in target tissues.⁴⁹ It may well be that reactive oxygen species are the central stimulators of growth of initiated cells, although the crucial genetic targets of oxidative stress are not well characterized. It is clear that at least one product of oxidative stress can be prostaglandin.⁵⁰ These potent cell growth promoters and other eicosanoids may be central mediators of the promotion of carcinogenesis, and regulating them may be an underlying hallmark of the action of an antitoxicant as an antipromoter. Recently, the ability of phorbol ester to stimulate prostaglandin production in human keratinocytes has been demonstrated,⁵¹ giving further support to the general theory of the role of prostaglandins in tumor promotion. But much work remains to be done to firmly establish this theory and especially to work out the molecular mechanisms for eicosanoid-altered gene expression and initiated cell growth.

Antitoxicants as Antioxidants

In general, antitoxicants acting at the stage of promotion act by one or more of four mechanisms. An antipromoter may be an antioxidant, limiting oxidative stress and its consequences for cell growth. The evidence that antioxidative effects cause antipromoting effects is almost entirely circumstantial — at best the two effects coincide, and often the evidence must be pieced together from different studies that have separately identified antioxidative or antipromoting effects. There are many ways to measure antioxidant activity. The measurement of products of oxidative stress such as lipid peroxides; conjugated dienes; ethane/pentane exhalation; aldehydes and other secondary products of lipid damage by reactive oxygen species; certain types of DNA damage; alterations in amino acid side chains, such as protein-mixed disulfide or carbonyl formation; the direct measurement of free radicals by electron paramagnetic resonance; or the measurement of changes in tissue antioxidant status (glutathione reduction state, vitamin E, vitamin C, uric acid) can all provide evidence of an antioxidant's protective ability against damage due to oxidative stress.⁵² The coincidental measurement of tumor promotion and oxidative stress is difficult, but the development of such methods would be very useful. For example, measuring the flux of reactive oxygen species may be more telling than measuring end products, but performing such measurements *in vivo* is not currently possible.

Numerous antipromoting antioxidant food components have been identified, as reviewed by Slaga.⁵³ These components include (–) epigallocatechin gallate and flavonoids, both of which are also anti-initiators. Isoflavones are another class of antipromoters. Isoflavone-containing extract of soy flakes, containing approximately equal amounts of genistein and daidzein (total isoflavone dose 1 mmol/kg diet), inhibit the early stage of phenobarbital-induced promotion of rat hepatocarcinogenesis.⁵⁴ Because phenobarbital stimulates hepatic lipid peroxidation⁵⁵ among its many effects, and because isoflavones are antioxidants *in vitro*,⁵⁶ the antioxidant activity of isoflavones may be important in their antipromoting effect.

Several nonnutrient antioxidant food components have effects suggesting that they would act as antipromoters. General antitoxic effects of such compounds may indicate the potential for antipromoting activity. Silymarin, an antioxidant component of artichokes that inhibits toxicity of agents including allyl alcohol and carbon tetrachloride, inhibits phorbol ester-induced stimulation of mouse skin ornithine decarboxylase (ODC) activity, a well-studied hallmark of tumor promotion.⁵⁷ The flavonoids, apigenin and robinetin, also suppressed ODC in the same model system,⁴⁰ whereas the anti-initiator indole-3-carbinol stimulated ODC. Sesame components such as sesamol⁵⁸ also may be candidate antipromoters. The ability of food components to inhibit iron-induced lipid peroxidation also may suggest antipromoting potential. Turmeric fed to male Wistar rats for 10 weeks increased antioxidant enzyme activities (catalase, superoxide dismutase, and glutathione peroxidase) and inhibited oxidative damage due to iron overload.⁵⁹

In vitro iron-induced microsomal peroxidation was inhibited by thymol and carvacrol (from thyme), 6-gingerol (from ginger root), and hydroxytyrosol (from olives).⁶⁰ The flavonoid hispidulin, although not a food component but derived from an Indian flower, suppresses bromobenzene-induced lipid peroxidation in mouse liver when the flavonoid is given intraperitoneally.⁶¹ This lends further support to the general concept that flavonoid antioxidants are antitoxic. It would be extremely useful to determine which biological effects, antioxidant or otherwise, and which types of antioxidant activity, correlate most strongly with the relative antipromoting abilities of the many food component antioxidants so far identified.

Although antipromoting effects of antioxidants have received much attention, antioxidants also may be generally antitoxic because many if not most toxicants cause oxidative damage. Genotoxicity, which is central to initiation and progression stages of carcinogenesis, also may be inhibited by antioxidants. For example, ochratoxin-induced genotoxicity was blocked in mice by vitamin C.⁶² Flavonoids, indoles, aromatic isothiocyanates, ellagic acid, coumarins, and organosulfides inhibit initiation of carcinogenesis, as reviewed by Słaga.⁵³ The well-known antioxidant carotenoids can block later stages of chemically-induced gastric cancer.⁶³ Both β -carotene and canthaxanthin (a nonvitamin A precursor) inhibit 3-methylcholanthrene-induced transformation of 10T1/2 cells, suggesting that general antioxidant properties of these compounds are important in their antipromoting action.⁶⁴

Indirect antioxidant inhibition of genotoxicity also occurs in the specific case of the nitrosamines. Vitamin C can block nitrosamine formation preventing initiation of gastric cancer,⁶⁵ suggesting that antioxidants, in general, have this ability. Nonnutrient antioxidant inhibitors of nitrosamine formation include thymol, gallic acid, chlorogenic acid, tannins, soy foods, and tea.⁶⁶

Antitoxicants as Eicosanoid Suppressors

Inhibition of eicosanoid production in preneoplastic tissues or in tissues that control the growth of initiated cells is a second important function of antipromoting antitoxicants. Inhibition of eicosanoid production may converge with the antioxidant ability of antipromoting agents. Canthaxanthin, a carotenoid antioxidant, suppressed prostaglandin E_2 levels in human oral squamous carcinoma cells, whereas β -carotene stimulated PGE_2 production.⁶⁷ The antioxidant isoflavones, fed in a soy flake extract at 1 mmol/kg diet, inhibit fumonisin B_1 -induced rat hepatocarcinogenesis while significantly suppressing hepatic $PGF_{2\alpha}$ levels (Table 3.1). The chalcone-derived antioxidant, isoliquiritigenin, inhibited ODC induction in mouse ear, and inhibited DMBA-initiated and phorbol ester-promoted mouse skin carcinogenesis, while also inhibiting the production of PGE_2 in primary cultured mouse skin epidermal cells.⁶⁸ These studies show an important coincidence of suppression of cell growth stimulating prostaglandins with anticarcinogenic effects of several nonnutrient antioxidants.

TABLE 3.1

Soybean Isoflavone Extract Suppresses Hepatic Prostaglandin (PG) $F_{2\alpha}$ and Development of Placental Glutathione S-Transferase (PGST)-Positive Altered Hepatic Foci in Male F344/N Rats Fed Isoflavones (1 mmol/kg diet) and Fumonisin B₁ (FB, 0.07 mmol/kg diet) for 4 Weeks

Treatment ^a	n	Hepatic PGF ₂ (ng/g Liver)	PGST-(+) Focal Volume (% Liver Vol.)
Control	6	49 ± 3	0 ± 0
FB	6	77 ± 9 ^b	5.0 ± 2.6
Isoflavones	6	42 ± 5 ^c	0 ± 0
FB + isoflavones	6	52 ± 6	2.3 ± 0.8

^a Rats were initiated at 10 days of age with 15 mg diethylnitrosamine/kg body weight. At weaning at 21 days of age, rats were fed a basal diet based upon AIN-76A, or basal diets containing an acetone extract of soy flakes that provided 1 mmol total isoflavones/kg diet (approximately 1:1 genistein:daidzein) or fumonisin B₁ (0.07 mmol/kg diet) or both. After killing 4 weeks later, hepatic PGF₂ was analyzed by radioimmunoassay, and PGST-(+) altered hepatic foci analyzed according to the method of Lee et al.⁵⁴

^b Significantly different from the control group, $P < 0.01$, by analysis of variance.

^c Significantly different from the control group, $P < 0.05$, by analysis of variance.

Antitoxicants as Hormone Antagonists

Antipromoters may act as hormonal analogs, antagonizing growth promoting effects of certain endogenous factors, as has been hypothesized for soybean isoflavones and lignans. Genistein and daidzein, the major isoflavones in soybeans, and lignans found in whole grains, such as enterolactone, are estrogen analogs and may be of health benefit against breast and prostate cancer, among other diseases.⁶⁹ Traditional soy-containing Asian diets may be a significant factor in the much lower rate of breast cancer in these populations than in the U.S.⁷⁰ In individuals affected by such diseases, circulating hormones may be considered to act as endogenous toxicants. Soybeans inhibit mammary cancer in animal models, and this effect is largely due to their isoflavone content.⁷¹ Daidzein and genistein have 1000-fold less estrogenic activity than β -estradiol in a mouse uterine growth assay,⁷² but such compounds are able to bind to estrogen receptors and limit the action of estrogens.⁷³ Isoflavones also enhance levels of sex hormone-binding globulin,⁷⁴ which effectively lowers circulating estrogen levels. Antiestrogenic effects of numerous food components also may be due to inhibition of cytochrome P-450 (aromatase)-catalyzed activation of estrogens, which may lower circulating estrogen concentrations as well. The isoflavone (coumestrol), the flavonoids (luteolin and kaempferol), and enterolactone and other lignans have this ability in human preadipocytes in 1-20 μ molar concentrations,⁷⁵ as do the flavonoids chrysin and biochanin A,⁷⁶ and other

related compounds. The major isoflavones in soybeans, genistein and daidzein, are very weak aromatase inhibitors.⁷⁶ The relative importance of antiestrogenicity of isoflavones, considering all of the other potentially anticarcinogenic effects of these compounds (i.e., the ability to inhibit tyrosine kinases such as the ras oncogene product),⁷⁷ remains to be seen.

Other hormones may be modified by nonnutrient food components. A group of synthetic flavonoids has been found to inhibit deiodination of thyroxine in rat hepatocytes.⁷⁸ This suggests that food flavonoids might be capable of the same antagonist effects against toxicant-induced alterations in thyroid hormone metabolism. Anti-hormonal effects of numerous nonnutrient food components deserve further study, because toxicant-induced damage may be mediated hormonally. Numerous toxicants, including pesticides such as endosulfan and chlorinated hydrocarbons such as toxaphene, dieldrin, and many others are estrogenic,⁷⁹ and dietary nonnutrient antiestrogens may be an important counter to such environmental contaminants.

Antitoxics and Post-Translational Modification of Toxicant-Stimulated Proteins

The fourth mechanism of action of antipromoting antitoxics lies in altering post-translational modification of proteins which are induced or otherwise stimulated by toxicants. Mevalonate-derived (isoprenoid) nonnutrient food components act in this way, as reviewed by Elson and Yu.⁸⁰ It is theorized that when an initiator or promoter stimulates the expression of a ras family oncogene, the isoprenoid/monoterpene, *d*-limonene, can interfere,⁸¹ as an analog, with the isoprenylation-dependent activity of the ras gene product,⁸² thus blocking cell growth-stimulating effects of the ras tyrosine kinase. Isoprenoids such as geraniol, *d*-limonene, perillyl alcohol, β -ionone and tocotrienols inhibit chemical carcinogenesis.⁸⁰ The effect of isoprenoid food components to interfere with the action of endogenous isoprenoids also might alter toxicant response by inhibiting 3-hydroxy-3-methylglutaryl-coenzyme A (HMGCoA) reductase, the rate-limiting step in cholesterol synthesis, which depends upon isoprenoid condensation. HMGCoA reductase is inhibited by tocotrienols and monoterpenes.⁸⁰ Cholesterol is vital for the growth of cells, including, of course, tumor cells. Suppression of cholesterol synthesis is another potential mechanism for antitumor effects of dietary isoprenoids. Elevated blood cholesterol is a common response to hepatotoxicants. For example, phenobarbital and polychlorinated biphenyls increase total serum cholesterol in rats.⁸³ Fumonisin B₁-containing corn increases blood cholesterol in rats.⁸⁴ The mechanism underlying this general phenomenon is not clear and may differ with the toxicant studied. It is reasonable to hypothesize that suppression of cholesterol synthesis may be an important effect of dietary isoprenoids against hepatotoxicants.

TABLE 3.2

Functions and Food Sources and of Nonnutrient Antitoxinants

Function	Antitoxinant	Major Food Source
Sequestrants/diluents	Dietary fibers	Plant foods
	Phytic acid	Whole grains/legumes
Biotransformation enhancers	<i>d</i> -limonene	Citrus
	Indoles	Brassica vegetables
	Canthaxanthin	Shrimp
	Organosulfides	Onion/garlic
	Ellagic acid	Walnuts
	Isothiocyanates	Brassica vegetables
Biotransformation inhibitors	(-)-epigallocatechin gallate	Green tea
	Flavonoids	Plant foods
	Isoflavones	Soybeans
	Other phenolics	Plant foods/wine
	Caffeic acid	Coffee/tea
Antioxidants	Isothiocyanates	Brassica vegetables
	Flavonoids	Plant foods
	Isoflavones	Soybeans
	Other phenolics	
	(-)-epigallocatechin gallate	Green tea
	Silymarin	Artichokes
	Thymol	Thyme
	Carvacrol	
	Ellagic acid	Walnuts
	Caffeic acid	Coffee/tea
	Tannins	Plant foods
	Unknown	Turmeric
	Carotenoids	Fruits/vegetables
	Isothiocyanates (aromatic)	Brassica vegetables
	Organosulfides	Onion/garlic
Eicosanoid suppressers	Canthaxanthin	Shrimp
	Isoflavones	Soybeans
	Isoliquiritigenin	Green vegetables
Hormone antagonists	Isoflavones	Soybeans
	Lignans	Whole grains/legumes
	Flavonoids	Plant foods
Post-translational modifiers of toxicant-altered proteins	Mevalonate derivatives	
	Monoterpenes	Citrus
	Tocotrienols	Whole grains/palm oil

Conclusions

Antitoxinants are probably abundant in at least some human diets. Such components have been widely identified especially in plant foods (Table 3.2). The advice not only to consume five or more servings per day of fruits and

vegetables (the “five-a-day” plan), but more generally to incorporate generous amounts of all plant foods, including especially whole grains and legumes into human diets⁸⁵ is reasonable from the standpoint of what is known about antitoxinants currently. Such advice is very unlikely to be harmful, and may be beneficial, although much more work needs to be done to characterize food composition, biological effects of antitoxinants, and their fundamental mechanisms of action in model systems relevant to human physiology and conceivable dietary intakes. The application of this knowledge might eventually be made to genetic engineering of foods or food processing to enhance for certain components if, and only if, efficacy and lack of harm are already well established. A further implication of this field is in the use of food-derived antitoxinants to ameliorate harmful side effects of pharmaceuticals, but again, only after careful determination of efficacy and lack of harm. Traditional Chinese and other medicinal practices may have been employing this technique of using complex mixtures of effective but toxic substances combined with antitoxinants for thousands of years without benefit of molecular characterization of such components. The potential exists for significant benefits to human health from research into antitoxinant food components, especially with the molecular tools now available. This research may lead us back to some very simple advice, which can certainly serve us well as we progress in our understanding of life processes; the advice being to eat a very wide variety of foods.

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Safety of Genetically Engineered Foods

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Introduction

Biotechnology has been defined in a number of ways. The basic underlying definition is the use of living organisms and processes to enhance plants, animals, and microorganisms for useful purposes. The food industry is one of the largest users of biotechnology. Historically, one may find examples of bio-

technological applications, primarily microbial food production and preservation, that extend back thousands of years.¹ Microorganisms play a major role in our food supply; a few examples include the production of cheese, bread, wine, soy sauce, yogurt, vitamins, and food processing enzymes. In the past several years the science of biotechnology has expanded to include various novel techniques such as genetic engineering. Genetic engineering is one subset of biotechnology defined as the manipulation of genetic information by techniques other than traditional breeding. These primary techniques include genetic transformation and recombinant DNA technology.² The focus of this chapter will be on the safety of foods developed by genetic modification using recombinant DNA technology.

Genetic manipulations important to the food industry include those of microorganisms, plants, and animals. Safety issues have arisen in all of these areas. Many of these safety issues will be discussed below. In the case of microorganisms, most genetic manipulations have been for production of food ingredients or the production of enzymes critical to some aspect of food production. For example, cheese production is one of the first examples of the application of recombinant DNA technology in foods.^{3,4} Through the use of recombinant DNA, microbes are able to mass produce the enzyme chymosin, which is necessary for curd production in cheese.^{5,6} This makes the enzyme much more widely available and greatly reduces cost, considering the natural source is the lining of the stomach of weanling calves. The production of cheese using recombinant DNA technology has proceeded virtually unquestioned. This technique of harvesting an enzyme from a bacterial source containing recombinant DNA differs in impact from those food products that are the direct product of incorporation of recombinant DNA into a plant/animal to be used as a food source or ingredient. This genetic engineering of plants/animals to serve as a food or food component will be the focus of this chapter. The first food product to be released into the marketplace that is a result of the genetic engineering technology was the Flavr Savr^{TM*} tomato, developed by Calgene, Inc.⁷⁻⁹ Since the introduction of this product, several other genetically modified plants have been introduced. Examples include herbicide-resistant Roundup^{®**} Ready soybeans and insect-resistant *Bacillus thuringiensis* corn and cotton. Field trials are being conducted on a number of genetically modified plants including tomatoes, potatoes, corn, cotton, soybeans, and rapeseed.

This chapter will present an overview of some of the issues relevant to the safety of genetically engineered foods, including regulation, technology, issues relevant to the safety of consumption, environmental concerns, and labeling. Specific examples and potential benefits also will be discussed. The focus will be mainly on the issue of development of genetically modified

* Registered trademark of Calgene, Inc., Davis, CA.

** Registered trademark of Monsanto Co., St. Louis, MO.

plants as they are of the major current focus in the marketplace with respect to genetically modified organisms.

Establishment of the Safety of Genetically Modified Foods

Regulatory Aspects

Currently, U.S. agencies involved in the review/regulation of genetically modified organisms include the Food and Drug Administration (FDA), the U.S. Department of Agriculture (USDA), the Environmental Protection Agency (EPA), and state governments. The roles of each of these in the regulatory process of genetically modified foods has been reviewed by Wilkinson.¹⁰ In 1992, the FDA stated their policy about new plant varieties, that foods created by genetic engineering will be regulated in a similar manner to foods created by conventional means unless special circumstances apply.¹¹ With regards to labeling, there currently is no specific regulation for labeling genetically engineered foods/products unless there is an alteration in the identity of a product or its safety. There continues ongoing controversy over the issue of mandatory labeling to notify consumers of genetically modified organisms; most of the arguments for labeling are based strictly on the process itself.

With regards to transgenic plants, there is the concept of substantial equivalence that was advocated by the Organization for Economic Cooperation and Development (OECD) in order to address the issue of safety of foods developed by biotechnology.¹² This concept basically states that if a new product of biotechnology is substantially equivalent to an existing food or food component it can be considered as safe as that food/food component. This concept was applied extensively in the development of the Flavr Savr tomato. There has been extensive testing conducted in order to establish that the tomato produced is equivalent to a “standard” tomato. Calgene, Inc. provided documentation for all the testing which was conducted prior to release of their product.¹³

Comparison of Genetically Modified Foods to Those Developed by Traditional/Classical Breeding vs. Genetic Engineering

Traditional plant breeding is a classical technique that has been conducted for years and is widely accepted without controversy. The basics of breeding are

that there is exchange of genetic material of sexually compatible plants, usually of the same species.^{14,15} Several techniques are available to breeders that help enhance development of new varieties by introducing new combinations of genetic material, including hybridization between plants of same and different species and genera, chemical and physical mutagenesis, protoplast fusion, somaclonal variation, and *in vitro* gene transfer techniques.¹¹ The process of traditional breeding involves the crossing of two plants that each contains thousands of genes. The actual process of mixing the genetic information is random, imprecise, and uncontrollable.² The progeny of the cross are selected in the field and back-crossed or selfed. Offspring containing the majority of desirable traits are identified. Sometimes as many as 7 to 12 generations are necessary to achieve the goal. This process is very laborious and time-consuming. The basic premise here is that the mixing of two genomes is complicated and not very direct. Many pathways could be altered in the process. This requires extensive testing and selection to ensure safety of newly generated food plants.

Generally, the structure and biology of the transgene of interest is well known prior to genetic engineering.¹⁶ This is not necessarily the case, however, with traditional breeding where breeders select organisms with the desired phenotype but often may not know the nature of the gene or combination of genes introduced into progeny. Knowledge of the transgene allows one to focus attention on specific gene products and systems, allowing a direction of safety studies.

The process of genetic engineering, in contrast to conventional breeding, is much more direct and generally less time-consuming. Typically, genetic engineering involves the insertion of one, or a small number of genes, avoiding the mixing of all the genetic material from two plants as seen above. This also avoids many undesirable changes that may occur under traditional breeding. The introduction of a gene by genetic engineering also is more efficient, requiring much less time. The opportunity to insert beneficial genes from sources that may not be amenable to traditional breeding is a way to improve plants and to increase diversity as well. One concern with genetic engineering is that the insertion of the gene into the chromosome of the plant is random. This is a valid concern; just as with traditional breeding, there is randomness associated. Since there are few genes transferred in genetic engineering, the degree of variability in the whole plant may be less than that with traditional breeding. However, any new variety developed needs to be evaluated for potential positive and negative effects regardless of whether it was developed by traditional breeding or genetic engineering. Effort is being made to achieve directed placement of genes into the genome through the genetic engineering process.^{17,18}

Technologies Used in Genetic Modification of Foods

This section contains a description of the basics of introduction of DNA into plants for genetic engineering. The technologies are developing rapidly;

thus, it is impossible to cover all of them in depth. Two commonly used protocols will be briefly reviewed as a general guide for understanding the basic technologies.

Once the biochemical pathway for a desirable component is known and understood, a particular enzyme(s) key to production of the compound of interest is identified. A gene is obtained which will encode that particular enzyme. Introduction of the gene into the chromosome of a plant can be accomplished by a number of techniques, which will be described briefly, including *Agrobacterium tumefaciens* transformation and bombardment using a gene/particle gun.

Transformation Systems

The introduction of DNA into plant chromosomes is referred to as a transformation; thus these plants are often referred to as transgenic plants. Many plant transformations have been conducted using a common soil bacterium, *A. tumefaciens*. This transformation system has been successfully applied to many dicotyledonous plants and some monocotyledonous plants.^{15,19,20} This soil bacterium, when infecting a plant, results in the production of crown gall tumors on the infected plant. These tumors were found to be the result of transfer of genes from the bacterium into the plant cell genome. The *Agrobacterium* has a large plasmid referred to as the TI (tumor-inducing) plasmid, which was responsible for the production of crown gall tumors via introduction of a segment of DNA from the TI plasmid (called the T-DNA) into the genome of the plant. This system has been adapted to the transformation of plant cells. First, genes responsible for the production of the crown gall disease have been removed and the desired transformation is created by inserting the gene of interest into the T-DNA region of the plasmid. All the genetic material between the borders of the T-DNA is inserted into the plant chromosome. The *Agrobacterium*-infected plant material is then selected to indicate which cells have taken up the desired transgene. Often this involves the co-introduction of a marker gene for antibiotic resistance with the gene of interest. *Agrobacterium* transformations are quite routinely used with certain crops due to their ease and efficiency.

Another technology available that has been applied successfully to the introduction of DNA into monocots is the gene gun/particle bombardment technology, sometimes referred to as "biolistics."^{15,19,21} In the earlier days of plant transformations, this technology was applied to plants for which *Agrobacterium* was not successful. In this system DNA is coated onto small particles of metal such as gold or tungsten and delivered into plant cells through cell walls using a microparticle gun.²¹ Plant regeneration then involves the proper tissue-culturing strategies and hormones to regenerate plants from bombarded tissues. This also involves selection of transgenics through the use of antibiotic-resistance selection techniques.

Gene Expression

There are multiple approaches to the manipulation of gene expression in plants that are to be used as sources of food and food products. Expression of an endogenous gene can be enhanced so as to achieve more of the particular product of the gene or pathway. Likewise, expression of an endogenous gene can be reduced by technologies such as antisense and cosuppression.²² Antisense technology is what was used in the production of the Flavr Savr tomato, the first released, genetically engineered food to the marketplace. Antisense techniques involve the isolation of a gene of interest that naturally occurs in the plant, followed by transformation of the plant with the gene in reverse orientation.²³ This causes mRNA to be produced that will complement that of the original gene. It is believed that a complex is formed between the endogenous mRNA and from the antisense gene that will be rapidly degraded, preventing translation into protein.^{24,25} Cosuppression is another technique that reduces gene expression, although the mechanism is unclear.²⁶⁻²⁹ Transformation of a plant with an endogenous gene (overexpression) results, in certain cases, in a reduction of expression of that gene.

Although manipulation of endogenous gene expression is often times quite desirable, one of the beneficial aspects of plant genetic engineering over traditional crop breeding is the ability to introduce a segment of DNA from a sexually incompatible organism. This would allow for expression of a gene from another organism that is not naturally found in that plant. This is quite beneficial in several aspects. First, it enhances the diversity of a plant species to produce compounds not normally present. Another benefit would be to be able to increase flux through a pathway by using a gene from another source that may not be subject to the same regulation as in the targeted plant.

Potential Benefits of Transgenic Plants

As mentioned above, there are different technologies that can be applied to the genetic manipulation of plants to be used as dietary components. Genes from similar organisms or widely different species are being manipulated to achieve desirable effects. These benefits are widespread and include improvements in food quality such as those that may impact nutritional value, shelf life, flavor and aroma, functional properties of food components, economic potential, and processing characteristics. Other potential agronomic benefits include increased yield, drought and salinity tolerance, temperature tolerance, insect and herbicide resistance, to name a few. These benefits should lead to increases in yield and improved crop performance to be able to feed a rapidly growing world population. A few examples of transgenic plants and their potential are discussed here.

The first genetically engineered food to be introduced to the marketplace was the Flavr Savr tomato, in 1994. This is an example of the use of antisense technology to reduce expression of the enzyme polygalacturonase, which acts to degrade pectin leading to softening of cell walls. By reducing softening one can leave the tomato on the vine longer allowing the accumulation of color and flavor, thus allowing a higher quality tomato to be presented to the consumer. Shelf life of these tomatoes has almost doubled that of traditional tomatoes, so that the fruit stays fresh for 2 weeks after ripe harvesting.³⁰ Other efforts also have been directed at improvement of the tomato.^{31,32} Disruption in ethylene biosynthesis, by using antisense technology with a gene responsible for coding for the rate-limiting enzyme in its biosynthesis, has been attempted for lengthening shelf life and, thus, higher quality product.³³

Quality traits of crops being manipulated currently also include manipulation of fatty acid composition to present healthier oils to consumers and to improve animal feeds. One area is the manipulation of saturation of oils. By reducing numbers of double bonds in a vegetable oil, one can increase the oxidative stability and also take advantage of the important health benefits of monounsaturates. Increasing the saturation can lead as well to less hydrogenation of oils, resulting in less expensive processing and less exposure of consumers to trans fatty acids.³⁴

One of the beneficial aspects of the research being devoted to the development of crops that are more resistant to adverse conditions such as drought and salinity is that they may help grow crops in areas otherwise unsuitable. For example, Apse et al.³⁵ has increased the activity of a salt transporter in *Arabidopsis* plants, increasing the tolerance of the plant to salinity in the environment. This technology will be implemented in crop plants with the goal of being able to grow them in higher salt environments and/or to be able to irrigate with salt water. This may be beneficial in various parts of the world where population growth is great and there is not adequate land and/or water for crop growth.

Moffat³⁶ reviewed the genetic engineering of tropical plants. Most of the focus has been on the production of crops of importance in the developed world. However, since the population often expands rapidly in lesser-developed areas, more focus is currently being directed at crops of importance to these developing countries, such as tropical areas. Cassava, for example, supplies the world's third largest source of calories, just behind rice and corn. Because of diseases and pests there has been little recent increase in the yield of these plants. Biotechnological manipulations are being conducted in an effort to increase yields, such as transformation with a truncated protein produced by the gemini virus to make the plants resistant to the African cassava mosaic virus; another transformation with replicase to disrupt the life cycle of invading viruses also is being attempted. The estimate is that cassava yields might increase tenfold if these strategies are successful. This would greatly benefit the population of developing countries.

Insect resistance and herbicide tolerance are two of the most widely known, commercially available genetic modifications to date. These include genetic engineering of cotton and corn with the *B. thuringiensis* toxin gene. *B. thuringiensis* toxin has previously been demonstrated to be effective in application to crop plants in the form of a spray. This bacterial toxin is only toxic to a few species of insects, some of which are the most destructive.^{37,38} The protein responsible has been genetically engineered into corn and cotton and dramatic improvements in yield are noted.¹⁰ One of the most significant arguments in favor of this manipulation is that 90% of all U. S. insecticide usage is targeted towards pests of corn and cotton.¹⁰ Thus, the expectation is that far less chemical insecticide would be applied. Applications also have been directed so that herbicides may be used throughout the growing season, by creating plants tolerant to the herbicide glyphosate.³⁸ This creates the situation where herbicide can be applied after emergence and, thus, better control of weeds is established. These plants are referred to as Roundup Ready and include such crops as soybean and maize. Proponents argue that the application of herbicide also can be more directed and, thus, the amount applied should be reduced. Also, this allows the selection of a less toxic herbicide, such as glyphosate.

Safety Concerns with Technologies and Resultant Foods

Toxicity/Allergenicity

One primary source of concern is the introduction of toxic or allergenic proteins, the product of gene expression. The fear of a toxic component resulting is legitimate; however, toxic components associated with foods are well known and are tested for before the release of any genetically modified food or food product resulting from classical breeding. One should always be aware, however, that there is the potential for new toxic compounds to arise in the food supply, and feeding studies to assess this possibility should be conducted. This applies equally to genetically enhanced foods and classical breeding. Allergenicity is another issue of extreme importance. Since allergens are proteins there is always the potential for introduction of allergens when introducing new genes into an organism. One example of a potential problem was the transformation of soybean with a gene encoding a Brazil nut protein in an effort to enhance the amino acid profile of the soybean.^{39,40} This transformation was effective at greatly enhancing the sulfur-containing amino acids in soybean. However, nuts are a great source of allergens and it was discovered that this protein from the Brazil nut is associated with allergenicity. This was detected through thorough testing before the transgenic plants ever were released into the marketplace and the development of these

plants was stopped. Something to point out in relationship to these concerns of toxicity/allergenicity of proteins is that if genetic manipulations are done to enhance oil composition, for example, the protein does not become a component of the resultant oil. Also, if a food is processed by heat, thermal denaturation of proteins can lead to alterations of enzyme activity and protein structure in such a way that might make these concerns less of an issue. One must consider differences in food processing techniques applied to foods, particularly if the gene of interest is obtained from another food source and transferred into a food plant that is not processed similarly.⁴¹

Safety of Recombinant DNA

Foods are by nature composed of DNA, as it is a basic constituent of living tissues. Human consumption of DNA of all species has occurred safely over time. Even human DNA is consumed, as it is a component of sloughed cells from the nasopharynx and the gastrointestinal tract.¹⁶ The fact that DNA is degraded by nucleases in the digestive tract should make the origin of the DNA of minor concern. Calgene, Inc. had done considerable research with *in vitro* digestibility studies of both the DNA and the protein associated with the NPTII gene, prior to release of the Flavr Savr tomato.¹³ *In vitro* data were calculated to estimate an intake of only 0.1% of DNA could be detected as fragments of 1000 base pairs or longer after exposure to simulated stomach fluids for 10 min and to simulated intestinal fluids for another 10 min.⁴² McAllen et al.⁴³ have conducted studies as well on the stability of nucleic acids to the digestive processes associated with ruminant animals demonstrating that there is rapid and complete degradation to nucleotides and nucleosides.

The possibility that some of the foreign DNA may survive digestion exists and that it is possible that it is absorbed intact or transferred to a microorganism in the digestive tract. However, these possibilities are not probable.⁴¹ Studies to evaluate these concerns need to more closely mimic an *in vivo* situation.

As mentioned above, many of the alterations of plants by introduction of recombinant DNA technology raise the same considerations in terms of safety as those alterations resulting from traditional plant breeding.

Safety of Selectable Markers

In the process of transferring genes to plants, only a small percentage of recipient plant cells take up the introduced gene. It is difficult to assess whether the intended effect has been achieved until the plant is fully developed. Therefore, it is desirable to use a selectable marker, linked to the desired gene, to determine which plant cells have been transformed.

Several selectable markers are available; many of them confer antibiotic resistance. One of the most commonly used marker genes is that for kanamycin resistance. It is responsible for production of the enzyme aminoglycoside

3-phosphotransferase II, commonly known as neomycin phosphotransferase II (NPTII). This enzyme chemically modifies and inactivates a group of aminoglycoside antibiotics, including kanamycin and neomycin. As a result, plant cells containing the desired gene linked to the marker gene become resistant to the antibiotic and, thus, can easily be selected by screening on agar plates containing kanamycin.

One of the safety concerns raised in transgenic plant development and commercialization is regarding the use of the antibiotic resistance selectable markers. Theoretically, after the transgenic plant has been selected, there is no need for the antibiotic resistance gene. However, there continues to be a small amount of protein produced and the gene also will be present in the plant. Thus, transgenic plants that are to be used for food will contain both the protein and the gene. Some of the major concerns expressed will be briefly addressed. These have been reviewed in a draft on the Internet submitted by the FDA to the industry application of selectable markers.⁴¹

Potential toxicity of the protein product from the resistance gene is one concern, which may be estimated by evaluating the digestibility of the protein by digestive enzymes, the homology in sequence to other toxic proteins, and other literature reports of potential toxicity of the protein. Another related concern to consumption of the antibiotic resistance gene product is the potential allergenicity. Demonstration that the protein does not have properties of other allergenic proteins is an important first step.

Development of more widespread antibiotic resistance in the environment as a whole is a recognizable concern. Fears of inactivation of orally administered antibiotics are one example, since a person consuming foods produced by genetic engineering would ultimately consume the protein conferring the antibiotic resistance. Would this protein inactivate any orally administered antibiotics? One might easily solve this problem by not taking antibiotics while consuming food, particularly genetically engineered foods. Fuchs et al.⁴⁴ showed that NPTII is inactivated by stomach acid and degraded by digestive enzymes. Also in 1993, Redenbaugh et al.⁴² presented a numerical assessment of the intake of the kanamycin resistance gene. For example, the human estimated intake of the resistance gene from fresh tomatoes was estimated at approximately 3.3×10^{-4} ng/day. The enzyme activity levels under simulated gastric conditions were essentially abolished after 20 min. They estimate 1% of enzyme remaining after consumption. The techniques used were similar to those established by the FDA when estimating the intake of recombinant bovine growth hormone. Similar considerations were done with the gene itself, considering DNA denaturation in the acid environment and the presence of nucleases. Due to degradation in the acidic environment of the gastrointestinal tract there was not much of a concern of the consumption of antibiotic resistance genes and gene products.

Within the gastrointestinal tract there are other possibilities, one of which is the transfer of the antibiotic resistance marker gene to gut epithelial cells. This issue was addressed in Calgene, Inc.'s petition for the Flavr Savr tomato. The gene for kanamycin resistance is not likely to survive degradation by

nucleases in the digestive tract as well as transfer to the epithelial cells, integration, and expression.⁴² If there were some of the gene expressed in this tissue, it is unlikely that it would be an issue, since the epithelial cells are sloughed off and replaced regularly.⁴¹ Another possibility is the transfer of the antibiotic resistance gene to gut microflora. This is again unlikely for some of the same reasons. One major reason is that the transfer of plant genes directly to microorganisms is not a simple process. Also, digestive enzymes, particularly nucleases, should degrade these genes. Bacteria are equipped with processes to destroy foreign DNA. In addition to the remote possibility of transfer directly to microorganisms, there is already the distinct possibility of transfer of antibiotic resistance occurring in microorganisms naturally, from one microbe to another, such that any possible transfer from plant to microbe would be negligible in comparison.⁴¹

Inactivation of orally administered antibiotics is another issue. There seems to be protection against this built in by the fact that enzymes such as neomycin phosphotransferase II (for kanamycin resistance) are broken down by proteases in the digestive tract, making it difficult to act on antibiotics in the gastrointestinal tract. Also, many of the enzymes are inactivated by food processing protocols and, thus, would not be capable of inactivating an orally administered antibiotic. Cofactor requirements also could prevent selectable marker gene products from inactivating antibiotics. One seemingly unrealistic way to avoid the problem is to not consume antibiotics during the consumption of genetically modified foods.

Other suggestions made by the FDA guidance report⁴¹ is the elimination of the use of antibiotic resistance genes for those antibiotics that are commonly encountered, for example, in the treatment of infectious agents. Most of the antibiotic resistance markers that are currently used have limited clinical applications. Selectable markers other than antibiotic resistance markers are being developed as a means of removal of the selectable marker gene once the transgenic plants have been selected.^{45,46}

Environmental Concerns

One should not ignore the potential impact of the technology of genetic engineering on the environment. Several concerns have been raised; a few are discussed here. We have seen issues raised about the creation of super weeds, widespread insect resistance, overuse of herbicides and insecticides, and deleterious effects on monarch butterflies (not to mention other possible negative effects on other wildlife). Losey et al.⁴⁷ reported preliminary findings on the effects of pollen from *B. thuringiensis* transgenic maize on the monarch butterfly. These researchers reported experiments of dusting milkweed plants with transgenic maize pollen and comparing the survival rate of monarch larvae to those surviving on milkweed without transgenic maize pollen.

This study was preliminary, but does point out some of the considerations that we need to address. We should consider the effects of any plant manipulations if they might affect bees or other creatures. Another issue is the development of insect resistance. Insect management strategies are being implemented in order to avoid potential widespread insect resistance; for example, with *B. thuringiensis* corn and cotton. Overuse issues are often negated by the fact that most of the genetic modifications will actually allow the use of fewer chemicals, which is desirable environmentally and economically. *B. thuringiensis* protein engineered into corn and cotton, for example, is designed to reduce chemical usage. Approximately 90% of insecticide applications are estimated to be applied to corn and cotton.¹⁰ Corn and soybeans in this part of the world also have no wild relatives and, therefore, are not likely to cross with neighboring plants, creating “superweeds.” However, all of these concerns are valid and will need to be addressed on a case-by-case basis. Environmental effects are quite complicated and, thus, are difficult to study and evaluate.

Labeling Issues

There are currently no regulations in the U.S. to impose mandatory labeling of genetically modified foods. This is a subject of great controversy. The controversy stems over the argument of whether the consumer needs to know how the food was produced. Several groups are pushing for mandatory labeling of genetically modified foods. One of the arguments of the proponents of mandatory labeling is that consumers have the right to know that the food they are consuming is a product of genetic engineering. The other side of the argument is that one need not label a process if there are no alterations in the safety or the identity of the food produced. Traditional breeding often involves a similar, less direct method of modification of foods, yet no labeling is required. If the argument is made that consumers have the right to know, for religious, cultural, or other reasons, how the food is processed then the argument has been made that labeling should be applied consistently.⁴⁸ Most foods subject to any kind of processing might, under these conditions, have to be labeled. However, consumers should be aware of the impact that mandatory labeling would have upon them. These outcomes include higher prices for the foods associated with the economics of alteration of labeling and with the need for segregation of crops. This segregation also would have to extend to the food processors receiving ingredients from different suppliers, thus increasing the effort and potential for higher prices. The labels themselves might provide no other safety or health information to consumers and might imply a safety hazard where there is no known hazard.⁴⁹ Another impact may be a reduced selection of products that otherwise may be available. If they are genetically engineered perhaps a consumer may never see them?⁵⁰

Archer Daniels Midland (ADM) announced last year its intention to segregate genetically modified crops from those that are not genetically modified. This should provide us with much more information about the practicality and realistic challenges that have been speculated due to the concern over the possibility of mandatory labeling.

Summary

It is not possible to say that the process of genetic engineering for the production of food materials is completely safe. There are very complicated issues that must be dealt with on a case-by-case basis. The technology for genetic engineering has already significantly advanced our knowledge of plant physiology and metabolic processes and will continue to assist us in understanding the complexity of life. This tool of genetic engineering is very powerful and should be investigated thoroughly as to its ability to feed an expanding world population. There are many concerns with respect to consumption, ecological issues, and labeling that also will require addressing on a case-by-case basis so that full consideration is given. There will continue to be many potential benefits of this technology, some already experienced and others yet to be explored.

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5

Microbial Toxins in Foods: Algal, Fungal, and Bacterial

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Introduction

The focus of this chapter is on aquatic (marine and freshwater) toxins, fungal toxins, and bacterial toxins. In order of economic significance, bacterial toxins are the most important, followed by mycotoxins and aquatic biotoxins. Bacterial toxins have the highest significance with respect to public health as well. Although mycotoxins have been responsible for individual acute poisoning outbreaks, primary concerns regarding mycotoxin contamination in foods and feeds are related to both human illnesses due to long-term, low-level exposure and animal health. On the other hand, although there is a paucity of information on aquatic biotoxins, contamination of seafoods with these toxins has the highest potential human health risks because of the widespread distribution of susceptible seafoods and the etiology of toxin production and subsequent accumulation.

Aquatic Biotoxins in Seafood and Fresh Water

Microscopic planktonic algae are used as a source of food for filter-feeding bivalve shellfish. When planktonic algae proliferate, i.e., form algal blooms, a beneficial effect for aquaculture and wild fisheries operations can be expected. However, these algal blooms may become harmful, affecting the economy of surrounding areas and causing human health impacts.¹ From the estimated 5000 species of marine phytoplankton, only around 300 can discolor the surface of the sea and around 40 can produce potent toxins that can enter the food chain through fish and shellfish to humans.² The term “red tide” is used when the algae grow in such abundance that they change the color of the seawater to red, brown, or green; however, the term is misleading because not all water discolorations are toxic. Therefore, the proper term is “harmful algal blooms” (HABs).^{1,3} Although the organisms are often referred to as harmful algae, they include cyanobacteria as well as the almost animal-like *Pfiesteria piscicida*.⁴

HABs are entirely natural phenomena which have occurred for years. However, the past 2 decades have been marked by increased frequency, intensity, and geographic distribution. This apparent increased in HABs can be explained by the following:

1. Increased scientific awareness of toxic species.
2. Increased utilization of coastal waters for aquaculture.
3. Stimulation of plankton blooms by cultural eutrophication and/or unusual climatological conditions.
4. Transport of dinoflagellate resting cysts either in ships' ballast water or associated with translocation of shellfish stocks from one region to another.^{1,5}

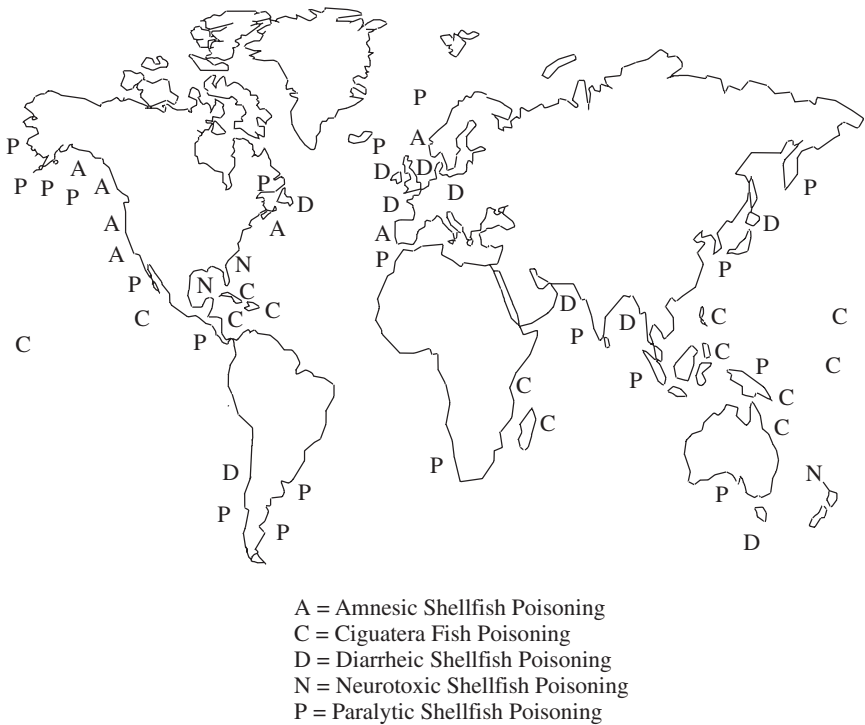


FIGURE 5.1
 Reported locations of some seafood-related diseases of nonmicrobial origin. (Adapted from Ledoux, M. and Fremy, J. M., *Recueil de Médecine Vétérinaire*, Feuier/Mar 1994.)

Marine toxins occur most significantly in shellfish and finfish. Paralytic shellfish poisoning, diarrheic shellfish poisoning, amnesic shellfish poisoning, and neurotoxic shellfish poisoning fit into the first category. Ciguatera fish poisoning and pufferfish poisoning are associated with marine fish toxins. Although circumstances leading to human exposure to cyanobacterial (blue-green algae) toxins through drinking water do not follow the etiology of seafood poisoning listed above, these toxins can be a serious public health concern.

Figure 5.1 summarizes the location of the most common seafood-related diseases associated with aquatic biotoxins.⁶

Marine Toxins

Shellfish

Paralytic Shellfish Poisoning

Paralytic shellfish poisoning (PSP) has been known in Europe and America since the 17th century.^{7,8} Cases of PSP have been documented in the Philippines, Argentina, Japan, the Mediterranean, the Atlantic coast of Spain, Gulf

of California, Gulf of Mexico, Portugal, and the Northeastern and Western coastlines of the U.S.⁹⁻¹⁴

Dinoflagellates identified as PSP-toxin producers can be found in the genera *Alexandrium* (formerly *Gonyaulax*), *Pyrodinium*, and *Gymnodinium*.¹⁵⁻¹⁷ PSP toxins also have been isolated from freshwater blue-green algae, such as *Aphanizomenon flos-aquae*, which produces saxitoxin and neosaxitoxin.^{18,19} A red macroalga, *Jania* sp., can produce gonyautoxins 1-3 (GTX) and saxitoxin as well.^{19,20} PSP toxins are accumulated by the phytoplankton consumers filter-feeders which, in turn, pass the stored toxin to their predators.^{15,21}

Saxitoxin (STX) and its derivatives are well known for their involvement in this fatal poisoning.¹⁹ Twenty-one different PSP toxins have been involved.^{22,23} These toxins can be classified in four different groups:

1. The most toxic carbamoyl toxins STX, neo-STX, GTX-1, GTX-2, GTX-3, GTX-4.
2. The intermediary toxic decarbamoyl toxins dc-STX, dc-neo-STX, dc-GTX-1,-2,-3,-4.
3. The less toxic N-sulfocarbamoyl toxins GTX-5(B1), GTX-6(B2), C-1, C-2, C-3, C-4.
4. The newly isolated deoxydecarbamoyl toxins do-STX, do-GTX-2, do-GTX-3.

STX is a tetrahydropurin with an LD₅₀ to mouse (IP) of 9-11.6 µg/kg. Its molecular formula is C₁₀H₁₇N₇O₄2HCl.²⁴⁻²⁶ The other PSP toxins are derived from STX by combination of radicals. These toxins are polar, water-soluble, and heat resistant.⁶ Carbamoyl toxins dominate in shellfish, while N-sulfocarbamoyl toxins are the dominating group in phytoplankton (dinoflagellates).²⁷ Newer members of the STX family do not possess the carbamoyl moiety of STX.¹⁹

Detection of STXs is difficult due to their complex chemistry, variations in composition, and their low concentrations.¹⁵ Currently, the most widely applied test method for PSP toxins is the classic mouse bioassay.^{28,29} However, the method's narrow dynamic range, variability of dose/response, and logistic constraints have stimulated the development of alternative methods.³⁰

HPLC techniques are the most widely used nonbioassay methods for determination of PSP compounds. Assays are generally based on separation of the toxins by ion interaction chromatography with fluorescence detection following post-column oxidation under alkaline conditions.²⁸ The method most commonly used for routine determination of PSP toxins is the Sullivan method and its variants, all of which involve HPLC using a reverse-phase technique with a resin-based column.^{31,32} An improved liquid chromatographic method for quantitative PSP toxins determination in shellfish has been developed using prechromatographic oxidation of the toxins to fluorescent purines.³³

A simple and fast radioimmunoassay (RIA) for STX has been developed.³⁴ For this method, solutions of ³H-STX are necessary, thereby limiting the general use of the method and adding to its cost.³¹ The most popular approach to the development of a rapid semiquantitative immunochemical method for PSP toxin analysis is based on specific antibodies.³¹ Indirect enzyme-linked immunosorbent assay (ELISA) and enzyme immunoassay (EIA) were developed for the detection of STX.³⁵ An absorption-inhibition ELISA technique based on polyclonal antibodies to STX (Saxitoxin test) shows broad antigen specificity and cross reacts with at least two GTXs (GTX-2 and GTX-3). This test yielded comparable results to HPLC-FD method and AOAC mouse bioassay; however, the kit is no longer produced commercially due to incomplete collaborative studies.³⁶ A direct EIA using polyclonal anti-STX antibodies and STX-horseradish peroxidase shows to have high sensitivity (3 to 4 ng/g tissue).³⁷

A microtiter plate-based neuroreceptor binding assay was developed, exploiting the highly specific interaction of PSP toxins with voltage-dependent sodium channels, and, thus, is based on functional activity. This is a competitive binding assay in which ³H-STX competes with unlabelled STX and/or its derivatives for a given number of available receptor sites in a preparation of rat brain synaptosomes. The percent reduction in ³H-STX binding is directly proportional to the amount of unlabelled toxin present. The reported limit of detection is 5 ng STX/ml sample extract.³⁸

The PSP complex comprises a group of neurotoxins, and the toxicity data on PSP are mainly restricted to acute toxicity in mammals. The mechanism of action of PSP is well defined; the toxins block the sodium channels associated with nervous conduction, affecting the respiratory, neuromuscular, and cardiovascular systems. The binding site of paralytic toxins is the same as that of tetrodotoxin, but different from that of brevetoxins and ciguatoxins. The STX dose at which the first symptoms appear in humans range from 150 to 1600 µg STX.¹ The limit dose for consumption is 400 MU (80 µg/100 g); however, some European countries have reduced this level to 40 µg STX/100 g.^{6,39} The lethal dose for humans is 1 to 4 mg expressed as STX equivalents.⁴⁰ Symptoms usually observed in humans include tingling and numbness around the lips and extremities leading to respiratory paralysis. No antidote is currently known for PSP.³¹

Diarrheic Shellfish Poisoning

Diarrheic shellfish poisoning (DSP) is a gastrointestinal disturbance resulting from ingestion of shellfish infested with dinoflagellate toxins. Unlike PSP, the predominant human symptoms of DSP are gastrointestinal disturbances.⁴¹ Cases of DSP have been reported in the U.S., Canada, Japan, Chile, and Europe.^{40,42-47} DSP is caused by the consumption of contaminated mussels, oysters, and/or scallops that ingest the organism during normal filter feeding.^{48,49}

Several dinoflagellates have been implicated in DSP toxin production, notably phototrophic species of *Dinophysis* and two species of

Prorocentrum.^{27,42,43,45,46,50,51} *P. lima* has been found to produce both okadaic acid (OA) and dynophysistoxin-1 (DTX-1). *Dinophysis fortii*, *D. rotundata*, *D. tripos*, *D. acuta*, *D. noruegica*, and *D. acuminata* produce either OA or DTX-1 or both.^{42,52}

DSP toxins are a group of polyether carboxylic acids.¹⁶ Three different groups of toxins have been isolated from various Japanese shellfish involved in DSP phenomena. One group consists of OA (LD₅₀ in mouse IP 200 µg/kg) and its derivatives, DTX-1 (LD₅₀ in mouse IP 160 µg/kg), DTX-2, and the acyl derivative DTX-3 (LD₅₀ in mouse IP 500 µg/kg). The other two groups consist of macrocyclic polyethers of the pectenotoxin (PTX) family (LD₅₀ in mouse IP are 250 µg/kg for PTX-1, 230 µg/kg for PTX-2, 350 µg/kg for PTX-3, 770 µg/kg for PTX-4, and 500 µg/kg for PTX-6), and yessotoxins (YTXs, LD₅₀ in mouse IP 100 µg/kg).^{19,53,54} The DTXs are structurally related to OA.⁵⁵ The structure of PTX-1 was found to be a novel polyether lactone. YTXs have been isolated from scallops and elucidated to be a brevetoxin-type polyether.¹⁹ DTX-1 is the most common DSP toxin in mussels in Japan, while OA is reported to be the major DSP toxin in Europe.²⁸

The mouse bioassay has been used worldwide to determine the distribution of DSP contaminated shellfish.^{28,48} To date, one of the most promising approaches for the determination of DSP toxins is a method based on HPLC using fluorescence detection after esterification of the carboxylic acid functionality with 9-anthryldiazomethane (ADAM) to produce a highly fluorescent 9-anthrylmethyl derivative.⁵⁶ However, the instability of ADAM causes problems in the HPLC determination of DSP toxins. Therefore, a reaction with 4-bromo methyl-7-metoxycoumarin for derivatization was proposed. The coumarin derivatives of the DSP toxins are stable, and an additional cleanup step after the derivatization is not required.⁵⁷ For the detection of OA, a fluorometric method was developed by Lee and colleagues.⁵⁸ The fluorometric HPLC procedure permits the determination of plankton and shellfish toxins with accuracy and rapidity. A combined liquid chromatography atmospheric pressure ionization mass spectrometry (LC-MS) method has been developed and appears to be one of the most sensitive and rapid methods of analysis for DSP toxins.^{59,60}

Some quantitative immunochemical methods have greater sensitivity than HPLC, with the lowest detection limit of 0.02 µg OA/g hepatopancreas for immunoassays and 0.4 µg for HPLC.⁶¹ A monoclonal antibody prepared for OA has been utilized to assess extracts of shellfish in a competitive ELISA for detection of OA.⁶² Another ELISA procedure for DSP toxins (DSP-Check) is currently being marketed by UBE Industries, Inc., Tokyo, Japan.⁴⁸ The monoclonal antibody to OA used in this ELISA cross reacts with DTX-1, but PTXs and YTXs are unreactive.⁶³ Another ELISA test kit (Rougier Bio-Tech) uses an anti-OA monoclonal antibody and an anti-idiotypic antibody which competes with OA for binding sites on the anti-OA antibody. This antibody exhibits a higher sensitivity (10- to 20-fold) for OA than either DTX-1, DTX-2, methyl-, diol-, and alcohol-derivatives of OA.⁶⁴

A rapid assay test kit for the detection of ciguatoxin, OA, and related polyether compounds based on solid-phase immunobead assay (S-PIA)

technology was developed by Park and coworkers.⁶⁵ This methodology (Ciguatect™) can be used to monitor shellfish beds for DSP toxins, and shellfish depuration operations for elimination of DSP toxins and to screen for toxic shellfish in the marketplace.^{61,65}

A novel rapid cytotoxicity assay for the detection of OA and related compounds has been developed using Buffalo green monkey kidney cell cultures. The basis of the assay is that DSP toxins induce morphological changes (cell rounding and vacuolization followed by complete disruption of the cellular monolayer) in this cell line, an event which can be evaluated by direct microscopic observation of the cells. A high correlation ($r = 0.95$) was found between this new assay and the mouse bioassay. It is reported that OA concentrations of $0.005 \mu\text{g}/\text{ml}$ are enough to provoke moderate toxic effects after only 5 to 6 h of exposure.⁶⁶

A new method for detection of DSP toxins, micellar electrokinetic chromatography, has been used for the determination of nonderivatized toxins. A detection limit of 40 pg of OA was achieved. The ultraviolet (UV) intensities of this toxin measured at 200 nm showed good linearity in the range 40 to 640 pg.⁶⁷

The DSP illness in humans usually begins within 30 minutes to a few hours after consumption of toxic shellfish. It is characterized by incapacitating diarrhea, nausea, vomiting, abdominal cramps, and chills. Recovery occurs usually within 3 days, with or without medical treatment.⁶⁸ Experiments on animals have shown that the nondiarrheic toxins in the DSP complex exert toxic effects in liver (PTXs) and heart (YTXs).²⁸ No human fatalities have ever been reported; however, OA and DTX-1 may be tumor promoters, producing stomach tumors and chronic problems in shellfish consumers.⁶⁹ Shellfish containing more than $2 \mu\text{g}$ OA and/or $1.8 \mu\text{g}$ DTX-1/g hepatopancreas are considered unfit for human consumption causing closure of harvesting and marketing operations.⁵⁸

Amnesic Shellfish Poisoning

A new type of seafood toxicity was reported in 1987 where 107 individuals exhibited symptoms following consumption of mussels from Prince Edward Island, Canada.^{70,71} The name amnesic shellfish poisoning (ASP) understates the severity of the problem, as it is known that domoic acid (DA), the principal toxin responsible for ASP, also accumulates in fish and in crab viscera along the west coast of the U.S.⁶⁸ Chemical analytical surveys have revealed the presence of DA from Southern California to Alaska.⁷² Several Eastern Canadian locations have reported ASP outbreaks.^{40,47} Only insignificant concentrations have been detected in other parts of the world such as Europe, Australia, Japan, and New Zealand.¹

The diatoms *Nitzschia pungens* f. *multiseriata*, *Pseudonitzschia australis* (*N. pseudoseriata*), and *N. pseudodelicatissima* have been implicated in the production of DA.^{40,49,71,73} DA, isodomoic acids A to H, isodomoic acid C5' diastereomer, and domoilactones A and B are involved in ASP.^{72,74-76} DA is an analog of the excitotoxic amino acids glutamate and kainic acid.^{71,77,78}

A method for detection of DA is the AOAC ASP mouse bioassay with a longer observation time (up to 40 h instead of 15 min).^{77,79} The Atlantic Research Laboratory of the National Research Council of Canada has developed an analytical method for DA based on a reverse-phase HPLC.⁸⁰ DA can be determined easily in shellfish by direct HPLC with UV detection at levels greater than 1 ppm.⁸¹ In 1989, Pocklington and collaborators developed a reverse-phase gradient HPLC method based on fluorometric detection. A detection limit of 750 pg of DA in seawater after derivatization with fluorenylmethoxycarbonyl was reported.⁸²

A receptor binding assay using ³H-kainic acid and freshly prepared kainate receptors has been reported. This method is reported to be extremely sensitive.⁸³ A radioimmunoassay has also been developed for DA.⁸⁴ Assay kits for these methods are still at the research stage and are not commercially available yet.

The neurotoxicity of DA arises from its effect as a potent glutamate agonist. It can be considered to be a conformationally restricted form of glutamic acid that disrupts normal neurochemical transmission in the brain by binding to certain glutamate receptors of normal cells.⁷² Glutamate and analogs like kainic acid have an excitatory effect to stimulate the neurons through release of endogenous glutamate. However, excessive amounts of these kinds of amino acids can cause neurotoxicity.⁴⁷

Symptoms of ASP include vomiting, abdominal cramps, diarrhea, confusion, disorientation, and memory loss.⁸⁵ The latter is the most persistent symptom and can last over a year in several cases. Shellfish containing more than 20 µg DA/g are considered unfit for human consumption.⁸⁶

Neurotoxic Shellfish Poisoning

Neurotoxic shellfish poisoning (NSP) is caused by ingesting shellfish that have fed on the dinoflagellate *Ptychodiscus brevis* (formerly *Gymnodinium breve*).^{6,40,87} This organism is responsible for HABs along the Gulf of Mexico coasts of Florida and Texas.^{87,88} Unexpectedly, in early 1993 more than 180 human shellfish poisonings were reported in New Zealand, caused by an organism similar but not identical to *P. brevis*.¹ Brevetoxins (PbTx) are responsible for massive fish kills and accumulate in bivalves during bloom conditions. An unusual feature of this organism is the formation by wave action of toxic aerosols which can lead to respiratory asthma-like symptoms.^{89,90} The shellfish involved with NSP are primarily clams, but the toxins have been found in other bivalves.⁶

NSP is caused by PbTx which include a group of 9 phycotoxins composed of two skeletons of polycyclic polyethers of 42 to 47 carbon atoms. Both types belong to the polyether toxic group similar to ciguatera fish poisoning and diarrhetic shellfish poisoning toxins:

- Type 1 (Brevetoxin A): PbTx-1, PbTx-7, PbTx-10
- Type 2 (Brevetoxin B): PbTx-2, PbTx-3, PbTx-5, PbTx-6, PbTx-8, PbTx-9²²

The PbTx_s can be readily detected in seawater and in shellfish using the standard mouse bioassay, isocratic HPLC, RIA, and ELISA, each method possessing distinct advantages and disadvantages.^{6,91} Even though excellent separations of PbTx_s can be achieved by silica gel TLC, the sensitivity (>1 ppm) remains a problem. The success of the soft ionization techniques (desorption chemical ionization, fast atom bombardment, and cesium ion liquid secondary ion mass spectrometry) presents several possibilities for detection of PbTx_s in complex matrices.⁹² The RIA for PbTx_s is based on the competitive displacement of ³H-PbTx-3 by PbTx_s in the test samples from binding with the polyclonal antibodies to PbTx-3. Then, bound antibodies are separated from free antibodies using a suspension of dextran/charcoal followed by centrifugation, and the radioactivity is measured in the supernatant.⁹³ In the ELISA method, the same antibodies are used and are detected with rabbit antigoat IgG-horseradish peroxidase.⁹⁴

Kogure et al. developed a sensitive cell-based assay for the detection of sodium channel blocking toxins based on the ability of the toxins to antagonize the effects of veratridine and ouabain on neuroblastoma cells.⁹⁵ The original method was simplified by incorporating a colorimetric method based on the ability of metabolically active cells to reduce a tetrazolium compound, MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium), to a blue-colored formazan product.^{96,97} This assay was further modified to be able to detect sodium-enhanced ouabain/veratridine-dependent cytotoxicity. The detection limit of this assay is about 10⁻⁴ MU (1 MU = LD₅₀ dose for a 20 g mouse over 48 h).^{98,99} This cell assay is several orders of magnitude more sensitive than the mouse bioassay.⁹⁹

The PbTx_s induce a long-lasting excitation of the sodium channel. The action site is the same as that of ciguatoxins.⁶ Symptoms include tingling and numbness of the lips, tongue, throat and perioral area, muscular aches, gastrointestinal upset, and dizziness.⁴⁰ No deaths have been reported from this relatively mild form of poisoning.

Fish

Ciguatera Fish Poisoning

Ciguatera fish poisoning (CFP) is a disease associated with the ingestion of fish living in tropical and subtropical regions contaminated with one or more polyether toxins of the ciguatoxin (CTX) class.^{100,101} The illness is now widespread in the tropical Caribbean, subtropical North Atlantic, and the Pacific regions.¹⁰²⁻¹⁰⁷

More than 400 species of fish have been reported to be associated with CFP outbreaks, but the toxicity fluctuates widely from one species to another. The most common species implicated with CFP are the moray eel, snapper, grouper, Spanish mackerel, barracuda, parrot fish, surgeon fish, amberjack, and dolphin fish.^{107,108}

The major source of the toxins is a group of benthic and epiphytic dinoflagellates discovered within the last decade.¹⁰⁹ More than 20 species of

benthic dinoflagellates have been implicated in the production of the toxins. They include *Gambierdiscus toxicus*; *Prorocentrum* spp. (*P. lima*, *P. concavum*, *P. emarginatum*, *P. mexicanum*); *Amphidinium carterae*, *Ostreopsis* spp. (*O. ovata*, *O. siamensis*, *O. lenticula*, *O. heptagona*); *Thecadinium* sp.; and *Coolia monotis*.¹¹⁰⁻¹¹² However, only *G. toxicus* has been proved to produce the toxins that cause CFP.¹¹³

There are at least five groups of toxins which have been implicated with CFP: CTXs and its congeners from the Pacific and Caribbean — maitotoxin, scaritoxin, okadaic acid, and a recently named toxin, prorocentrolide.^{110,114,115} Recent studies suggest that an excess of 20 toxins may be involved in CFP.^{107,116,117} Pacific CTX-1 (P-CTX-1) is considered the principal toxin involved in CFP.¹¹⁸ It is a highly oxygenated, white, solid lipid.¹¹⁹ Its LD₅₀ was determined to be 0.45 µg/kg IP in mice. Two less polar toxins, P-CTX-2 (LD₅₀ in mouse IP of 2.3 µg/kg) and P-CTX-3 (LD₅₀ in mouse IP of 0.9 µg/kg), were isolated from moray eel.¹²⁰ Five toxins were separated by reverse-phase HPLC from a Caribbean (C) ciguatoxic fish: C-CTX-1 and C-CTX-2 which are diastereomers that differ from the Pacific family of CTXs, and three C-CTX-1 related compounds. The presence of different families of CTXs in ciguateric fish from the Caribbean Sea and Pacific Ocean probably explain the clinical differences in each area.¹²¹ Several CTX congeners named gambiertoxins (GTXs) have been identified in wild and cultured *G. toxicus*, although none identical to that found in Pacific moray eel.¹²²⁻¹²⁵ It has been proposed that CTXs may arise from the oxidative biotransformation of GTXs.¹²³ Other CTX congeners include gambierol, CTX-3C, and gambieric acids A to D.¹²⁴⁻¹²⁶ Maitotoxin (MTX, LD₅₀ = 0.13 µg/kg, IP against mice) presumably plays a role in diversifying CFP symptoms, particularly in the poisoning caused by implicated herbivorous fish.¹²⁷ Scaritoxin is a lipid-soluble toxin isolated from parrot fish.¹²⁸ It manifests symptoms physiologically similar to CTX in mice, but is chromatographically different.¹²⁹ It is likely a CTX or GTX, but its structure has not been characterized as of yet.¹³⁰ OA is a lipid-soluble, polyether carboxylic acid with a lethality to mouse IP at 200 µg/kg.^{131,132} It has been suggested that it could be present in barracuda harvested from the Caribbean and implicated in CFP; however, Lewis and Holmes suggested further confirmation and an estimate of whether the detected levels were sufficient to induce signs of human intoxication.^{130,133,134} Prorocentrolide has been isolated from the ciguatera-related *P. lima*. The structure was revealed to be a new type of nitrogenous polyether lactone.¹³⁵

Ciguatera toxins are odorless, tasteless, and cannot be detected by any simple chemical test. A major area of concern has been the development of simple assay methods for the detection of CTX, especially for use in mass screening of fish from endemic areas.¹³⁶ Since CTXs do not possess a distinctive UV chromophore, it is not possible to separate CTX from other lipids present in a crude lipid extract from fish by monitoring the HPLC eluant with a UV detector. HPLC coupled to fluorescence detection provides a high sensitivity method that has the potential to detect natural levels of CTXs in crude

extracts.¹³⁷ Yasumoto and coworkers have reported promising results by labeling CTX with novel coumarin-based fluorescent reagents or the fluorescent 1-anthrolylnitrile, prior to HPLC separation and fluorescence detection.¹³⁸ HPLC coupled to ionspray MS is an alternative to fluorescent detection of CTX in HPLC eluants. If sufficient toxin is available, nuclear magnetic resonance (NMR) and ionspray MS can be used for structure confirmation or for the characterization of unknown toxins once purified.¹³⁷

An immunological approach to examine CTXs was initiated in 1977 with the development of a modified RIA employing sheep anti-CTX antibody.^{139,140} An enzyme immunoassay (EIA) using the same anti-CTX was initiated in 1983.^{141,142} Later, a simplified stick enzyme immunoassay (S-EIA) was performed using correction fluid-coated skewered bamboo sticks.¹⁴³⁻¹⁴⁵ In 1990, Hokama adopted the particulate solid-phase immunobead assay (S-PIA) approach, known as the "paddle test," in dealing with the detection of CTX and related polyethers.¹⁴⁶ Based on the same principle, HawaiiChemtect International developed a commercial kit in which the original format was modified to an innovative rapid S-PIA (Ciguatetect™). This kit can be used for the detection of toxins associated with ciguatera and DSP with application to rapid screening programs of toxic fish and shellfish in harvesting areas and the marketplace.^{61,65,134,147}

CTXs appear to be stored for long periods in fish and humans.¹⁴⁸ Despite that more than 175 ciguatera symptoms have been reported, symptoms occur primarily in four categories: gastrointestinal, neurological, cardiovascular, and general symptoms.¹⁴⁹ This multiphase intoxication is thought to be due to different toxins. Although mortality from ciguatera is low, morbidity is high and symptoms may be debilitating and prolonged.¹⁰³ Diagnosis of ciguatera is based on clinical symptoms and it is sometimes supplemented by the bioassay of the fish involved.¹⁵⁰ Park and coworkers and Gamboa have used S-PIA and ELISA methods for the detection of ciguatera-related toxins and OA in human serum to confirm diagnosis of CFP and possible involvement with chronic fatigue syndrome (CFS).¹⁵¹⁻¹⁵³

An initial intoxication does not confer immunity. Repetition of multiple attacks of CFP results in a clinically more severe illness and more rapid onset of symptoms compared to that of patients experiencing the disease for the first time.^{103,154-156}

Treatment of CFP remains symptomatic.¹⁵⁷ In 1988, intravenous mannitol was reported as a successful treatment of patients exhibiting acute CFP, provided treatment was given within 48 h of the poisoning event.¹⁵⁸ Recent research has found that there may be a correlation between patients who test positive for CFP and those clinically diagnosed with CFS. Many of the symptoms for CFS mimic those for CFP.¹⁵¹

Pufferfish Poisoning

Pufferfish poisoning results from ingestion of the flesh of certain species of fish belonging to the Tetraodontidae family.⁴⁰ TTX occurs in such diverse

animal species such as pufferfish, starfish, Atelopid frogs, Taricha salamanders, octopuses, and two Japanese shellfish.¹⁵⁹ Throughout the world there are about 30 species of pufferfish, distributed widely along the coastline of Japan and in the Pacific Ocean, China Sea, Indian Ocean, and Mediterranean Sea.¹⁶⁰

Marine bacteria which produce TTX have been isolated.¹⁶¹ These include *Vibrio*, *Alteromonas*, *Pleisiomonas*, *Bacillus*, *Micrococcus*, *Moraxella*, *Acinetobacter*, *Aeromonas*, *Alcaligenes*, *Flavobacterium*, *Caulobacter*, *Actinimycetes*, and *Pseudomonas* spp.¹⁶²

TTX is an aminoperhydroquinazoline compound, with a relative mass of 319 Da. It has a guanidium group and a unique intramolecular hemilactal bond. It is unstable at pH levels above 8.5 and below 3.⁵⁵ HPLC fluorometry, mass spectrometry, and capillary isotachopheresis have been applied successfully to identify or quantitate TTX in small volumes of toxin extracted from pufferfish. Using TLC, TTX and derivatives are visualized as a pink spot by spraying the Weber reagent, or as a yellow fluorescent spot under a UV lamp (365 nm).¹⁶³ An assay using neuroblastoma cells for detection of sodium channel-specific marine toxins has been developed by Manger and coworkers.¹⁶⁴

Toxic signs in humans generally appear within 60 min after the ingestion of fish. Nausea and vomiting are common early symptoms. Other symptoms include initial tingling and numbness of lips, tongue, and fingers leading to paralysis of the extremities, ataxia, difficulty in speaking, and finally, death due to respiratory paralysis.⁴⁰

Pfiesteria piscicida Poisoning

Pfiesteria piscicida is a toxin-producing dinoflagellate that has been responsible for killing more than a billion fish in the past decade in the estuaries of North Carolina. It was first identified in the early 1990s by Burkholder and coworkers from the National Marine Fisheries Service (NMFS).^{165,166}

Pfiesteria presents at least 24 flagellated, amoeboid, and encysted stages. It is a dinoflagellate that can photosynthesize if it has obtained the chloroplasts from true algal cells. It can change its eating habits, turning into a predator if fish are around. Although unknown, the cause of this change in behavior is likely to be a substance present in fish oil or excrement. Then, *Pfiesteria* releases its toxins making the fish lethargic. When the fish are dead, flagellated stages transform to amoeboid stages which feed on the fish remains or, if conditions are unfavorable, cells turn into dormant cysts.¹⁶⁵⁻¹⁶⁷ Even though the identity and mechanism of action of the toxins are unknown, two fractions have been isolated from *Pfiesteria* cultures. A water-soluble fraction isolated by a group of the NMFS has been found to be responsible for initially stunning and killing fish. This compound is heat stable and of low molecular weight. The University of Miami isolated a small lipophilic compound which causes the epidermis of fish to slough off. It seems that water eutrophication also plays a role in turning this organism into a predator. It may be due to the stimulation of growth of algae that nontoxic *P. piscicida* feed on.⁴

The human health impacts of *Pfiesteria* include narcosis, development of sores, headaches, asthma-like symptoms, kidney and liver dysfunction, acute short-term memory loss, and severe cognitive impairment, all of which reverse with time. The affected people were in contact with either contaminated water or inhaled toxic aerosols from the cultures.

Freshwater Toxins

Cyanobacterial Toxins

Since the first report of toxic cyanobacteria (blue-green algae) in the late 19th century, studies in several countries have revealed a wide occurrence of toxic cyanobacteria waterblooms.¹⁶⁸ All continents except Antarctica have reported toxic blooms. In the U.S., 27 states have reported the presence of toxic cyanobacteria waterblooms, as well as 16 countries in Europe.¹⁶⁹

There are no known vectors, such as shellfish, that concentrate toxins of freshwater cyanobacteria in the human food chain. However, the decreasing water quality and increasing eutrophication of our freshwater supplies mean that large growths or waterblooms of cyanobacteria are becoming more common.¹⁶⁹

Toxins of cyanobacteria are a relatively new group of biotoxins produced by several genera of fresh water and marine forms.¹⁷⁰ They fall into four classes: hepatotoxins, neurotoxins, nonspecific toxins, and lipopolysaccharides.¹⁶⁹ The main genera responsible for freshwater toxic blooms are the cyanobacteria *Anabaena*, *Aphanizomenon*, *Oscillatoria*, *Gloeostrichia*, *Nodularia*, and *Microcystis*. More than one species within these genera can be toxic and all toxic species can produce waterblooms.¹⁷¹⁻¹⁷⁴

Worldwide, the most common toxins of cyanobacteria involved in acute toxicoses are the hepatotoxins of *M. aeruginosa*. Research indicates that these toxins are peptides with a molecular weight ranging from 500 to 2800 Da.^{175,176} Microcystins are monocyclic heptapeptide hepatotoxins that have been isolated from *Microcystis*, *Anabaena*, *Nodularia*, *Nostoc*, and *Oscillatoria*.¹⁶⁹ To date, at least 40 microcystins have been discovered.¹⁷⁷ Nodularin is structurally similar to microcystins. It is a monocyclic pentapeptide hepatotoxin with a molecular weight of 824 Da.¹⁷⁸

Strains of *Anabaena flos-aquae* and *Aphanizomenon flos-aquae* from the U.S. and Canada continue to be the only proved sources of alkaloid neurotoxins produced by cyanobacteria.¹⁶⁹ Five chemically defined neurotoxins are now known from species of these genera: anatoxin-a, anatoxin-a(s), saxitoxin, neosaxitoxin, and homoanatoxin.^{169,179-182}

There are several similarities between marine toxins and freshwater toxins:

1. Both are waterbased.
2. Both are produced by microorganisms.
3. While retained within the cells to varying degrees, both groups are exotoxins.

4. Both groups of toxins are fast-acting neuro- or organ-compounds absorbed via the oral route.
5. Certain cyanobacteria toxins have structural/functional similarities to certain paralytic shellfish toxins (saxitoxin and neosaxitoxin).¹⁸³

The mouse bioassay is used for a simple determination of cyanobacterial toxins.²⁹ A major disadvantage is that it cannot detect low amounts of toxins or distinguish between different types of neurotoxins or hepatotoxins.^{169,185} Due to these limitations, attempts have been made to develop other types of bioassays using various invertebrates, bacteria, and cultured cell lines.¹⁸⁶⁻¹⁸⁸

The chemical methods developed for the detection of these toxins include HPLC, gas chromatography-electron capture device (GC-ECD), TLC, and fast atom bombardment mass spectrometry (FABMS).^{171,189-191} All of these methods are highly sensitive, but require expensive, sophisticated equipment, and can only be performed in specialized laboratories. Brooks and Codd developed an antibody to microcystin-LR but it had low cross-reactivity with other microcystin variants.¹⁹² Chu and coworkers developed an antibody to all microcystins and nodularin which was used to develop an ELISA.^{193,194}

Direct human contact with toxic blooms has been rare and the present threat to humans is through drinking water supplies, recreational water, and the increasing use of cyanobacteria as a source of single-cell protein.¹⁸³ Cyanobacteria can cause allergic reactions in sensitive persons.¹⁷⁰ In addition to acute lethal poisonings, episodes of dermatitis and/or skin irritation from contact with freshwater cyanobacteria are occurring with greater frequency.¹⁹⁵ Treatment and therapy for cyanobacterial poisoning is limited and largely unavailable for these neurotoxins.¹⁸⁴

Microcystins show tumor-promoting activity through inhibition of protein phosphatases 1 and 2A. Microcystin-LR and -RR were easily decomposed by chlorination with sodium hypochlorite, and the decomposition depended on the free chlorine dose. No noxious products using hepatotoxicity, mutagenicity (Ames test), and protein phosphatase inhibition assays were detected from the chlorination process.¹⁹⁶

Mycotoxins

Animal and human health problems related to food products contaminated with toxic metabolites produced by fungal growth have long been recognized. The Food and Agriculture Organization has estimated that at least 25% of the world's food crops are affected by mycotoxins annually.¹⁹⁷ Thus, many scientific reports have been published concerning the occurrence of mycotoxins in foods and feeds, and their impact on human and animal health. Recently, there have been reports on cocontamination of various toxins, i.e.,

aflatoxin B₁/fumonisin B₁ and ochratoxin A/aflatoxin B₁, among others.¹⁹⁸⁻²⁰⁰ Although more documentation is needed on the levels and effects of mycotoxin cocontamination, it is important to consider that food commodities are a complex environment and that the individual effect of each toxin might be affected by the presence of other toxins or food constituents. This section will briefly discuss the individual mycotoxins that have been considered of primary health significance.²⁰¹

Aflatoxins

Historically, aflatoxins have undoubtedly been the group of mycotoxins of most concern because they have been shown to be both potent hepatotoxins and carcinogens in many species.²⁰² Aflatoxin contamination of foods and feeds occurs when aflatoxigenic species of *Aspergillus* sp. successfully colonize a commodity, grow and find conditions appropriate for toxin production. The three species of *Aspergillus* that produce aflatoxins are *A. flavus*, *A. parasiticus*, and *A. nomius*.²⁰³

A. flavus is a common constituent of the microflora in air and soil throughout the world. It is prevalent in stored wheat, corn, cottonseed, rice, barley, bran, flour, peanuts, soybeans, sorghum, chili peppers, copra, millet, tree nuts, and green coffee beans, among other commodities. Growth can occur even when products are stored under relatively low moisture, which eliminates the growth of competing species such as *Penicillium* and *Fusarium*. However, storage in hot or humid conditions can aggravate toxin formation. Aflatoxin contamination also may be severe when developing crops are exposed to drought conditions.²⁰⁴

Chemically, aflatoxins are defined as a series of 18 known bisulfuran polycyclic compounds that fluoresce strongly in ultraviolet light (ca. 365 nm). Aflatoxins B₁ and B₂ produce blue fluorescence, whereas G₁ and G₂ produce green fluorescence. Four other aflatoxins, M₁, M₂, B_{2A}, and G_{2A} are produced in small amounts. In some animal species, such as dairy cattle, aflatoxins B₁ and B₂ are partially metabolized to give the hydroxylated derivatives: aflatoxins M₁ and M₂, respectively. Other metabolic derivatives are aflatoxin P₁ and Q₁.²⁰⁵

The effect of aflatoxins on animals is quite different depending on age, sex, species, nutritional condition of the animal, dosage level, frequency, and composition of the diet. Sensitivity to the toxins varies greatly from species to species (i.e., the LD₅₀ ranges from 0.5 mg/kg for the duckling to 60 mg/kg for the mouse). Rats, poultry, and trout are highly susceptible to the effects of aflatoxin, whereas sheep, hamsters, mice, and pigs are fairly resistant.²⁰⁶ The organ primarily affected is the liver, but changes can be seen in most organs. The carcinogenicity, mutagenicity, teratogenicity, and acute toxicity of aflatoxins have been well documented.²⁰⁷ AFB₁ is the most important in terms of occurrence and toxicity, and the most potent of the naturally occurring carcinogens.²⁰⁸ In susceptible experimental animals, cancer has been induced in

low doses that are comparable to levels present in contaminated human diets.²⁰⁹

Preharvest prevention of aflatoxin formation is difficult; therefore, aflatoxins in foods and feeds are considered a continuous risk. Discontinuing the use of aflatoxin-contaminated grains and oilseeds is not always practical. There is a need to manage the risks associated with aflatoxin contamination before using these products as animal feed or human food. Thus, several methods for decontamination and postharvest control have been reported.²¹⁰ The use of ammonia-heat treatments has shown effective reduction of aflatoxin.^{211,212} Other chemicals such as monomethylamine, sodium hydroxide, sodium hypochlorite, and hydrogen peroxide also have resulted in acceptable detoxification in several commodities.^{213,214} During fermentative production of ethanol, little degradation of the toxin was achieved.²¹⁴ Other decontamination approaches include food and feed processing such as thermal inactivation, irradiation, solvent extraction, mechanical separation, density segregation, and reduction in bioavailable aflatoxin by selective chemisorption. Biocontrol methods and microbial inactivation have been suggested as well as decontamination procedures.²¹⁵

Aflatoxins have become generally accepted to be poisonous and deleterious, and are now widely regulated in foods. In the U.S., the Food and Drug Administration (FDA) regulates feed and food containing aflatoxins at regulatory levels of 20 ppb of AFB₁ for human foods and selected animal feed. Levels up to 300 ppb are permitted for specific commodities and under selected animal feeding operations, and 0.5 ppb of AFM₁ in milk.^{216,217} In a recent survey, it was determined that 77 countries around the world have regulations for mycotoxins. Most of the existing international mycotoxin regulations are for aflatoxins.²¹⁸

Ochratoxin A

Ochratoxins are a group of related compounds that are produced by *Aspergillus ochraceus* and related species, as well as *Penicillium verrucosum*, and certain other *Penicillium* species.^{219,220} These toxins have been found in corn, wheat, barley, flour, rice, oats, rye, beans, peas, green coffee beans, pancake mix, and mixed feeds.²²⁰

Chemically, ochratoxin A, the main toxic component of the group, is classified as a chlorine-containing pentaketide dihydroisocoumarine linked to L- β -phenylalanine.²²¹ It is a colorless, crystalline compound soluble in polar organic solvents and dilute sodium bicarbonate solution. It is also slightly soluble in water.²²⁰

Ochratoxin A has been associated with porcine nephropathy, and also has been reported to be teratogenic to mice, rats, and chicken embryos. Despite the lack of conclusive evidence, ochratoxin A has been suggested as a possible causative factor in a human disease known as Balkan Endemic Nephropathy.²²²

General stability of ochratoxin A is high. It is not eliminated from grain by cleaning, and upon milling, it is distributed equally between flour and bran.²²³ Studies on the thermal stability of ochratoxin A have shown that there was a 76% reduction of the toxin when samples of white flour were heated to 250°C for 40 min. These studies have reported that ochratoxin A is more stable in humid environments as opposed to aflatoxin B₁.²²³ Paster and coworkers have reported that gamma irradiation (up to 7.5 Mrad) causes no decomposition of ochratoxin A in methanol.²²⁴

Patulin

Patulin is a highly reactive unsaturated lactone (4-hydroxy-4 H-furo [3,2-c] pyran-2 (6H)-one) produced by certain species of *Penicillium*, *Aspergillus*, and *Byssoschlamys*.^{225,226} It is a colorless, crystalline compound soluble in water and polar organic solvents.^{227,228} It is of public health concern because of its potential carcinogenic properties.²²⁹ Patulin contaminates numerous agricultural products that are commonly consumed by both humans and animals. Strains of patulin-producing mold have been isolated from grain, chick starter, malt feed, flour, moldy bread, bakery goods, sausage, cheese, and fruit, but the most common sources have been apples and apple products.²²⁵

Recent efforts have addressed the immunosuppressive action of patulin that has been related to adverse health effects such as ulceration, congestion, and hemorrhagic lesions, particularly in the gastrointestinal tract.^{230,231}

Some of the approaches that have been reported for patulin control include trimming moldy parts, addition of ascorbate to apple juice, alcoholic fermentation, and addition of SO₂. Physical removal of visible moldy spots from apples prior to processing is the best method to reduce patulin in apple products. Some juice processors add a mixture of ascorbate and ascorbic acid to the juice to reduce patulin. This toxin is highly reactive and has been shown to bind to sulfhydryl groups such as cysteine, thioglycolic acid, and glutathione. When patulin is bound to a sulfhydryl group, it becomes biologically unavailable; thus, the potential health risk is reduced.^{223,232}

Deoxinivalenol

Deoxinivalenol (DON) is the most common of over 50 identified trichothecenes toxins. Trichothecene mycotoxins are mold metabolites produced by *Fusaria* sp. They are chemically defined as sesquiterpenes characterized by a double bond at position C-9, an epoxide ring at C-12, and various patterns of hydroxy and acetoxy substitutions.²³³ These compounds are of concern because of their frequent presence in agricultural commodities such as wheat, corn, barley, and oats.²³⁴

Some of the adverse health effects for contaminated crop consumption include reduced weight gain and feed consumption, feed refusal, diarrhea,

emesis, immune suppression, gastrointestinal irritation, oral lesions, and death.²³⁵ Epidemiological data associated with human mycotoxicoses in Japan, China, and India determine deoxinivalenol-contaminated grain products as the probable causative agent.²³⁶

It has been reported that cleaning and polishing can remove approximately 25% of DON in wheat, but 60 to 80% of the toxin remains in the flour.^{237,238} In a corn wet milling study, it was shown that most of the DON went into the steep liquor, although detectable amounts remained in the starch.²²³ Sodium bisulfite has proved to be effective in reducing deoxinivalenol concentrations in contaminated grains; other effective chemical treatments include 30% chlorine gas and ammoniation.²³⁹ Current regulatory guideline levels for deoxinivalenol are 2.0 µg/g in uncleaned soft wheat used for nonstaple foods including bran, except for soft wheat destined for infant food where the guideline level is 1.0 µg/g.²⁴⁰

Citrinin

Citrinin is a secondary metabolite produced by *Penicillium citrinum* and *P. viridicatum*, that usually accompanies ochratoxin A; it is also a metabolite of some *Aspergillus* species. This metabolite is an optically active, yellow crystalline compound fairly heat stable in solution in 95% ethanol or *n*-hexane, but not in acid or alkaline solution. Citrinin is an unstable mycotoxin in grains and apple juice, so it degrades at a fast rate.²²⁰ The most commonly affected commodities are mixed barley, oats, corn, and yellow peanut kernels.²²¹

Citrinin has been shown to bind to human serum proteins *in vitro*. However, there is no evidence that it interacts with DNA. Citrinin has been related to kidney damage in laboratory animals and may be involved in cases of swine nephropathy. Some studies have addressed its potential for immunotoxicity.²³⁴

Fumonisin

Fumonisin are the most recently characterized toxins produced by *Fusarium moniliforme*. Although other *Fusaria* sp. produce fumonisins, *F. moniliforme* section *Liseola* is the most toxigenic. Reports of fumonisin naturally contaminated animal and human feeds occur worldwide. In the U.S., it has been estimated that *F. moniliforme* contaminates between 80 to 100% of all corn harvested.²⁴¹

Fumonisin are a group of diester compounds with different polyhydric alcohols and tricarboxylic acids of which fumonisin B₁ has been reported to be the most toxic. These toxins contain a primary amine moiety and are water soluble and heat stable.²⁴²⁻²⁵¹ There are several fumonisins; however, only fumonisins B₁, B₂, and B₃ have been found in significant amounts in both natural and laboratory conditions.^{245,246}

Fumonisin B₁ has been associated with a wide range of syndromes such as equine leucoencephalomalacia (ELEM), porcine pulmonary edema (PPE), hepatocarcinogenicity in rats, hepatotoxicity in poultry, and acute congestive heart failure in baboons and monkeys.²⁴³⁻²⁴⁸ In humans, fumonisins have been epidemiologically linked to human esophageal cancer. Recently, the International Agency for Research on Cancer classified *F. moniliforme* toxins as potential carcinogens (class 2B carcinogens) to humans.²⁵²

The toxicity of fumonisin B₁ has been related to its effect on sphingolipid metabolism. Some of the phenomena related to fumonisin toxicity include the inhibition of *de novo* sphingosine biosynthesis, accumulation of free sphinganine, depletion of complex sphingolipids, increase in degradation products from catabolism of free sphingoid bases, increase in lipid products derived from the increase in sphingoid-base degradation products, and increase in sphingosine.²⁵³ All of these effects lead to a cascade of diverse biochemical events which eventually result in a wide variety of toxicoses. After a fumonisin outbreak of ELEM in Virginia, the Virginia Department of Agriculture suggested some preventive measures that include (1) avoid feeding corn as a sole ration to horses; (2) if corn is the sole ration, buy from dealers who test for fumonisins; and (3) if a supply of corn on hand is to be fed to horses, it should be tested for fumonisins.²⁵⁴

Some industrial processes hydrolyze the tricarboxylic acid chains of fumonisins; however, it has been reported that hydrolyzed fumonisin (HFB₁) presents higher toxicity than fumonisin B₁ itself.^{251,255} Furthermore, cocontamination with aflatoxins has been reported, and the methods that are commonly used for aflatoxin control, i.e., ammoniation and fermentation, are not as effective for fumonisin detoxification.²⁵⁶⁻²⁵⁸ Physical separation has shown good potential in reducing fumonisin. In a dry milling study, Katta et al. found that dry milling of corn resulted in a concentration of fumonisins in fractions such as germ, bran, and fines. Some of these fractions are frequently used to produce animal feed; therefore, they present an increased risk for animal health. This same study showed that the flaking grits that are widely used as breakfast cereal and snack foods presented lower amounts of fumonisin, so there is a decreased risk for humans.²⁵⁹ As mentioned before, aflatoxins and fumonisins have been found to cocontaminate corn, so potential toxic interactions are currently under study.

T-2 Toxin

T-2 toxin, a trichothecene mycotoxin produced by *Fusaria* sp., is primarily associated with moldy millet, but also with wheat, rye, oats, and buckwheat. Due to its lipophilic nature, T-2 toxin appears to be transmitted to milk when dairy cattle are fed contaminated grains.²⁶⁰ This toxin has been shown to be an inhibitor of protein and DNA synthesis in mammalian cells, a potent dermal irritant, and an impairing immune function agent. It is cytotoxic and has a radiomimetic effect on rapidly dividing cells.^{235, 261-263}

T-2 toxin has been implicated in alimentary toxic aleukia disease (ATA) outbreaks in the former Soviet Union, and in cases of pellagra.²³⁶ It has been reported that repeated exposure of experimental animals to T-2 toxin or diaeoxyscirpenol results in markedly increased susceptibility to Gram-negative bacteria and herpes simplex virus. When T-2 toxin is coadministered with lipopolisaccharides (LPS), there is a marked increase in susceptibility to LPS. This effect suggests that impaired resistance to LPS might be one mechanism to increased susceptibility to Gram-negative bacteria.²³⁴

Experimental wet milling of corn contaminated with T-2 toxin showed that 67% of the toxin was removed by the steep and process water.²⁶⁴ Studies with mycotoxin-binding agents have shown that bentonite and spent canola oil bleaching clays appear to be effective in decreasing the toxicity of feed containing T-2 toxin. These agents adsorb the toxin present in the diet and inhibit its absorption in the gastrointestinal tract.²⁶⁵

Zearalenone

Zearalenone (ZEN) is produced by *Fusarium graminearum* and *F. sporotrichoides* in the field and during storage of commodities such as corn, barley, pig feeds, silage, sorghum, and hay. Chemically, zearalenone is (R,S)-2, 4-dihydroxy-6-(6'-(6'-oxo-10'-hydroxy-1-undecenyl) -benzoic acid unlactone.²⁶⁶

Hyperestrogenism is the most common biological effects associated with zearalenone. Swine appear to be the most sensitive of the domestic animals. However, cattle, other ruminants, and sensitive species of poultry have been reported to present ZEN-related hyperestrogenism.²⁶⁷ The results of carcinogenicity bioassays for ZEN in rats and mice demonstrate "positive evidence of carcinogenicity."²⁶⁷ However, further studies are required to confirm whether ZEN should be considered a potential human carcinogen.²³⁸ Although ZEN and its metabolites have been shown to be transferred to milk, there is limited evidence that the carryover levels pose a potential risk.²⁶⁸

Simple cleaning can physically remove ZEN from contaminated grains. It has been reported that removing the outer portion of the kernel in a dehuller results in a 40 to 100% reduction.²³⁸ Also, simple washing using distilled water reduced 2 to 61% ZEN from contaminated barley and corn. Chemical treatments such as the use of formaldehyde treatment, calcium hydroxymonomethylamine, or calcium hydroxide also reduce ZEN.^{269, 270}

Bacterial Toxins

In the U.S., the cost due to foodborne diseases is \$8.4 billion (84% represented by bacterial and viral diseases). It has been estimated that campylobacteriosis costs \$156 million; *Clostridium perfringens* enteritis, \$123 million; and *E. coli*

infections, \$223 million. Botulism has a high cost per case, but its total impact is only \$87 million because relatively few cases occur.²⁷¹

This section discusses only pathogenic bacteria where the human illness is associated with foodborne toxins. According to the Council for Agricultural Science and Technology, *Bacillus cereus* (diarrheal type), *Clostridium perfringens*, enterohemorrhagic and enterotoxigenic *Escherichia coli*, and *Vibrio cholerae* are the toxicoinfecting bacteria frequently documented as foodborne disease agents in the U.S.²⁷² The toxins produced by these organisms require linkage to and/or invasion of the intestinal epithelial cells and damage to specific cells. On the other hand, *Bacillus cereus* (emetic type), *Clostridium botulinum*, and *Staphylococcus aureus* are toxicogenic bacteria which synthesize toxins in the food, and their presence is not required to cause illness. Other bacteria associated with foodborne diseases such as *Salmonella* species are not discussed in this chapter.

Toxicoinfections

Bacillus cereus, a Gram-positive, facultative aerobic, spore-forming rod produces two types of toxins. One is a heat-labile, large molecular weight protein which produces effects that are similar to those caused by *Clostridium perfringens*.^{273,274} Diarrhea is the primary symptom and the disease is a toxicoinfection. The other toxin is a heat-stable, low molecular weight peptide which produces a severe emetic (vomiting) reaction, referred to as *B. cereus* emetic intoxication. *B. cereus* has caused foodborne diseases in Europe since the 1950s, but it was not thought to be the cause of food poisoning in the U.S. until the 1970s.²⁷³

B. cereus (diarrheal type) causes toxicoinfection through toxins produced in the intestinal tract. It is a mild, self-limiting disease of a 1-day duration which involves nausea, abdominal cramps, diarrhea, and some vomiting.^{273,275} Two enterotoxins have been purified. Hemolysin BL (HBL) (described by Beecher and MacMillan)²⁷⁶ and a single protein (described by Shinagawa et al.) are both cytotoxic.²⁷⁷ While a serological method has been developed for the diarrheal type toxin, animal models or cell culture methods are better suited for the vomiting type toxin.

Campylobacter jejuni is recognized as a major cause of human diarrhea throughout the world. It is the most common cause of diarrhea in children of developing countries.²⁷⁸ Implicated vehicles of transmission are undercooked chicken, processed turkey, cake icing, raw clams, and drinking water.²⁷³ Symptoms vary from insignificant enteritis to enterocolitis with abdominal pain and profuse diarrhea, usually malaise, fever, vomiting, and, in extreme cases, grossly bloody stools. It is suggested that *C. jejuni* is composed of numerous clones distributed worldwide.²⁷⁹ Some strains of *C. jejuni* produce a cytotoxin.²⁷³

C. perfringens, a Gram-positive, nonmotile, spore-forming, anaerobic rod, is a normal inhabitant of the large intestine of man and animals. Spores of the

organism persist in soil, dust, and foods (raw meat, poultry, fish, and vegetables) subject to fecal pollution.²⁸⁰ Symptoms are nausea, abdominal pain, and acute diarrhea, and are the result of the fluid accumulation in the intestinal lumen when an enterotoxin is released in the gut during sporulation of the consumed cells.^{273,275,280} Sporulation in the digestive tract is associated with toxin production. There are five types of *C. perfringens* (A, B, C, D, E), distinguished by the toxins they produce. The A-type toxin (phospholipase C) is the most important and the agent causing gastroenteritis.^{275,280} *C. perfringens* A-toxin is a cytolysin that produces hydrolysis of membrane phospholipids. Cytolysins are produced by a wide variety of Gram-positive and Gram-negative bacteria. They damage cell membranes causing lysis and cell death. The A-toxin is dermonecrotic, hemolytic, and lethal to cells in culture. Highly purified A-toxin has been shown to cause platelet aggregation and to increase vascular permeability in guinea pig skin.²⁸¹ Molecular weight values reported for purified A-toxin by different authors differ between 30,000 and 54,000. Diagnosis of *C. perfringens* by its symptoms is confirmed by detecting the toxin in the feces.

Escherichia coli is part of the normal flora of the intestinal tract of humans and other warm-blooded animals. Foodborne diarrheagenic *E. coli* are grouped into four categories according to virulence properties, clinical syndromes, differences in epidemiology, and distinct O:H serogroups. They are enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enterohemorrhagic *E. coli* (EHEC), and enteroinvasive *E. coli* (EIEC). It is known that some EPEC strains produce toxins, particularly verotoxins.²⁸² Verocytotoxin, which can create a life-threatening situation, is thought to be the causative agent that results in bloody diarrhea and severe abdominal pain. Vomiting may occur but fever is rarely seen. ETEC strains produce toxins associated with mild to severe diarrheal illness. ETEC adhere to small intestinal epithelial cells by means of fimbrial adhesins (named "colonization factor antigens") and producing one or more enterotoxins belonging either to the heat labile family (LT-1, LT-2) or the heat stable family (STa, STb).^{282,283} Analysis of foods may involve enrichment and plating for isolation of toxigenic strains with confirmation by toxin assays or direct analysis for ETEC by gene probes.

EHEC strains, including *E. coli* O157:H7 and *E. coli* O26:H11, produce toxins that cause severe damage to the lining of the intestine. The toxins produced are verotoxin and Shiga-like toxin.^{282,284} They are closely related or identical to the toxin produced by *Shigella dysenteriae*. Manifestations of illness related to *E. coli* O157:H7 toxicoinfection include hemorrhagic colitis, hemolytic uremic syndrome, and thrombotic thrombocytopenic purpura.²⁸⁵ The mechanism by which this microorganism causes illness has not been completely defined, but verotoxins and adhesion of the organism to intestinal cells have been associated with *E. coli*'s virulence.²⁸² Transmission of the illness is primarily due to food, but person-to-person transmission also has occurred in some *E. coli* O157:H7 outbreaks. The organism is heat sensitive, but it can survive in ground beef during frozen storage for several months.²⁸⁶ Hemorrhagic colitis can be diagnosed by isolation of the

verotoxin-producing *E. coli* from diarrheal stools or by testing the stools for the presence of verotoxins (VT1 and VT2). Use of DNA probes that detect verotoxin-encoding genes is the most sensitive approach.

The genus *Vibrio* contains 28 species, 10 of which may cause illness in humans.^{273,287,288} The infection, cholera, is usually spread by human excrement that contaminates food and water.^{273,288} Persons affected with this microorganism may be asymptomatic, may have only mild diarrhea (often mistaken), or may have profuse, watery diarrhea due to the production of an enterotoxin (cholera toxin (CT)) excreted in the small intestine where colonization occurs.²⁸⁷ CT binds to mucous membranes lining the intestinal tract, stimulating secretion of fluid and electrolytes from the intestinal walls.²⁸⁸ *V. cholerae* neuraminidase plays a subtle but definite role in the pathogenesis of this organism, enhancing the biological effect of CT when CT production is low.²⁸⁹ An evolutionary relatedness between the enterotoxins of *Salmonella typhimurium* and *V. cholerae* was suggested by Prasad et al.²⁹⁰

An enterotoxic *V. cholerae* strain (*V. cholerae*-01 and some non-01) must be able to survive the acidity of the stomach and small intestine before colonization and excretion of CT which produce the diarrhetic response associated with cholera.²⁸⁷ Non-01 gastroenteritis has been associated with the consumption of seafood, exposition to polluted freshwater, brackish water, or seawater.²⁹¹ With the non-01 type, diarrhea occurs in all cases and is far more common in the U.S., but less severe than illness associated with 01 strains.^{273,287} Shellfish are more likely to be a source of infection than water, since shellfish are filter-feeding organisms, thereby capable of concentrating bacteria.²⁹² The easiest and most meaningful way to demonstrate the pathogenic potential of a *V. cholerae* food isolate is demonstration of CT production. There are several methods developed to determine CT, including ELISA, solid-phase sandwich radioimmunoassays, and DNA methodologies.²⁸⁷

V. vulnificus is a dangerous vibrio associated with marine environments. Its primary vehicles are raw or undercooked seafood, particularly oysters and clams. The organism is highly invasive in humans, releasing both a hemolysin and a cytotoxin, and can result in primary septicemia.^{273,275}

Intoxications

Bacillus cereus (emetic) produces a heat-stable toxin of severe emetic (vomiting) reaction accompanied by gastric pain between 1 to 6 h after food ingestion. The illness usually lasts 6 to 24 h.²⁷³

Clostridium botulinum produced 50% of foodborne mortality from outbreaks between 1899 and 1973. The mortality rate decreased substantially later with the use of antitoxin and better recognition of the symptoms. The U.S. food industry insists that foods involved in botulism (sausages and other meats, fish, vegetables, and fruit products) be handled with extreme care.^{273,293} Neither the organism nor its spores are harmful, but the toxin, a heat-labile high molecular weight protein produced during growth of the organism under anaerobic conditions, is very lethal. Just a few nanograms of

the toxin can cause illness. The toxin can be destroyed if heated (80°C for 10 min). Seven immunogenic types (A to G) of the toxin have been identified, with the most common blocking the release of acetylcholine at the synapse. They represent the most potent poisons known, and have neuro-, entero-, and hemotoxic properties with varied susceptibility according to the animal species. Types A, B, and E toxins often cause human botulism, while types C and D cause animal botulism. Type F has rarely been involved in human botulism.²⁹³ A monoclonal antibody-based ELISA was developed for detection of *C. botulinum* type E toxin in implicated foods.²⁹⁴ The most sensitive and common method for detecting the toxin is the mouse neutralization test.

Staphylococcus aureus is the microorganism responsible for the second most commonly reported foodborne disease in the U.S. One third of all foodborne illness in this country is attributed to this microorganism.^{273,295} The very unpleasant, but not fatal, staphylococcal food poisoning (staphyloenterotoxigenosis, staphyloenterotoxemia) is an intoxication which results from the ingestion of enterotoxins produced by this pathogen within various foods.²⁹⁵ A toxin dose of less than 1.0 µg produces the symptoms. Staphylococcal enterotoxins are a family of structurally related 28,000 molecular weight proteins.²⁹⁶ *S. aureus* produces five different cytolysins (designated SEA through SEE).²⁹⁷ B-toxin (sphingomyelinase C) generates hydrolysis of membrane phospholipids, D-toxin acts as a detergent-like compound, and the precise mechanisms of A-toxin and leucocidin are not yet completely established.²⁸¹ B-toxin was the first bacterial toxin that was shown to be an enzyme. It is a Mg²⁺-dependent phospholipase C with a substrate specifically confined to sphingomyelin and lysophosphatidyl choline. The toxin is a cationic protein with a molecular weight of approximately 30,000.²⁸¹ Hot-cold hemolysis is one of the most remarkable features of *S. aureus* B-toxin. Incubation at 37°C of small quantities of B-toxin with sensitive erythrocytes in the presence of Mg²⁺ ions results in little or no lysis. However, if the treated erythrocytes are taken to temperatures below 10°C for a short period of time, lysis occurs quickly.²⁸¹ Reverse phase HPLC has been shown to rapidly purify SEB, which may alter epithelial cell transport by direct effects or by indirect mechanisms mediated via the submucosa or some other gastrointestinal-associated cell type.²⁹⁸

Staphylococcal A-toxin, a very heat-resistant protein, is the most potent of the four hemolytic membrane-damaging toxins produced by this organism. The LD₅₀ dose in mice is 1 µg and in rabbits is 4 µg. In addition, A-toxin is a hemolytic and dermonecrotic and has been shown to affect many cells and tissues. Estimates of molecular weight vary from 26,000 to 30,000.^{275,281} Heating the food after the toxin is present does not ensure safety. It is impossible to detect the toxin in the food by appearance, taste, or smell.²⁷³ Although the precise mode of action of A-toxin on natural and artificial membranes is still unknown, there is general agreement regarding the following aspects:

1. Binding of the toxin to the cell membrane and the formation of functional lesions in the membrane are separate events.

2. Release of marker molecules from toxin-treated cells is prevented in osmotically stabilized media.
3. Toxin possesses an intrinsic surface activity. It forms solid films on aqueous media, penetrates lipid monolayers and lyses liposomes prepared from either mixtures of lecithin and cholesterol or the extracted lipid from erythrocytes.
4. At high concentrations (10 to 100 $\mu\text{g}/\text{ml}$) the toxin forms hexameric ring structures on erythrocyte ghosts and liposomes.
5. Specificity of the toxin for certain membranes probably does not depend solely on the lipid composition of the membrane, as liposomes prepared from lipids extracted from sensitive rabbit and resistant human erythrocytes exhibit similar sensitivity to the toxin.²⁸¹

D-toxin, a "surface agent," is cytolytic in a wide variety of membrane systems, including bacterial protoplast and spheroplasts, erythrocytes, tissue culture cells, lysosomes, and liposomes. D-toxin action is characterized by a rapid rate of lysis and the absence of a lag phase.²⁸¹ The primary structure of the toxin consists of 26 amino acid residues with a largely hydrophobic core. Symptoms may occur within 1 to 6 h after eating. They include nausea, vomiting, retching, abdominal cramping, diarrhea, headache, weakness, chills, and fever. The illness lasts until the toxin is expelled from the system, usually in 24 h. The toxins show an immunosuppressive effect *in vivo*. It has been postulated that immune problems such as systemic lupus erythematosus, rheumatoid arthritis, or allergic reactions may be caused even by subemetic amounts of toxin.²⁷³

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6

Natural Toxins and Chemopreventives in Plants

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Introduction

Our food contains, in addition to the many well-known major (protein, fat, carbohydrate, and fiber) and minor (vitamins, minerals, and nonessential compounds) nutrients, thousands of naturally present toxic plant compounds. Some are known or strongly suspected to cause cancer in laboratory animals and, thus, may be potentially carcinogenic in people. Many of these compounds are commonly termed “nature’s pesticides” because they are often toxic to predators, such as insects and animals, thereby conferring a competitive advantage to the plant that produces them. Other natural toxins in plants have no known role. Although these chemicals are in every meal we eat, they have received little attention compared to that given to minute residues of synthetic chemicals such as PCBs and pesticides. Our food contains significantly greater amounts of natural plant toxins and carcinogens than the synthetic kind, and our bodies aren’t able to distinguish between the two. Still, while popular notion remains that “natural is good,” it is clear that natural toxins pose a far greater health risk than that posed by synthetic chemicals in our foods.

Fortunately, our food also contains natural chemicals that can counteract the adverse effects of many natural and synthetic toxins. While much more work on these “antitoxins” or “chemopreventives” is needed, the data thus far are very encouraging that some plant foods can actually reduce the incidence of certain types of cancer. Hundreds of animal and epidemiological studies have identified several foods or specific compounds that offer protection against the carcinogenic effects of a wide variety of natural and synthetic chemicals. A few compounds have been shown to actually reverse the carcinogenic process in animals. As might be imagined, the field of anticarcinogenesis is one of the most exciting areas in nutritional toxicology and cancer research.

Natural Plant Toxins in Foods

The following is a survey of some of the most well-studied and characterized plant toxins.

Canavanine

Despite the notion that they are the ultimate health food, alfalfa sprouts contain up to 15,000 ppm canavanine. Canavanine is produced in other legumes as well, such as the jack bean. It is an analog of arginine and, as such, can substitute for this amino acid in cellular proteins, thereby compromising their function. Canavanine inhibits the enzyme nitric oxide synthetase¹ and

induces heat-shock proteins in human cells *in vitro*.² Due to its action as an antimetabolite, it is under current consideration as an antitumor drug in combination with other antimetabolites such as 5-fluorouracil,³ but has not yet been tested for carcinogenicity. Canavanine is suspected of causing autoimmune disorders in people, such as lupus erythematosus.⁴ Primates fed alfalfa sprouts develop a severe toxic syndrome resembling human lupus.

Cyanogenic Glycosides

Cyanogenic glycosides are cyanide-containing compounds naturally present in seeds from apples, apricots, cherries, peaches, pears, plums, quinces, and also in almonds, sorghum, lima beans, cassava, corn, yams, chickpeas, cashew nuts, and kirsch. High cyanide varieties, distinguished by their bitter taste, may contain over 600 ppm cyanide on a dry weight basis, while “sweet” varieties contain much less.⁵ There are several such cyanogenic glycosides, of which linamarin, amygdalin, and dhurrin are examples (Figure 6.1). In the 1970s, amygdalin, as laetrile, gained notoriety as a fad remedy and preventative for cancer and other ailments. Cyanogenic glycosides are toxic by virtue of the release of free hydrogen cyanide which occurs when the plant tissue is disturbed as during chopping, processing, or ingestion. These conditions initiate the hydrolysis of the glycoside by the action of β -glucuronidases and other enzymes naturally present in the plant tissue and in the intestinal lumen. The process also can be initiated by acid, but this doesn't appear to occur in the digestive tract to any great extent despite the acid environment in the stomach. Hydrolysis by β -glucuronidases produces the sugar and a cyanohydrin, the latter spontaneously or enzymatically degrades to form free hydrogen cyanide. The scheme of release of hydrogen cyanide is depicted in Figure 6.1.

Cyanide is one of the most acutely toxic chemicals. It binds to and inactivates heme enzymes, the most critical of which is mitochondrial cytochrome oxidase, resulting in an acute, life-threatening anoxia. The two-step therapy is initiated with sodium nitrite, which induces methemoglobinemia permitting the release of cyanide from heme proteins, followed by sodium thiosulfate, which acts as a substrate for rhodanese, an endogenous hepatic enzyme that catalyzes the conversion of free cyanide to the less toxic thiocyanate.

Cases of acute human poisoning from the cyanide released from certain varieties of lima beans, cassava, and bitter almonds are a regular occurrence.⁶ Due to the importance as a subsistence crop in Africa and South America, cyanogenic glycosides in cassava probably represents the greatest health risk. Traditional methods of processing cassava, such as sun-drying, soaking, boiling, and fermenting, eliminate most of the cyanide.⁷ In addition to regular cases of human deaths, cyanogenic glycosides in cassava may be responsible for birth defects, endemic goiter,⁵ and “konzo,” an upper myelopathic motor neuron disease endemic to East Africa.⁸ Cyanogenic glycosides also have been implicated as a causative agent of diabetes. The risk associated with

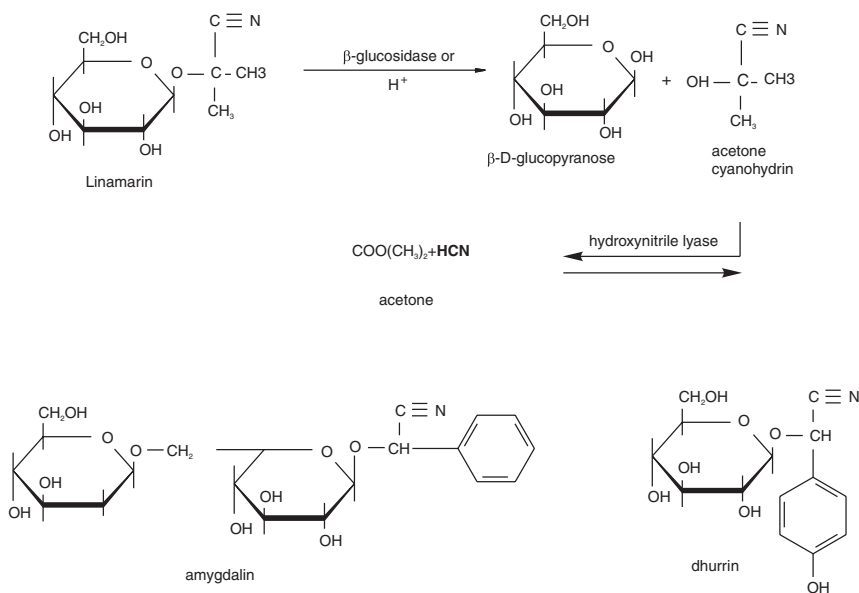


FIGURE 6.1

Mechanism of formation of cyanide from linamarin. Chemical structures of amygdalin and dhurrin.

cyanide poisoning due to cassava is negligible in the U.S. because associated products (such as tapioca pudding) are rarely consumed here.

Allyl Isothiocyanates

Allyl isothiocyanates are a group of major naturally occurring compounds that confer a pungent flavor to foods, such as mustard and horseradish, where it is present at about 50 to 100 ppm. It is also present at much lower levels in Brassica vegetables such as broccoli and cabbage, and in cassava and other tropical staple foods. In high doses, it is carcinogenic in rats, but it is nonmutagenic in bacteria. Isothiocyanates occur in cruciferous vegetables as glucosinolate conjugates that are hydrolyzed when the plant releases enzymes such as during chewing (Figure 6.2). Isothiocyanates are toxic goitrogens which inhibit binding of iodine in the thyroid gland. Because iodine is required for the formation of the critical thyroid hormones thyroxine (T_4) and triiodothyronine (T_3), isothiocyanate-induced hyperthyroidism (goiter) mimics iodine deficiency. Hyperthyroidism is a physiological response as the thyroid attempts to compensate for reductions in both T_4 and T_3 production.

Normal dietary exposures to isothiocyanate-containing foods releases milligram amounts of isothiocyanates. As in the case of cyanogenic glycosides, normal processing steps (chopping, rinsing, milling) results in a safe product.

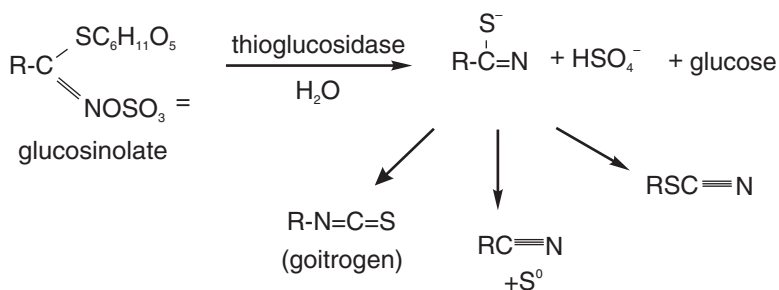


FIGURE 6.2

Enzymatic formation of goitrogenic isothiocyanate from glucosinolate.

Endemic goiter is seen in geographical areas like India and Africa, where consumption of poorly processed foods is coincident with iodine deficiency.

Hydrazines and Other Toxins in Edible Mushrooms

The three most commonly eaten mushrooms are the cultivated mushroom (*Agaricus bisporus*), the shiitake mushroom (*Cortinellus shiitake*), and the false morel (*Gyromitra esculenta*). All contain substantial amounts of compounds in the hydrazine family (Figure 6.3), many of which are potent liver toxins and animal carcinogens. N-methyl-N formylhydrazine is commonly found in concentrations of 500 ppm and causes lung tumors in mice. It is carcinogenic in hamsters as well. People consuming a 100 g serving and, therefore, ingesting 50 mg would be getting very nearly the same dose on a per kilogram (kg) body weight basis as that giving cancer to mice upon sustained daily exposure.

Shiitake mushrooms and the cultivated mushroom contain up to 3000 ppm agaritine. A metabolic product of agaritine (a diazonium derivative) is a

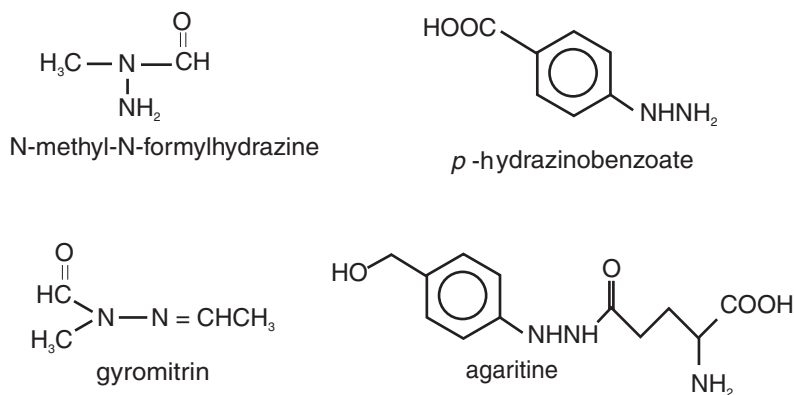


FIGURE 6.3

Carcinogenic hydrazines in commercial mushrooms.

potent carcinogen and a mutagen. Gyromitrin (acetaldehyde-N-methyl-N-formylhydrazine), the major carcinogenic hydrazine in the false morel, also is present in similar concentrations. Other carcinogenic hydrazines include *p*-hydrazinobenzoate (present in *A. bisporus* at 10 ppm) and 4-(hydroxymethyl) benzenediazoate (HMBD), the latter shown to induce DNA strand breaks presumably through a carbon-centered, free-radical intermediate, a possible mechanism of the carcinogenic action of hydrazines in general.⁹ Another carcinogenic hydrazine, methylhydrazine, is present in smaller concentrations (14 ppm). Whole mushrooms have been shown in numerous studies to cause cancer in laboratory animals, but whether they are a significant cause of cancer in people is uncertain. Recently, a diet of whole *A. bisporus* mushrooms (30% of total diet) did not cause a significant increase in tumors compared to controls in rats.¹⁰

Toxic Substances in Spices and Flavoring Agents

Safrole, estragole, myristicin, β -asarone, piperine, and isosafrole (Figure 6.4) are closely related alkenylbenzenes found in many spices, essential oils, and herbs. They also are present, in much lower levels, in parsnips, parsley, and sesame seeds. All are weak to moderate rodent hepatocarcinogens.

Safrole is found in sassafras tea and makes up 85% of oil of sassafras (*Sassafras albidum*),¹¹ which was once used to flavor root beer. It has been banned as a flavor additive since 1960, but is a minor, natural component of nutmeg, mace, star anise, cinnamon, and black pepper. Sassafras bark is an ingredient in filé powder used to make gumbo, a spicy Cajun dish. Estragole, a related aromatic flavor agent, is found in tarragon, basil, and fennel, and is likewise a weak carcinogen. Safrole is bioactivated to a DNA-binding species via hydroxylation of benzyl carbon, conjugation with sulfate, and then alkylation of DNA with displacement of the sulfate group.¹² Another route of bioactivation involving a rearrangement to electrophilic quinone methides has been identified for safrole and is presumed to occur with related flavor compounds.¹³ Epoxidation at the allylic side chain is another activation route identified for safrole, estragole, and eugenol. Epoxide intermediates of these compounds degrade to form covalent adducts with guanine *in vitro*.¹⁴

Isosafrole, a component of ylang-ylang (*Cananga odorata*) oil, a flavorant and scent, is carcinogenic in mice. Many of these alkenylbenzenes interact with cytochrome P-450 (CYP) mediated metabolism. For example, both isosafrole and safrole are powerful inducers of 1A family CYP enzymes. Safrole and isosafrole also inhibit CYP 2E1 enzymes and, in so doing, protect against carbon tetrachloride liver toxicity in mice.¹⁵ Piperonyl butoxide, a related synthetic alkenylbenzene, is a commercial CYP inhibitor used as a synergist with pyrethroid and carbamate insecticides.

β -asarone is a major component of oil of calamus (derived from the *Acorus calamus* root which is a folk remedy for indigestion), and was once used to flavor vermouth and bitters. It causes intestinal tumors in rats.

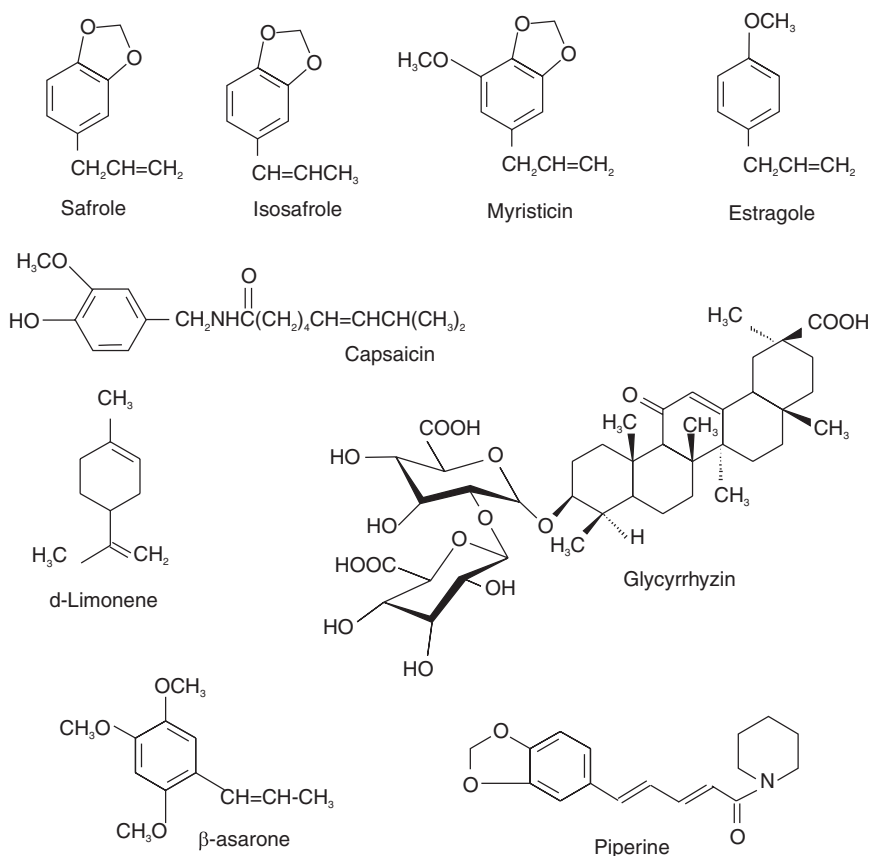


FIGURE 6.4
Natural toxins in spices and flavoring agents.

Myristicin is a major flavor component of nutmeg, derived from the dried, ripe seed of the tree *Myristica fragrans*. Approximately 2% of nutmeg is myristicin, which is present in the volatile oil distilled with steam from the dried seeds. Mace, a closely related spice, is derived from the arilloide, or outer coating of the seed. The world's principal commercial supply of nutmeg is grown in the Malay peninsula. Myristicin is found in black pepper, parsley, celery, dill, and carrots as well. While not thought to be carcinogenic, large amounts of nutmeg, equivalent to two whole nutmeg seeds (ca. 15 g) are intoxicating and allegedly hallucinogenic. However, large doses also are associated with undesirable side effects, such as tachycardia, flushed skin, and dry mouth. Pure myristicin is not as hallucinogenic as nutmeg. Thus, it is assumed that other components in nutmeg may contribute to its potential psychoactive properties.

Piperine, an alkaloid present in high concentrations (10%) in black pepper (*Piper nigrum* and other sp.), is largely responsible for the pungent "bite" of

this condiment. Powdered *P. cubeba* berries are added to cigarettes and smoked as a remedy for throat irritation, and oil derived from these berries is added to some throat lozenges. Reports of the cancer-causing ability of this compound are conflicting. Extracts of black pepper caused cancer in mice at several sites in skin painting tests, while orally injected piperine did not.¹⁶ Furthermore, piperine is not mutagenic in a number of *in vitro* screening assays.¹⁷ However, under appropriate conditions, piperine is chemically converted to potentially carcinogenic intermediates. In the presence of nitrite, piperine is nitrosated to form highly mutagenic nitrosamine intermediates *in vitro*, which may have potential carcinogenic activity. Like the related alkenylbenzenes, piperine also affects CYP expression and activity. For example, piperine specifically inhibits CYP 2E1, while specifically inducing the expression and activities of CYP 1A and 2B.¹⁸

Capsaicin is the extremely pungent ingredient (up to about 0.5%) in red and yellow chili peppers: *Capsicum frutescens*, *C. conoides*, and *C. annum*. Due to its irritating qualities to the eyes and mucous membranes, a solution of capsaicin in an aerosol spray is a popular dog repellent for mail carriers. Topical creams containing capsaicin (0.025%) are commercially available as an analgesic. Although its pain relieving qualities are debatable, it has been shown to cause a local depletion of *substance P*, an endogenous neuropeptide known to transmit pain impulses. Thus, even though the physiological conditions causing pain may persist, capsaicin prevents pain impulses from reaching the brain.

Some evidence suggests that capsaicin is a weak carcinogen. It is a bacterial mutagen in the Ames test and causes benign digestive tract adenomas in mice with life-long dietary exposure at 0.03%.¹⁹ Intraperitoneal injections of capsaicin causes the formation of sister-chromatid exchanges and micronuclei in mice.²⁰ Sister-chromatid exchanges and micronuclei are genotoxic endpoints presumably associated with cancer risk. One possible toxic mechanism is that CYP 2E1 converts capsaicin to an active phenoxy radical intermediate that has the potential for alkylating tissue macromolecules such as DNA and protein.²¹

Glycyrrhizin is a saponin-like glycoside derived from the dried roots of *Glycyrrhiza glabra*, popularly known as licorice. Licorice is one of the oldest folk medicines traditionally used as an expectorant, flavoring agent (also used to mask the bitter taste of medicines), and demulcent. Cuneiform tablets dating to about 4000 B.C. mention the medicinal use of licorice by the Sumerians, and pieces of licorice root was found in King Tut's tomb. The one caveat to the many benefits of licorice is that it promotes hypertension. Glycyrrhizin is thought to be responsible for the hypertensive properties of licorice, which is brought about by the inhibition of the enzyme 11 β -hydroxysteroid dehydrogenase. This enzyme acts as a protective modulator in certain mineralocorticoid receptor-rich tissues — particularly kidneys, colon, and salivary gland — by metabolizing receptor-active glucocorticoids such as cortisol to 11-keto derivatives (e.g., cortisone) which are not receptor agonists. A condition of excess glucocorticoids brought about by inhibition

of the dehydrogenase leads to severe sodium retention, hypokalemia, and hypertension.²² Licorice reportedly has been responsible for fatal episodes of acute hypertension in people. Consequently, people with heart problems or hypertension should avoid licorice; as little as 100 to 200 g/day can cause persistent, heightened mineralocorticoid activity.

d-Limonene is a major constituent of citrus oils and also is found, in much lower amounts, in other fruits and vegetables. The major sources of d-limonene are oils of orange, grapefruit, and lemon. Citrus peel oil can contain as much as 95% d-limonene. d-Limonene per se or citrus oils where d-limonene is the major constituent have been widely used as flavoring agents and/or as fragrances in perfumes and soaps, and in a variety of foods such as ice cream, soft drinks, baked goods, gelatin, chewing gum, and puddings. It is also the active ingredient in “natural” citrus-based degreasing solvents and in insect repellents. Animal studies show that d-limonene is nephrotoxic to male animals. d-Limonene binds specifically, but reversibly to α_{2u} -globulin which is the major low molecular weight protein produced by the renal proximal tubules and, hence, excreted in the urine of the male rat. Female rats excrete much less α_{2u} -globulin. Accordingly, male rats that do not excrete α_{2u} -globulin (NBR strain) do not exhibit nephrotoxicity following d-limonene treatment.

Some animal studies indicate that d-limonene causes renal tumors in rodents. When administered orally, d-limonene induced renal adenomas and carcinomas in male rats, but not in mice. Oral d-limonene also was shown to significantly promote the development of N-nitrosoethylhydroxyethylamine-induced renal tumors in male rats. However, the toxicity and carcinogenicity of d-limonene appear to be absolutely species- and gender-specific due to the specific binding of this natural compound with α_{2u} -globulin. Because humans do not excrete α_{2u} -globulin, d-limonene is not thought to be harmful to people.²³ Indeed, several studies have shown d-limonene to possess chemoprotective properties.²⁴

Pyrrolizidine Alkaloids

Pyrrolizidine alkaloids (PAs) are common plant toxins produced by over 200 species of flowering plants, from genera such as *Senecio*, *Crotalaria*, and *Cynoglossum*. They are often present at very high levels — as much as 5 % of the plant’s dry weight. Pyrrolizidine alkaloid-containing plants pose significant health hazards to people who consume some kinds of “natural” herbal teas and traditional folk remedies and those who eat grain-based foods contaminated with PA-containing plant parts. Some PAs have been investigated in clinical trials for their anticancer potential.

These chemicals are often carcinogenic, mutagenic, and teratogenic and chronically hepatotoxic. Pyrrolizidine alkaloids are derivatives of a necine base like retronecine, otonecine, heliotriine esterified to various necic acid substituents (Figure 6.5). Pyrrolizidine alkaloids are activated by CYPs primarily of the 3A4 subfamily to reactive bifunctional pyrrolic electrophiles that form covalent cross-links to a variety of cellular nucleophiles, such as DNA and

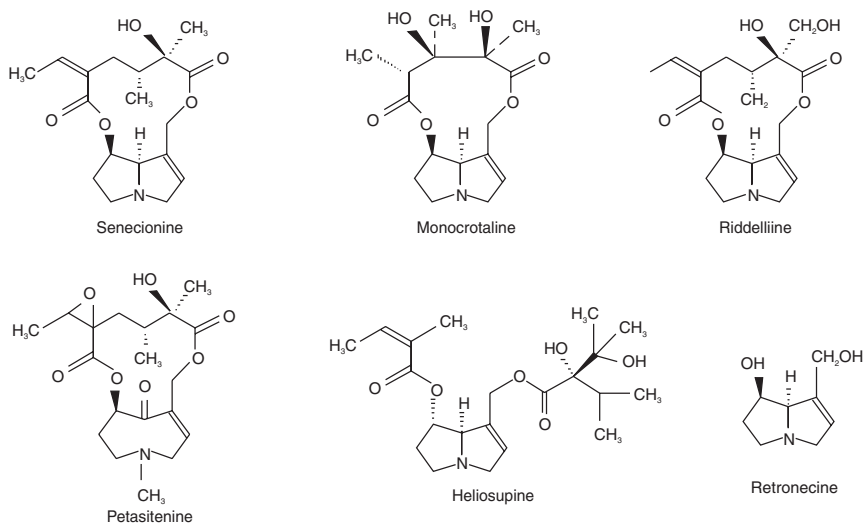


FIGURE 6.5
Chemical structures of selected pyrrolizidine alkaloids.

proteins. Pyrrolic intermediates then reportedly form electrophilic carbonium ions at atoms 7 and 9 and cross-link cellular nucleophiles at these positions.²⁵ Cytochrome P450s also convert PAs to the less toxic and more easily excreted N-oxides that do not interact with cellular constituents (Figure 6.6). Accordingly, animals that metabolize PAs to produce proportionally more N-oxides (such as sheep) appear to be relatively resistant to the toxic effects of PAs

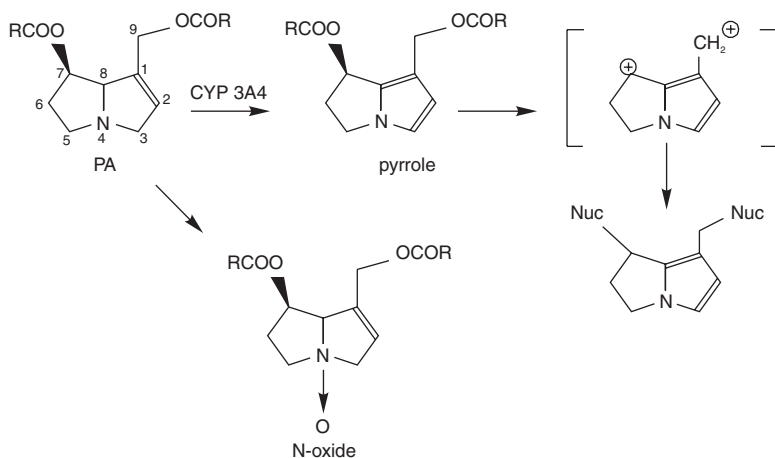


FIGURE 6.6
Metabolic fate of pyrrolizidine alkaloids to N-oxides and electrophilic pyrroles (nuc = nucleophile).

compared to animals that produce more of the pyrrole (rats and horses). Other hydrolysis reactions also may occur that decrease the toxicity of PAs.

DNA cross-links are probably a critical event in PA bioactivity in that the cytotoxic, antimitotic, and megalocytic activity of PAs closely correspond with the formation of cross-links *in vitro*.^{26,27} Pyrrolizidine alkaloids form both DNA interstrand and DNA-protein cross-links in equal amounts *in vitro*.^{26,27} Structure-activity studies have revealed that the presence of a continuous macrocyclic diester and α,β -unsaturation are important structural determinants for DNA cross-link formation.²⁷ Thus, PAs like senecionine are more potent cross-linkers than monocrotaline, which is more potent than open diesters such as latifoline and heliosupine. Of those examined, the simple necine retronecine is the least active cross-linker. The pattern of proteins cross-linked by PAs is similar to those cross-linked by other bifunctional compounds, such as cisplatin and mitomycin C; actin has been postulated to be one of the proteins involved in the PA-induced cross-links.²⁸

Petasitenine is found in *Petasites japonicus*, a medicinal herb used as an expectorant and cough suppressant. The flower stalks of this herb are used as a food or herbal remedy. When incorporated into the diet, dried stalks are hepatocarcinogenic to rats. Purified petasitenine is also hepatocarcinogenic in rats as well as mutagenic in bacteria.

Tussilago farfara (coltsfoot) is a common herb used for centuries as a medicine for coughs and bronchitis in Europe and Asia. (*Tussilago* is the ancient Roman name for "cough suppresser.") The plant contains the pyrrolizidine alkaloid senkirkine at concentrations as high as 150 ppm, as well as high concentrations of senecionine, another very toxic and carcinogenic PA. Again, both the dried buds of coltsfoot (when ground and mixed in the diet) and purified senkirkine or senecionine cause liver tumors in rats, and both are bacterial mutagens.

Human intoxication by PA-containing plants is well recognized and reported in the medical literature, and is endemic in Jamaica, India, and parts of Africa. Diseases, such as liver cirrhosis, veno-occlusive disease, and liver cancer, are linked to consumption of PA-containing plants. Hispanic and Native American populations in the west and southwest U.S. are at high risk for PA intoxications due to their traditional widespread use of herbs, occasional lack of confidence in traditional medicine, and, more commonly, lack of access to medical care.

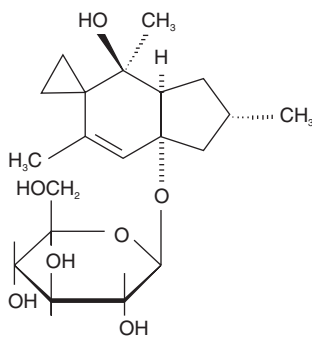
Comfrey (*Symphytum officinale*) is a nearly universal herb commonly sold not only in health food stores and by herbalists, but also in supermarkets. Since ancient Greek and Roman times, both leaves and roots have been used to make teas and compress pastes to treat a variety of external and internal diseases, such as healing of wounds, skin disorders, and respiratory diseases. Numerous vegetarian recipes call for comfrey leaves to make soufflés, salads, and breads. Comfrey leaves and roots contain up to 0.3% pyrrolizidine alkaloids such as intermedine, lycopsamine, symphytine, and others. Diets containing powder from dried leaves and roots caused liver tumors in rats.

Additionally, these pure pyrrolizidine alkaloids also are animal carcinogens and bacterial mutagens. There are several cases cited in the medical literature of comfrey-related intoxications in people. The well-demonstrated reported toxicity and carcinogenicity of comfrey is such a significant cause for concern that the governments of Australia, Canada, Great Britain, and Germany either restrict comfrey's availability or have banned its sale entirely. The U.S. Food and Drug Administration (FDA) has not yet acted to restrict the sale of pyrrolizidine alkaloid-containing foods.

Substances in Bracken Fern

Bracken fern (*Pteridium aquilinum*, *esculentum*, and others) is widely used as human food in greens or salads in many countries such as New Zealand, Australia, Canada, the U.S., and especially Japan. It is also a forage plant for sheep and cattle. It first attracted the attention of veterinary scientists who noticed severe toxicity — bladder cancer, bone marrow depression, severe leukemia, thrombocytopenia, and a hemorrhagic syndrome — in livestock grazing on this plant. When fed to rodents, bracken is a strong bladder, lung, and intestinal carcinogen. Lactating cows fed bracken fern produced milk that was carcinogenic to rats, showing that human exposure also may occur through cow's milk. Human consumption of bracken fern has been linked to an increased incidence of esophageal cancer in Japan.

The major carcinogen in bracken is believed to be *ptaquiloside* (Figure 6.7), a potent norsesquiterpenoid glucoside that is present at often high concentrations (up to 1.3% dry weight) in the plant. Ptaquiloside is a potent alkylator of DNA that appears to interact primarily with adenines at codon 61 in the *Ha-ras* oncogene in ptaquiloside-fed sheep.²⁹ The plant also contains quercetin, kaempferol, and other mutagenic compounds of the flavonoid family which may contribute to its carcinogenicity. It also contains toxic tannins.



Ptaquiloside

FIGURE 6.7

Chemical structure of ptaquiloside, the major carcinogen in Bracken fern.

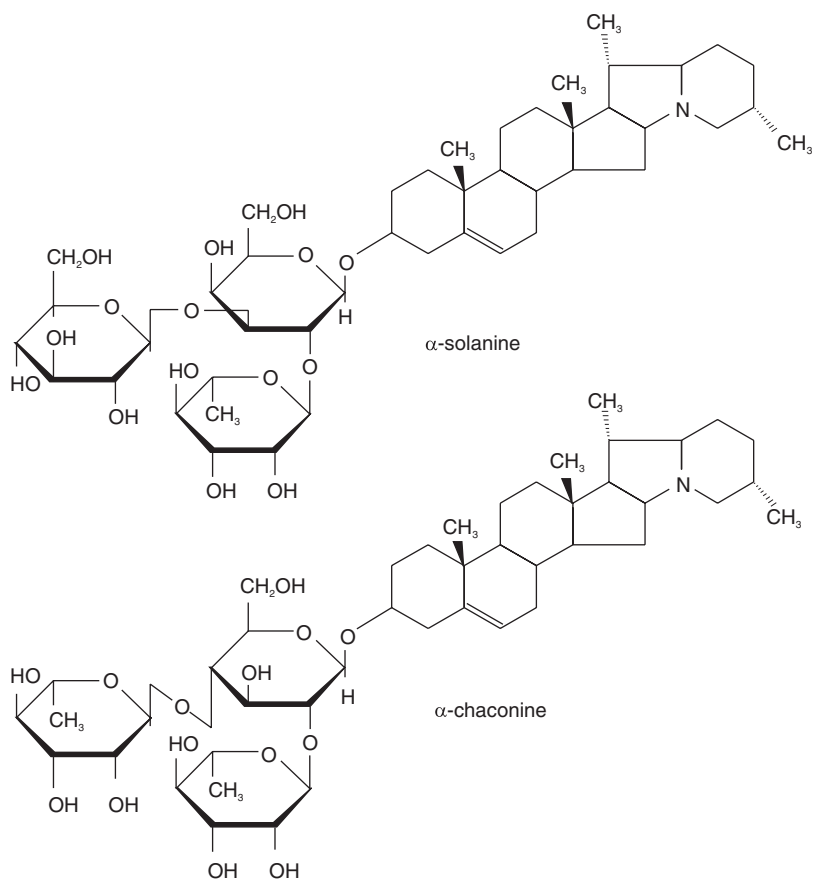


FIGURE 6.8
 Potato glycoalkaloids α -solanine and α -chaconine.

Acetylcholinesterase Inhibitors in Potatoes

Members of the family Solanaceae contain a variety of toxic glycoalkaloids. Potatoes (*Solanum tuberosum*) are an important food staple in many parts of the world and, under certain conditions, produce a variety of glycoalkaloids. Potatoes that have been damaged, exposed to light (green), or sprouted contain the glycoalkaloids α -solanine and α -chaconine (Figure 6.8) that can exceed concentrations of 100 ppm. Like physostigmine, solanine and chaconine are highly potent inhibitors of the enzyme acetylcholinesterase. Higher amounts of solanine and chaconine are present in the potato greens (tops). Healthy potatoes contain negligible amounts of these toxins. Episodes of human poisoning by green potatoes have been documented. Poisoning symptoms — gastric pain, weakness, nausea, vomiting, labored breathing — are consistent with acetylcholinesterase inhibition. These symptoms have been duplicated in clinical trials with human volunteers. Studies have

indicated that the acetylcholinesterase inhibitory activity of solanine is probably insufficient to cause these toxic effects, which are probably due to the combined toxicity of solanine with other cholinesterase inhibitors in the potato, such as chaconine.

Most cases of human poisoning and deaths have occurred in Europe, but are occasionally seen in the Western Hemisphere. Poisoning episodes are not infrequent in animals fed damaged potatoes or peel, greens, or trim. A small number of studies in which animals are fed toxic doses of blighted potatoes or pure glycoalkaloid have indicated that these compounds may have weak teratogenic activity. For example, solanine and chaconine (and their aglycone derivative, solanidine) induced craniofacial malformations (exencephaly, encephalocele, and anophthalmia) in Syrian hamsters.³⁰ In that study, solanidine was a much stronger teratogen than solanine and chaconine, which were classified as weakly teratogenic. As is the case with their anticholinesterase activity, the teratogenic and embryotoxic effects of solanine and chaconine appear to be synergistic.³¹

Tannins

Tannins long have been known as plant materials that confer a dark color when applied to animal hides thereby turning them into “tanned” leather. Although a precise definition is difficult due to their diverse and polymeric nature, one working definition is that tannins are a large group of water-soluble polyphenolic compounds with a molecular weight greater than 500 that have the ability to bind to and/or precipitate proteins. It is their ability to bind to proteins that is of toxicological and nutritional concern. Tannins also strongly bind to metals, such as iron, copper, and zinc, and reduce the gastrointestinal absorption of these metals. The two major classes of tannins are the proanthocyanidins (or “condensed tannins”) which are flavonoid polymers, and hydrolyzable tannins, which are polymers of gallic or ellagic acid esterified to either glucose or a polyphenol, such as catechin. As will be discussed later, some polyphenolic compounds also are beneficial in that they can prevent cancer in certain animals.

Tannins occur in nearly every plant-derived food, but they are particularly high in bananas, raisins, spinach, red wines, bracken fern, coffee, and tea. Tea is an especially rich source of tannins. Green tea has about 4%, while black tea may contain as much as 33% tannin; adding milk to tea will bind the tannins so that they will be less absorbable. A normal diet will provide several grams per week from fruits and vegetables. Tannins also are high in traditional herbal stimulant drinks such as those derived from Brazilian guarana (*Paullinia cupana*), betel nut (*Areca catechu*), and kola nut (*Cola nitida* and *C. acuminata*). In animal studies, tannins cause a diminished weight gain and lowered efficiency of nutrient utilization. The major biochemical basis for these effects appears not to be inhibition of dietary protein digestion but rather a systemic inhibition of the metabolism of digested and absorbed nutrients.³²

Tannins are liver carcinogens as well in rats and mice. Habitual chewers of betel nut (primarily in India, Pakistan, and Southeast Asia) have a high incidence of carcinoma of the mouth which has been linked to the high tannin content (10 to 25%) of this nut, although other components may be involved. An extract of betel nut causes cancer in hamsters. A high incidence of esophageal cancer in the Transkei in South Africa has been associated with the consumption of high-tannin varieties of sorghum. Some polyphenolic tannins are also anticarcinogenic (see below).

Caffeic Acid and Chlorogenic Acid

Caffeic and its quinic acid conjugate chlorogenic acid (Figure 6.9) occur in an extremely wide range of fruits and vegetables. Other minor conjugates of caffeic acid also are known to exist. Upon ingestion, chlorogenic acid is hydrolyzed in the gastrointestinal tract to yield caffeic and quinic acids. In humans, caffeic acid is metabolized to *o*-methylated derivatives, such as ferulic, dihydroferulic, and vanillic acids, and *meta*-hydroxyphenyl derivatives, which are excreted in the urine. Caffeic acid and conjugates are present in high concentrations (over 1500 ppm) in many seasonings (thyme, basil, anise, caraway, rosemary, tarragon, marjoram, sage, and dill); vegetables (lettuce, potatoes, radishes, and celery); and fruits (grapes, berries, eggplant, and tomatoes). Coffee is particularly rich in these phenolics, in addition to many other compounds (see below). A cup of coffee contains about 190 mg of chlorogenic acid. Caffeic acid inhibits 5-lipoxygenase which is a key enzyme in the biosynthesis of various eicosanoids, such as leukotrienes and thromboxanes. These eicosanoids are mediators of a wide variety of physiological and disease states and are involved in immunoregulation, asthma, inflammation, and platelet aggregation. At high doses (2% in the diet), caffeic acid caused a significant incidence of forestomach squamous cell papillomas and

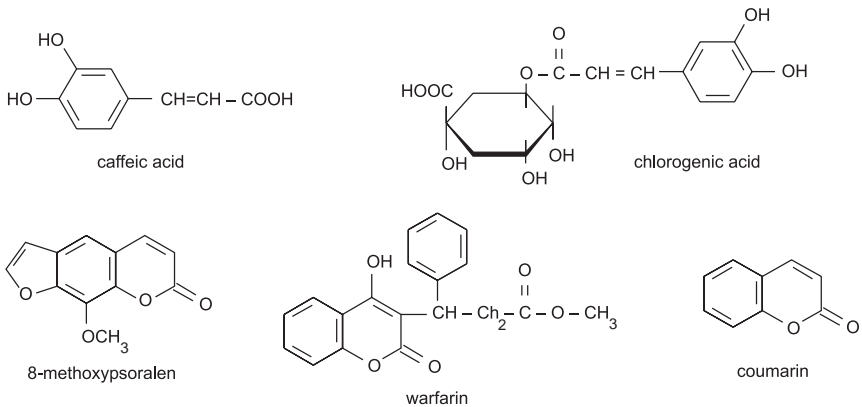


FIGURE 6.9
Chemical structures of caffeic and chlorogenic acids, 8-methoxypsoralen and coumarin.

carcinomas in both sexes of rats and mice, renal tubular cell hyperplasia in male rats and female mice, and alveolar type II cell tumors in male mice.³³ Oral caffeic acid also can enhance (or inhibit) the carcinogenic activity of known carcinogens. Chlorogenic acid has been shown to be mutagenic in bacteria, but has not been tested for carcinogenicity.

Coumarin and Psoralen

Coumarin (Figure 6.9) is widely found in plants such as cabbage, radish, and spinach, and in plants traditionally used as flavoring agents, such as lavender and sweet woodruff (*Asperula odorata*); the latter is an essential herb for making May wine, which is a popular German drink used to salute the coming of Spring. Coumarin is widely found in herb teas based on tonka beans (*Dipteryx odorata*) and sweet clover (*Melilotus albus* and *officinalis*) called “melilot.” The name “coumarin” originates from *coumarou*, the Carribean name for tonka beans. Purified coumarin was once used as a food additive, but this use was banned by the FDA after it was discovered that high doses caused liver damage in test animals. Coumarin is a powerful anticoagulant and is, in fact, the active ingredient in many brands of rodent baits. It also is used in human medicines as a blood thinning agent. Coumarin has been reported to cause bile duct carcinomas in rats as well.

Psoralens are a group of phototoxic furocoumarins widespread in a number of plant families such as Apiaceae (formerly Umbelliferae — celery and parsnips), Rutaceae (e.g., bergamot, limes, cloves), and Moraceae (e.g., figs). Celery contains 100 ppb psoralens, while parsnips contain approximately 40 ppm. When activated by sunlight, psoralens are mutagenic, presumably due to their ability to form interstrand and protein cross-links with DNA. Many members of this chemical family are carcinogenic as well, including 5-methoxypsoralen and 8-methoxypsoralen (also called methoxsalen, xanthotoxin, Figure 6.9). The latter, along with UV-A irradiation (PUVA) is used to treat skin disorders such as psoriasis and mycosis fungoides. However, psoriasis patients so treated exhibit a significant increase in premalignant skin lesions as well as malignant melanoma.^{34,35} Methoxsalen, in addition to forming DNA cross-links, causes a specific mutation in the tumor suppressor gene p53. Mice treated with PUVA exhibit signature missense mutations in exons 4 to 8.³⁶ Dietary exposure to psoralens is probably not a significant health risk; however, the margin of safety is thought to be narrow. Human volunteers who ingested 300 g of celery root (with a total phototoxic furocoumarin content of 28 ppm) experience no skin reactions after UVA exposure, and the blood levels of psoralen, methoxsalen, and 5-methoxypsoralen were below the analytical detection limit.³⁷

Miscellaneous Flavonoids: Quercetin, Ellagic Acid, Kaempferol, and Rutin

This family of chemicals is widespread in plant-derived foods, including fruits and fruit juices, vegetables, buckwheat, tea, cocoa, red wine, dill,

soybeans, bracken fern, and others. The estimated average daily intake of flavonoids is 1 g. None of these has yet been conclusively shown to be carcinogenic, but both quercetin and kaempferol are mutagenic. Rutin is not mutagenic in itself, but it can be metabolized by intestinal bacteria to yield quercetin. Quercetin also has some anticarcinogenic properties.

Natural Chemopreventives in Plants

Introduction

Cancer researchers have long ago discovered that a diet rich in some fruits and vegetables can prevent, reduce the severity, or delay the onset of cancer. A survey of approximately 200 studies that examined the relationship between fruit and vegetable intake and the incidence of several cancer types showed that an overwhelming majority (128 of 156) of these studies demonstrated that intake of fruits and vegetables statistically lowered cancer risk. The case was particularly striking for fruits, which showed a statistically significant protective effect in 28 of 29 studies against cancers of the esophagus, oral cavity, and larynx, and in 24 of 25 studies for protection against lung cancer.³⁸ Given these strong data already available, organizations such as the National Cancer Institute recommend that people eat a balanced diet with five servings of fruit and vegetables daily.

Isolating the individual compounds or *phytonutrients* with anticarcinogenic properties has proved to be difficult, but many have now been identified. These chemicals, known as *chemopreventives*, are not chemotherapeutics or cancer antidotes per se, but agents that have been shown in various experimental protocols to somehow interfere with the cancer process rather than cure advanced malignancies. Cancer is a multistage process, with a multitude of biochemical and molecular events that, left unchecked, culminate in cellular malignancy. Although the anticancer mechanisms of many chemopreventives have not been identified, several compounds have been shown to intervene at one or more of the stages of this process. Experimental protocols that have identified anticancer compounds from plants usually involve the administration of the chemopreventive either before, after, or concurrently with some chemical carcinogen such as aflatoxin B₁ (AFB₁) or benzo(a)pyrene (B(a)P) in laboratory animals. Chemopreventive action is manifested by one of several endpoints, such as a reduction in the tumor incidence of the animal group, a delay in the time in which tumors develop, or a reduction in the number or size of a malignant or premalignant lesion in an animal. Somewhat paradoxically, several of these chemopreventives, such as some of the tannins and isothiocyanates also are known to be toxic. Most chemoprotectives are minor nonnutrients, but others have nutritional values,

such as vitamins A (and its analogs), E, and C. The latter kind will not be discussed in this chapter. Many anticancer phytochemicals have been identified. Some of the more promising chemopreventives that have been shown in animal studies to inhibit cancer induced by a variety of chemical carcinogens are discussed below.

Isothiocyanates

Isothiocyanates are a large group of natural plant compounds (also discussed above as goitrogenic) that exhibit promising anticancer properties. Sulforaphane (Figure 6.10) is a recently discovered powerful chemoprotective found in broccoli and other cruciferous vegetables.³⁹ It is a powerful inducer of important phase II detoxification enzymes such as glutathione S-transferase and quinone reductase. Sulforaphane is a monofunctional inducer in that it increases activities of phase II enzymes without inducing carcinogen-activating enzymes such as CYP 1A. Phenethylisothiocyanate (PEITC) and benzyl isothiocyanate (BITC) are promising constituents shown to inhibit a wide variety of tumor types in experimental animals. These compounds are found also in certain cruciferous vegetables such as cabbage, brussel sprouts, broccoli, and cauliflower. They appear to be particularly effective against lung carcinogenesis in rats induced by the nicotine-derived tobacco carcinogen nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and B(a)P.^{40,41} In addition to enhancing detoxification by quinone reductase, PEITC and BITC also are thought to inhibit CYP-mediated enzymatic activation of these carcinogens.

Indole 3-Carbinol

Indole 3-carbinol (I-3-C, Figure 6.10), also present in cruciferous vegetables, is another promising chemopreventive. Indole-3-carbinol inhibits carcinogenesis caused by a number of chemicals in rodents and rainbow trout, most likely by multiple mechanisms. It is thought that I-3-C and derivatives thereof, produced under acid conditions of the stomach, are most likely to be the bioactive compounds. For example, the *in vivo* derivative of I-3-C, 3,3'-diindolylmethane, is a potent noncompetitive inhibitor of rat and human CYP1A1, human CYP1A2, and rat CYP2B1.⁴² Indole-3-carbinol and its acid derivatives also have been shown to inhibit AFB₁ mutagenesis in *Salmonella typhimurium in vitro* by scavenging the electrophilic AFB₁-8,9-epoxide.⁴³ However, another *in vivo* I-3-C derivative, indolo[3,2-b]carbazole (ICZ), is an arylhydrocarbon (Ah) receptor agonist and CYP 1A1 inducer with a potency similar to that of 2,3,7,8-tetrachlordibenzodioxin (TCDD).⁴⁴ This bifunctional action of I-3-C (or derivatives thereof) may explain why this phytochemical inhibits hepatocarcinogenesis in trout and rats when given prior to and with AFB₁, but actually promotes carcinogenesis in both species when given

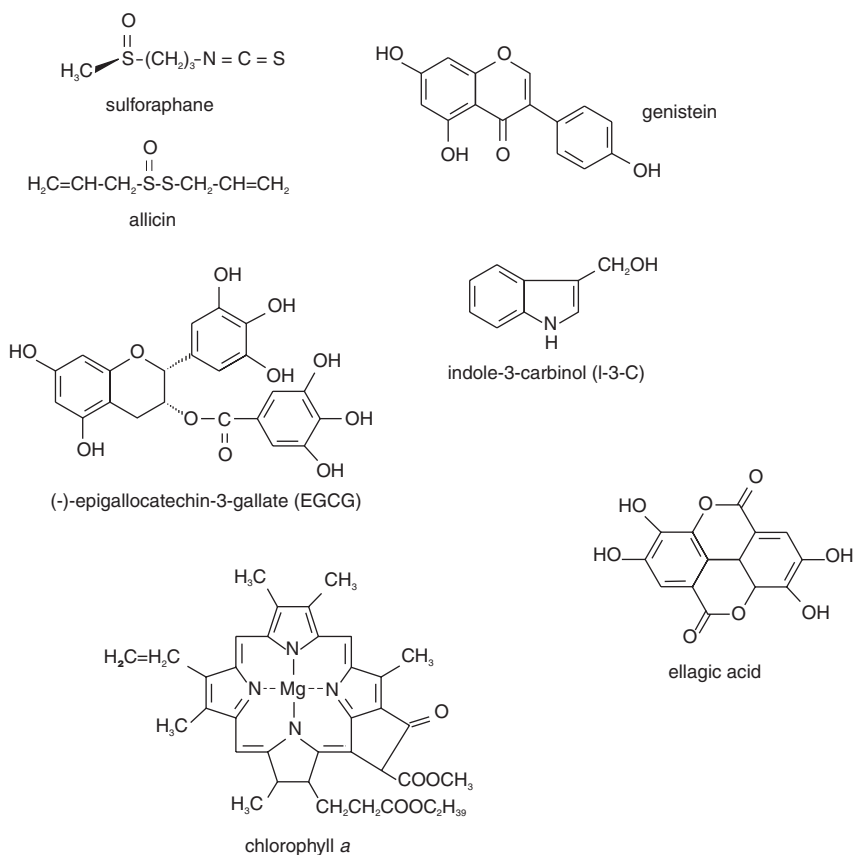


FIGURE 6.10
Natural cancer chemopreventives in foods.

continuously following AFB₁ initiation.⁴⁵ Such an observation is just one illustration why extreme caution should be exercised before chemopreventives are considered for human use. In any event, candidate chemopreventives should be rigorously examined in many experimental protocols to identify potentially adverse effects, such as cancer enhancement.

Polyphenols

Many foods are a rich source of chemopreventive polyphenolics, which are a type of plant tannin. Strawberries, blackberries, cranberries, walnuts, and pecans are a particularly good source of ellagic acid (Figure 6.10) which is the hydrolysis product of ellagitannins. Ellagic acid has been shown in numerous studies to be a versatile inhibitor of tumors at a number of sites induced by several compounds.⁴⁶ Some of the initial studies showed ellagic acid was

effective in preventing B(a)P-induced lung and skin tumors in mice. Ellagic acid also is active in reversing the skin tumor initiating and promoting activity of B(a)P and 12-O-tetradecanoylphorbol-13-acetate (TPA).⁴⁷

Tea is especially rich in several chemopreventive polyphenols that have been the object of intense study. Besides water, tea is the most commonly consumed drink in the world. While there is some, albeit equivocal, association between excessive tea consumption and some forms of human cancer, tea and tea components are now largely recognized to be chemopreventive.⁴⁸ A recent epidemiology study conducted in Shanghai associated green tea consumption with a reduction in esophageal cancer.⁴⁹ The chemopreventive properties of tea have been attributed to several polyphenols which are present in greater quantities in green compared to black tea due to differences in processing of the two products. The major polyphenols in green tea are the epicatechins, (-)epicatechin (EC), (-)epicatechin-3-gallate (ECG), (-)epigallocatechin (EGC), and (-)epigallocatechin-3-gallate (EGCG) (Figure 6.9). (-)epigallocatechin-3-gallate, which is thought to be the primary protective component in green tea,⁵⁰ accounts for over 40% of the total polyphenol content of green tea.⁴⁷ A 200 ml cup of green tea contains about 142 mg EGCG, 65 mg EGC, and 17 mg of EC, along with approximately 76 mg caffeine.⁴⁸ Black tea typically contains smaller amounts of these catechins because the majority of them are converted to epicatechin polymers, such as thearubigins and theaflavins, during processing. Aqueous extracts of green tea inhibit the mutagenic activity of several heterocyclic amines, in addition to reducing CYP-mediated metabolism of several substrates, suggesting that the chemoprotective properties of green tea are probably due to inhibition of enzymes which activate carcinogens as well as scavenging active metabolites.⁵¹ Green and black tea were both active in reducing aberrant colonic crypts induced by 2-amino-3-methylimidazo[4,5-f]pyridine (IQ), as well as reducing IQ-DNA adduct formation in rats.⁵²

Miscellaneous Chemopreventives

Chlorophylls (Figure 6.10) and their water-soluble salts called chlorophyllins are ubiquitous pigments found in green and leafy fruits and vegetables. Chlorophyllin (CHL), a copper/sodium salt of chlorophyll, has been given to people for a variety of purposes such as to reduce body, fecal and urinary odor; it has no known adverse side effects. Chlorophyllin derivative has been shown in a number of studies to reduce both *in vitro* and *in vivo* endpoints of the cancer process. For example, CHL powerfully inhibits the *in vitro* mutagenesis of AFB₁ in *Salmonella*, and inhibits the formation of AFB₁-DNA adducts in rainbow trout.⁵³ The mechanism of inhibition appears to be via complex formation with active AFB₁ metabolite, the AFB₁-8,9-epoxide. In addition to AFB₁, CHL also inhibits the carcinogenic action of other procarcinogens such as dibenzo[*a,l*]pyrene (DBP), B(a)P, 7,12-dimethylbenz[*a*]anthracene (DMBA), 1,2-dimethylhydrazine (DMH) and the heat-derived foodborne carcinogens

2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP), and IQ in trout, mice, or rats.⁵⁴

While much of the data on the chemopreventive properties of chlorophylls relate to CHL, recent data show that native chlorophylls are equally protective. Pure chlorophyll has equivalent activity as CHL to inhibit DPB-DNA adduct formation in rainbow trout (George S. Bailey, personal communication). The consistent protective properties of chlorophylls in animal and *in vitro* studies has provided the impetus for a newly initiated double blinded, placebo-controlled chemoprevention trial in Daixin, China, conducted by collaborators from Johns Hopkins and Oregon State Universities, to determine whether CHL can reduce biomarkers of AFB₁ (George S. Bailey, personal communication). People in this region of China have a particularly high intake of AFB₁ in their diet, and reductions of serum albumin-AFB₁ and urinary AFB₁-N⁷-guanyl DNA adducts would indicate that chlorophylls exert chemopreventive properties.

Allium plants, such as garlic, onions, leeks, and shallots contain a group of allylsulfur compounds, such as diallyl sulfide (Figure 6.10). Numerous studies have shown that these possess potent anticancer properties in a variety of species and organs caused by many carcinogens. Diallyl sulfide, the most potent of these, induces key detoxifying enzymes, such as GST. A related organosulfur compound from garlic, S-allylcysteine, induces GSTs in various tissues in mice and strongly inhibits DMH-induced aberrant crypts, but only when given in the initiation phase, further supporting its role as a detoxification promoter.⁵⁵ Allicin (Figure 6.10) is another organosulfur compound from garlic that possesses wide-ranging antimicrobial and anticancer properties.

Genistein (Figure 6.10) is an isoflavone found in soy beans and soybean products. Genistein appears to act through several mechanisms, but an important one may be through inhibiting angiogenesis or the process through which new blood vessels are formed. Because new blood vessels are important if a tumor is to grow, genistein may act by preventing tumors from growing.

Conclusions

The food supply in the U.S. can be regarded as among the world's safest, having high nutritional quality and extremely low carryover of agricultural chemicals. However, our food contains many naturally occurring plant compounds that have been shown to be toxic and/or carcinogenic in animals and people.

Because it is practically impossible to avoid all plant-derived toxins in a normal diet, the best way to minimize potential hazard would be to eat a wide variety of foods, but not too much of any one dietary item. Because natural chemopreventives are associated with a reduction in risk to many types of cancer, it is also important to include generous helpings of fruits and vegetables in the daily diet. There are several questions to be addressed before

chemopreventives can truly become a practical and safe protective therapy in people. For example, the anticarcinogenic benefits of at least some compounds are seen only when they are a natural part of the food from which they were derived. Thus, their benefits may not be seen when they are given as a supplement. Another concern is that animal studies have shown that under some experimental conditions, some chemoprotectives, such as indole 3-carbinol, may actually be carcinogenic in their own right or may *promote* the carcinogenic effects of another chemical. Lastly, some research has shown that the protective effects of a chemical may be specific to a given carcinogen or a closely related class of carcinogens.

Acknowledgments

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7

Pesticide Residues in the Food Supply

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Introduction

The use of pesticides in agriculture has undoubtedly led to increases in crop yield and reductions in crop loss due to pests such as insects, weeds, and plant diseases. According to one estimate, 40% of the world's food supply would be at risk without pesticides, and economic benefits from the use of pesticides in developed countries range from \$3.50 to \$5.00 per every dollar spent on pesticides.¹

The use of pesticides in contemporary agriculture also presents unique risks to human health and to the environment. Agricultural workers involved in the mixing, loading, or application of pesticides and those working in fields treated with pesticides face health risks that could result from excess exposure to the pesticides. Numerous cases of worker illness and injuries from pesticide exposure have been reported and epidemiological

evidence has linked occupational exposure to pesticides with an increase in the incidence of certain types of cancers.²⁻⁴ Pesticides have frequently been detected in surface water and in groundwater while other studies have indicated that pesticides also may be distributed throughout the environment by movement in the air and/or fog.⁵⁻⁸ Other potential risks from pesticides include the destruction of natural vegetation, reductions in natural pest populations, effects upon fish and wildlife, livestock losses, the evolution of pesticide resistance, and the creation of secondary pest problems.

Widespread public attention directed towards pesticides tends to focus more on consumer risks from exposure to pesticides in the diet rather than from worker or environmental concerns. Consumer surveys frequently indicate that 72 to 82% of Americans consider pesticide residues in foods to represent a major food safety concern.⁹ Public awareness of the presence and potential dangers of pesticide residues in the food supply has been raised by several events that have captured significant media attention in the past 15 years. These include the illegal use of the insecticide aldicarb on watermelons in California in 1985 that resulted in more than 1000 cases of probable or possible human pesticide poisoning, a report of the National Research Council (NRC) in 1987 that presented exaggerated estimates of potential human cancer risks from pesticides in the diet through the use of worst-case assumptions, and a widely publicized report of an environmental organization in 1989 alleging "intolerable" risks to children resulting from exposure to residues of cancer-causing and neurotoxic pesticides in food.¹⁰⁻¹² More recently, the potentially greater risks of infants and children to pesticides were comprehensively discussed in another NRC publication concluding that improvements in the risk assessment process were needed to take into account such differences.¹³ Controversial human epidemiological studies have indicated potential correlations between exposure to some chlorinated hydrocarbon insecticides while others have suggested that pesticides may be causing endocrine system disruption in the general population.¹⁴⁻¹⁷

Pesticides: Definitions, Classifications, and Use

According to the U.S. Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA), a pesticide is defined as:

any substance or mixture of substances intended for preventing, destroying, repelling, or mitigating any pest, any substance or mixture of substances intended for use as a plant regulator, defoliant, or desiccant, and any nitrogen stabilizer ...¹⁸

This comprehensive definition identifies a pesticide as an agent used specifically to control any of a wide number of different types of pests. While

TABLE 7.1**Pesticide Types and Targets**

Pesticide Type	Pest Controlled
Insecticide	Insect
Herbicide	Weeds
Fungicide	Fungi
Nematicide	Nematodes
Acaricide	Mites
Rodenticide	Rodents
Molluscicide	Snails
Algacide	Algae
Bactericide	Bacteria
Defoliant	Leaves

many have considered the term “pesticide” to be synonymous with “insecticide,” this broader definition also considers pesticides for use in control of weeds, fungi, and several other pests (Table 7.1).

Historically, pesticides have been used since as early as 1000 B.C., when sulfur was used by the Chinese to control powdery mildew on fruit.¹⁹ It is interesting to note that the most common pesticide used in California agriculture today, in terms of pounds of pesticide applied, is sulfur.²⁰ Arsenical insecticides were developed in the 16th century while chrysanthemum extract, rotenone, and nicotine insecticides trace their roots to the 17th century and are still used today.¹⁹

Insecticides

Insecticides exert their toxicity to insects in a number of ways, such as damaging nerves, poisoning muscles, or serving as desiccants or sterilants. The first major synthetic class of insecticides is known as the chlorinated hydrocarbons. This chemical family, developed during the 1930s and 1940s, includes insecticides such as DDT, aldrin, dieldrin, and chlordane. The introduction of these insecticides led to dramatic improvements in insect control due to their high insect toxicity and their environmental persistence. This persistence, while significantly contributing to the effectiveness of these insecticides, also resulted in environmental buildup and biological magnification leading to significant ecological and environmental damage. At the present time, very few chlorinated hydrocarbon insecticides remain registered for agricultural use because of their adverse environmental effects. Most recently, several chlorinated hydrocarbons have been associated with possible adverse effects on fertility and reproduction in humans and non-target organisms that may result from estrogenic or enzyme-inducing properties of the chemicals.¹⁷

The uses of most chlorinated hydrocarbon insecticides were replaced by the organophosphate and carbamate insecticides. These insecticides,

although derived as esters of two very different chemical families (phosphorothioic acid and carbamic acid, respectively), share a common mechanism of toxicological action resulting from inhibition of cholinesterase enzymes in both insects and mammals. While the acute toxicity of the organophosphates and carbamates is typically much greater than that of the chlorinated hydrocarbons, these newer insecticides are much less persistent in the environment. Presently, about 200 different organophosphate insecticides exist today and 39 are currently registered for U.S. food use. Examples of organophosphate insecticides include parathion, diazinon, mevinphos, chlorpyrifos, and azinphos-methyl. Fourteen carbamate insecticides are currently registered for U.S. food use, including carbaryl, aldicarb, methomyl, and carbofuran.

Pyrethroids represent a relatively new class of insecticides. Pyrethroids are synthetic derivatives of pyrethrins (natural extracts from chrysanthemums), but are more stable to light than their natural predecessors, allowing them to be more effective as insecticides. Pyrethroids typically are used as broad-spectrum insecticides and possess high insect toxicity while their mammalian toxicity is usually less than their organophosphate or carbamate counterparts. Their effectiveness is still limited somewhat to their significant environmental lability, their potential for resistance development, and their high cost.²¹

Herbicides

Herbicides play a major role in agricultural weed control throughout the world. They exist as a wide variety of different types including the triazine, sulfonyleurea, phenoxy, and quaternary ammonium herbicides.

Herbicides may exert their toxic action on weeds through a number of different mechanisms. Some (preplant herbicides) are applied before a crop is planted, others (preemergent herbicides) are applied after planting but prior to the appearance of weeds, while still others (postemergent herbicides) are used after the weeds have germinated. Some herbicides are broad-spectrum plant poisons that are toxic to virtually all types of plant material, including the crop being "protected;" an example of this type is the herbicide glyphosate. Genetically modified varieties of plants such as soy, corn, and cotton that have been engineered to be resistant to glyphosate recently have been introduced in agriculture. Other herbicides may be more naturally selective; the phenoxy herbicides (2,4-D, 2,4,5-T, MCPA) are toxic to broad-leaf plants but do not kill narrow-leaf plants like grasses. Herbicides also have a variety of ways in which they are introduced to the target weed. Some are applied directly to the plant material and cause their toxicity on contact while others may be applied to the soil or foliage and are translocated through the plant to their target site of action following absorption into the plant.

Fungicides

Fungicides control molds and other plant diseases by affecting the growth and/or metabolic processes of fungal pests. There are a wide number of

different fungicides available for agricultural use, including sulfur, aryl and alkyl-mercurial compounds, bisdithiocarbamates, and chlorinated phenols.

Pesticide Use

In 1997, through user surveys and sales records, the U.S. Environmental Protection Agency (EPA) estimated that approximately 4.5 billion pounds of chemicals were used as pesticides in the U.S.²² More than half of the amount of pesticide use (53%) involved chlorine or hypochlorites used for disinfection of potable and wastewater pools. “Conventional” pesticides (defined as those developed and produced exclusively or primarily for use as pesticides) accounted for 21% of the volume of pesticide use while “other pesticide chemicals” such as sulfur and petroleum were responsible for another 6% of pesticide use. The remaining uses involved wood preservatives (14%) and specialty biocides (6%).

With respect to the conventional pesticides and other pesticide chemicals, the EPA estimated that 77% of their use was in agriculture to produce food and fiber while industry/commercial/government uses accounted for 12% and the remaining 11% resulted from home and garden use.²²

The relative amounts of types of pesticides used in U.S. agriculture in 1997 are shown in Figure 7.1. The major uses, in terms of pounds applied, were for

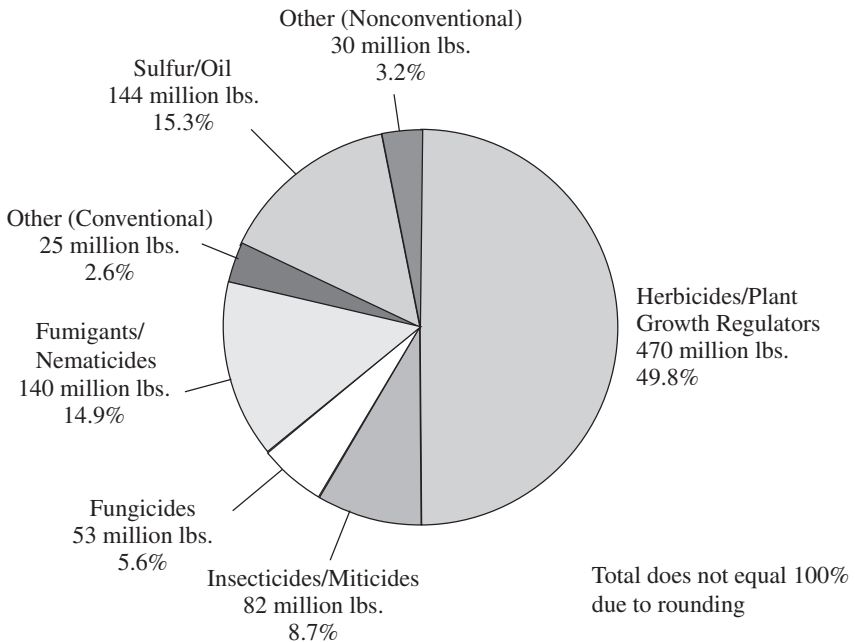


FIGURE 7.1
U.S. Agricultural use of pesticides, 1997.

herbicides/plant growth regulators, which accounted for nearly half of the total volume of agricultural pesticide use (470 million lbs). The use of sulfur/oils (144 million lbs) was next, followed by fumigants/nematicides (140 million lbs). Agricultural insecticide use represented 82 million lbs while fungicides accounted for 53 million lbs in 1997.²²

It was estimated that farm pesticide expenditures in 1997 were approximately \$8.3 billion, corresponding to 4.5% of total farm production expenses. Herbicides accounted for approximately \$5.6 billion, followed by insecticides at \$1.6 billion, and fungicides and other chemicals at \$1.1 billion in 1997.²²

Agricultural pesticide use patterns from 1979 through 1997 are shown in [Table 7.2](#). Total pesticide use has decreased slightly since 1979 with the largest drops shown for insecticide use and for other nonconventional pesticides such as sulfur and oils. The use of herbicides has been relatively steady, with a high of 516 million lbs in 1984 and lows of 425 million lbs in both 1987 and 1993.²²

As mentioned previously, the EPA estimates are based upon pesticide sales data and upon user surveys and are, therefore, only approximations of pesticide use and expenditures. In contrast, the state of California initiated in 1991 a much more accurate program requiring all agricultural pesticide use to be reported. A total of 189 million lbs of pesticides were reported to be used in California agriculture in 1996. Four pesticides (sulfur, oil, metam-sodium, and methyl bromide) accounted for 68% of the total pesticide use. Approximately 85% of the total pesticide use involved 31 specific pesticides while 19 commodities accounted for 83% of all agricultural pesticide use, 71% of all applications, and 82% of all acres treated in California in 1996.²⁰

It is important to understand that pesticide use does not necessarily imply pesticide residues. Many pesticides are applied prior to the planting and/or development of edible portions of the commodities while other pesticides may be sufficiently eliminated from a food crop prior to harvest.

Pesticide Regulation

In the U.S., the federal agencies responsible for regulation and monitoring of pesticides are the EPA, the U.S. Food and Drug Administration (FDA), and the U.S. Department of Agriculture (USDA). It is the responsibility of the EPA to register pesticides for use and to establish tolerances, representing the maximum legal allowable residues, for pesticides on food and feed crops. The FDA enforces tolerances by monitoring domestic and imported foods, while the USDA monitors meat and poultry in addition to operating the Pesticide Data Program (PDP) designed to more randomly analyze samples of fruits, vegetables, and processed food forms to aid in EPA's risk assessment efforts.

The EPA's authority to register pesticides and establish tolerances is provided in FIFRA, which became law in 1947 and has subsequently been

TABLE 7.2

U.S. Annual Volume of Pesticide Usage for Agriculture, 1979–1997

Pesticide	Year																		
	1979	1980	1981	1982	1983	1984	1985	1986	1987	1988	1989	1990	1991	1992	1993	1994	1995	1996	1997
<i>Millions of Pounds Active Ingredient</i>																			
Herbicides	492	504	513	503	455	516	501	481	425	450	460	455	440	450	425	485	461	481	470
Insecticides	188	163	152	142	135	129	126	121	90	100	95	90	85	90	80	90	91	84	82
Fungicides	57	59	62	59	59	56	59	59	52	54	54	50	47	45	47	48	49	51	53
Other conv.	106	100	104	101	100	100	94	94	91	95	113	133	144	150	154	163	170	190	165
Other chems.	246	227	215	207	196	194	194	188	180	177	161	164	140	161	166	163	168	152	174
Total	1089	1053	1046	1012	945	995	974	943	838	876	883	892	856	896	872	949	939	958	944

Source: U.S. Environmental Protection Agency, Pesticides Industry Sales and Usage, 1996 and 1997 Market Estimates, Office of Prevention, Pesticides, and Toxic Substances, Washington, D.C., 1999.

amended many times. FIFRA is a risk-balancing statute and it is the EPA's responsibility to determine if the benefits of the use of a specific pesticide (such as increased agricultural productivity, lower food costs, or public health protection) outweigh competing risks, such as adverse human health effects to consumers and/or workers or environmental effects. If the benefits are deemed to outweigh the risks, EPA has the authority to register the pesticide for specific uses and to determine appropriate use practices, including consumer, occupational, and environmental considerations. Such practices constitute the pesticide's "label" and failure to obey the label requirements constitutes a federal offense that could result in penalties and/or imprisonment.

Prior to registration, the manufacturer of the pesticide must submit to EPA the results of a full battery of toxicological tests performed on a number of animal species. Examples of some of the required tests include acute, sub-chronic, and chronic exposure, and carcinogenicity, metabolic fate, teratogenicity, and mutagenicity studies. The manufacturer also is required to provide studies on the effects of the pesticide on nontarget organisms, residue studies, and the environmental fate of the pesticide and its breakdown products. Completion of required toxicological and environmental studies for a new pesticide typically takes at least 10 years at a cost of approximately \$30 million.²¹

After the EPA registers a pesticide, individual states have the authority to restrict or deny the use of the pesticide for use within that state. California is one state that often provides more stringent use requirements for EPA-approved pesticides.

Setting Pesticide Tolerances

The processes EPA uses to establish pesticide tolerances are confusing and frequently misunderstood; these are described in significant detail in a review by Winter²³ and are summarized in the following paragraphs.

If the use of a pesticide on a food or feed crop presents the potential to leave a residue on the crop, the EPA usually requires the establishment of a tolerance representing the maximum amount of residue that is allowed for the particular pesticide/crop combination. Illegal residues result when residue levels exceed the established tolerance or in cases where residues are detected on crops for which the pesticide is not registered for use regardless of the level. Illegal residues may result in seizure of the offending crop, possible removal of the crop from the channels of trade, and possibly fines to the producer.

In general, tolerances are established to represent the maximum residues expected for a pesticide on a particular commodity resulting from adherence to the specified conditions of use.²³ To determine such maximum levels, pesticide manufacturers perform a series of field studies in a variety of geographical locations using the maximum application rate, maximum number of applications per growing season, and harvesting the commodity at the minimum preharvest interval. Tolerances should be considered enforcement

tools that indicate whether application practices have been followed, since it is highly unlikely that residue levels would exceed the tolerances if the applications were made under the conditions specified on the label. When a pesticide is detected on a commodity for which it is not registered, this could indicate that the pesticide was used on the wrong commodity or that efforts to prevent contamination of other commodities through factors such as drift or residual soil uptake were not adequately pursued.

Unfortunately, tolerances are often considered to be “safety standards” even though illegal residues rarely meet toxicological criteria as “unsafe” residues. While the EPA will make an assessment of the potential dietary risk from consumer exposure to the pesticide and will not grant a tolerance if the risk is deemed to be excessive, the tolerance will be established at a level high enough to accommodate the legal use of the pesticide as specified on the label if the EPA determines that the level of dietary risk is acceptable.²³

The EPA’s risk assessments consider the potential human exposure resulting from all registered (and proposed) uses of the pesticide. Frequently, as a first approximation of exposure, the EPA will calculate the theoretical maximum residue contribution (TMRC) representing the maximum legal exposure to the pesticide based upon the assumptions that (1) the pesticide is always used on all of the commodities for which it is registered, (2) residues will always result on the commodities and will be present at the tolerance level, and (3) no reduction will occur resulting from postharvest factors such as transportation, washing, peeling, cooking, and processing. The EPA compares the TMRC value with established toxicological criteria such as the reference dose (RfD) that represents, following analysis of animal toxicology studies and extrapolations to humans, the typical daily exposure level that is not considered to represent any appreciable level of risk.²⁴⁻²⁶ In cases where the TMRC is below the RfD, the EPA may deem the risks from the pesticide as negligible and will approve the manufacturer’s petition to establish the tolerance.

If the pesticide is considered to be oncogenic (a potential cancer-causing chemical), the EPA also will calculate the potential oncogenic risk posed at the TMRC. If the oncogenic risk is below the “negligible risk” level of 1 excess cancer per million, the EPA will typically approve the tolerance. The calculation of oncogenic risk relies upon conservative (risk magnifying) assumptions concerning low-dose extrapolations of human risks from the results of animal cancer studies performed at moderate and high doses.^{25,26}

In cases where the TMRC exceeds the RfD or when the oncogenic risk at the TMRC is greater than negligible, the EPA may refine its risk assessment practices by considering more realistic pesticide use, residue, and/or postharvest factors.^{23,24} It is clear from pesticide use studies that most pesticides are not used on 100% of crop acreage and, since tolerances are established to represent the maximum residues anticipated under legal conditions, normal residue levels are expected to be well below tolerance levels. Additionally, many postharvest factors such as washing, cooking, peeling, and processing may significantly reduce residue levels from those encountered at the field level. Studies have indicated that the TMRCs may frequently exaggerate exposure

by factors of 10,000 to 100,000 times.²⁷ As a result, the EPA may often refine its exposure estimates to represent an anticipated residue contribution (ARC) rather than the TMRC. If the ARC is below the RfD and the oncogenic risk at such an exposure level is below the negligible risk level of 1 excess cancer per million, the EPA will approve a tolerance.²³

At the international level, allowable levels for pesticide residues, known as maximum residue levels (MRLs), are established by the Food and Agriculture Organization of the World Health Organization (FAO/WHO) through the Joint FAO/WHO Meeting on Pesticide Residues.²⁸ Such standards are used in several countries throughout the world, although many countries, such as the U.S., develop their own standards for pesticide residue levels and enforce their sovereign standards on food entering their countries from other countries. In cases where the U.S. tolerances can be directly compared to the MRLs, the two sets of standards were found to be equivalent 47% of the time, while U.S. tolerances were lower (more stringent) 19% of the time and MRLs were lower 34% of the time.²⁹ The differences between tolerances and MRLs frequently exist due to different agricultural production and pest control practices, the use of different data sets, and differences in how pesticide breakdown products are regulated.²⁹

From the Delaney Clause to the Food Quality Protection Act

In 1958, an amendment to the Federal Food, Drug, and Cosmetic Act (commonly referred to as the Delaney Clause) specified that “no additive shall be deemed to be safe if it is found to induce cancer when ingested by man or animal, or if it is found, after tests which are appropriate for the evaluation of the safety of food additives, to induce cancer in man or animals.”³⁰ This controversial amendment has significantly affected the way pesticide residues in the U.S. have been regulated.

Interestingly, most pesticide residues were not directly subject to the Delaney Clause since they were not considered to be “food additives.” Exceptions occurred in the cases where pesticides were added directly to processed food forms or where pesticide residue levels were shown to increase as the raw commodity was converted into processed forms. According to the EPA’s so-called coordination policy, however, the EPA also applied the Delaney Clause to the raw commodities when residues concentrated in the processed forms.¹¹ For example, if residues of pesticides used on grapes or tomatoes (raw commodities) concentrated when going from the raw to processed forms (i.e., raisins, tomato paste), EPA’s interpretation of the Delaney Clause would not allow the use of the pesticide on the raw commodities even though “food additive” tolerances subject to the Delaney Clause were only established on the processed foods.

The Delaney Clause has been the subject of significant policy and legal debate. In 1985, the EPA commissioned the NRC to examine the legal issues concerning the EPA’s methods for establishing pesticide tolerances and to

consider the current and future impacts of the Delaney Clause on the availability of pesticides and public health protection. The NRC issued a 1987 report recommending that the Delaney Clause be replaced by a negligible risk standard that applied equally to both raw and processed food forms.¹¹ Such a recommendation was adopted by the EPA in 1988 in cases where the human dietary risks from pesticide residues were considered to be less than one excess cancer per million persons exposed over a 70-year lifetime.³¹ The calculation of the one cancer per million risk involved conservative assumptions from high-to-low dose extrapolations from animal studies to human exposure and statistical corrections including the upper 95% confidence interval of the slope of the dose/response curve obtained from the animal studies.²⁵

The *de minimus* policy adopted by the EPA in 1988 was challenged in court in 1989 through a petition that EPA revoke several food additive tolerances of seven oncogenic pesticides.³² The lawsuit was based upon the premise that the Delaney Clause represented an absolute “zero risk” statute rather than a “negligible risk” statute. Significant legal activity followed and was capped by a 1992 ruling of the U.S. Ninth Circuit Court of Appeals that overturned the EPA’s *de minimus* policy. The U.S. Supreme Court declined to review the Court of Appeals decision and the EPA in 1995 signed a consent decree with one of the petitioners, the Natural Resources Defense Council, that set a 5-year timetable to phase out uses of pesticides subject to the Delaney Clause. Such a phase-out was developed irrespective of the magnitude of theoretical oncogenic risk posed by the pesticides. Specific uses of 37 pesticides were subject to revocation by 1997 while uses of as many as 80 pesticides might have been affected by 2000.³²

The timetable hastened legislative activity to eliminate the Delaney Clause as it pertained to pesticides since several existing uses of pesticides were scheduled to be eliminated on statutory grounds rather than from excessive health risks. In July of 1996, Congress unanimously passed the Food Quality Protection Act (FQPA) that was signed into law by President Clinton on August 3, 1996. Under FQPA, pesticides were no longer considered, under any circumstances, to be food additives and were, therefore, no longer subject to the Delaney Clause, although the Delaney Clause still applies to other food additives.

The FQPA established one law for all pesticide residue tolerances on raw agricultural commodities and processed forms as well as standards that applied to all risks, both oncogenic and nononcogenic. The EPA is responsible for the determination that tolerances are “safe,” with safety defined as a “reasonable certainty that no harm will result from aggregate exposure” to the pesticide. Historically, as has been described previously, a “reasonable certainty of no harm” has been interpreted as an oncogenic risk below 1 in 1 million and, for nononcogenic risks, exposure below the RfD.

Many of the provisions of FQPA were based on the recommendations of the NRC in its 1993 report, *Pesticides in the Diets of Infants and Children*.¹³ This report was critical of the scientific and regulatory procedures that the federal

government used to assess the risks of infants and children from exposure to pesticides. The report's major conclusion was that the government frequently took a "one-size-fits-all" approach that treated infants and children as "small adults" and ignored potential differences of sensitivities to pesticides and to exposure levels. Such NRC-derived provisions found in the FQPA include the potential use of an additional 10-fold uncertainty factor when extrapolating from the results of animal toxicology studies to provide greater protection for infants and children (the 10× factor), consideration of exposure from water and residential exposure to pesticides in addition to dietary exposure (aggregate risk provision), and consideration of exposure to entire families of pesticides (such as the organophosphate insecticides) that possess common mechanisms of toxic action (cumulative risk provision). The FQPA also encourages the expedited approval of safer pesticides, requires periodic reevaluation of pesticide registrations and tolerances, requires the EPA to develop mechanisms to study possible risks of endocrine disruption, and provides a consumer right-to-know provision.

The development of suitable risk assessment models to comply with the requirements of the FQPA presents a formidable scientific challenge. The provisions of the FQPA, in combination with improved database management and computational skills of the past decade, have shifted much of the risk assessment focus from examining long-term (chronic) risks to more sophisticated examinations of shorter-term risk. As an example, traditional "deterministic" approaches simply required developing estimates of food consumption and residue levels that would be multiplied together to yield an exposure estimate. This exposure estimate was used to determine the oncogenic risk and also compared with the RfD to determine nononcogenic risk. This approach is being replaced with "probabilistic" approaches in which both food consumption and residue values are given as distributions rather than as point estimates. Through statistical convolution, it is possible to develop distributions of exposure that allow determination of what fraction of a population group of interest is exposed to greater than a particular level of exposure to a pesticide (i.e., RfD) on a given day.²⁵ Interpretation of the results of such distributional analyses presents its own challenges; notable questions of interest include what fraction of the population (i.e., 95% or 99% or 99.9%?) should be protected to meet the "reasonable certainty of no harm" provision and how accurate the tail of the exposure distribution really is. The EPA has identified dozens of science policy issues that need to be addressed as a result of the complexities of risk assessment practices prescribed by the FQPA, and EPA's interpretation of such issues may dramatically influence the future availability of pesticides in U.S. agriculture.

Pesticide Residue Monitoring and Enforcement

The primary U.S. federal agency involved in the monitoring of pesticide residues and enforcement of pesticide tolerances is the FDA, which is charged

with enforcing tolerances in domestic and imported foods shipped in interstate commerce. The FDA monitors foods and feed for illegal pesticide residues and may take regulatory action when tolerances are exceeded or when residues are detected on commodities that do not have tolerances established for the specific pesticides.³³

The majority of samples taken by the FDA in its monitoring efforts are in the FDA's surveillance monitoring program, where the types of commodities chosen to be sampled and the origins of the samples are targeted to enhance the FDA's ability to identify violative residues. The FDA also has a smaller-scale compliance monitoring program where samples are frequently drawn as a follow-up to illegal residue detection or where similar problems may have occurred previously for a specific shipper, grower, or in a particular geographic area or country.³³

In the FDA's surveillance program, domestic samples are usually collected near the production source or at the wholesale level, while imported foods are typically sampled at the point of entry into the U.S. Most of the samples involve raw agricultural commodities, although some processed foods also are analyzed for pesticide residues. Analytical methods commonly rely on multiresidue techniques capable of simultaneously determining several pesticide residues. The multiresidue methods used by the FDA are capable of detection of approximately 200 individual pesticides in addition to many pesticide metabolites, impurities, and pesticide alteration products. The multiresidue methods are frequently supplemented by single residue methods for important pesticides that are not detected using multiresidue techniques. The analytical methods used by the FDA are normally capable of detection of pesticides at a level of 0.01 ppm or below.³³

In 1998, the FDA analyzed 7457 food samples in its surveillance program for pesticide residues, including 3597 samples of domestic foods and 3860 samples of imported foods. Domestic samples (Figure 7.2) showed a violation rate of 0.8%, while no residues were detected in 64.9% of the samples. For imported samples (Figure 7.3), no residues were detected on 68.1% of the foods analyzed, while violative residues were determined in 3.0% of the samples.³³

The USDA is responsible for two programs that analyze foods for pesticide residues. In the USDA's National Residue Program, samples of meat, poultry, and raw egg products are monitored for pesticide residues, animal drugs, and environmental contaminants. In this program, multiresidue analytical methods are used that detect residues of the major insecticide classes as well as 40 other individual pesticides.³⁴

In 1991, the USDA initiated its Pesticide Data Program (PDP) that collects, in cooperation with many state agencies, several thousand food samples that are analyzed for pesticide residues each year. In contrast with the FDA's surveillance program that focuses primarily upon tolerance enforcement and is not particularly useful for risk assessment purposes, PDP's sampling procedures are designed to capture actual residues in foods that more accurately reflect residue levels near the time of consumption of the food items.³⁴

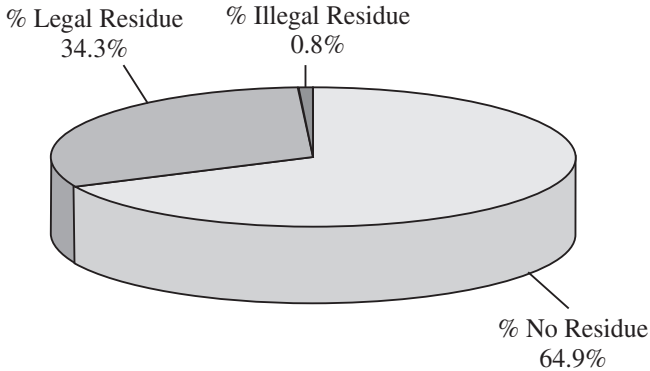


FIGURE 7.2
 FDA surveillance monitoring program, 1998: residue findings, domestic samples.

In 1997, 10 states participated in PDP activities. The 10 states (California, Florida, Michigan, New York, Ohio, Texas, Washington, Colorado, Maryland, and Wisconsin) represented about 50% of the U.S. population and all geographical regions of the country.³⁴

Samples in the 1997 PDP program were collected from 15 different commodities. Among fresh fruit and vegetables, samples of pears, potatoes, spinach, sweet potatoes, tomatoes, and winter squash were collected. Samples also were collected from six processed fruit and vegetable products: apple juice (ready-to-serve and concentrate), canned/frozen green beans, orange juice (ready-to-serve and concentrate), canned peaches, canned spinach, and frozen winter squash. Samples also were collected for whole milk, wheat, and soybeans.³⁴

PDP collected and analyzed 8177 food samples in 1997; these samples originated from 43 different states and 23 foreign countries. At least one residue was detected in 70% of the fresh fruit and vegetable samples, in 45% of the processed fruit and vegetable samples, in 80% of the wheat, in 87% of the

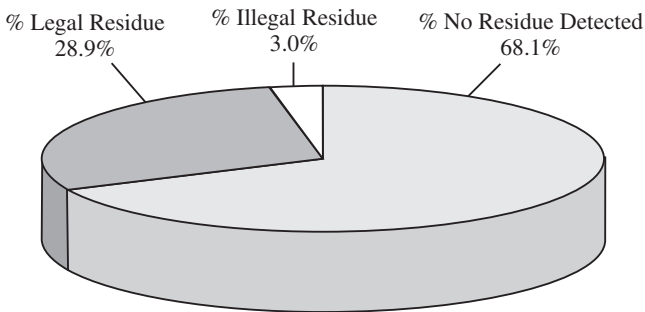


FIGURE 7.3
 FDA surveillance monitoring program, 1998: residue findings, imported samples.

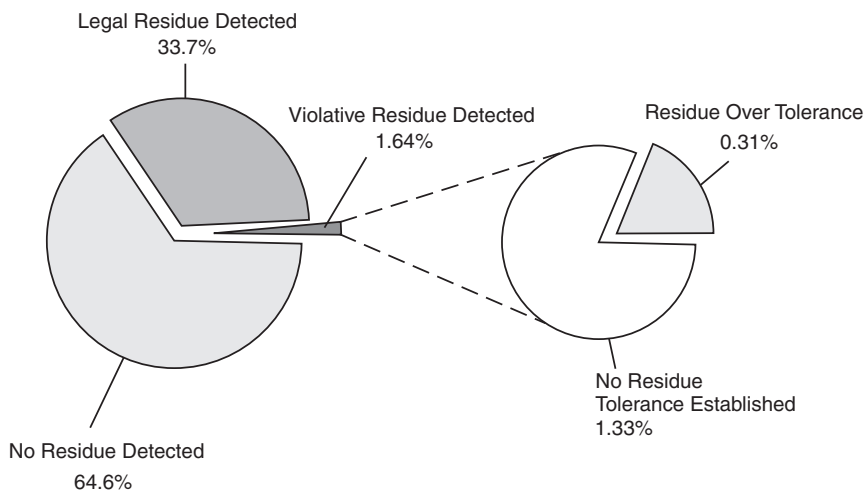


FIGURE 7.4

California routine marketplace surveillance program, 1995: residue findings.

soybeans, and in 15% of the milk samples. For the fruit and vegetable samples, only four of the 6321 showed residues in excess of tolerance, although a total of 422 other presumptive violative residues were detected where tolerances for the detected pesticides were not established on these commodities.³⁴

In addition to the federal monitoring programs, individual monitoring programs are also conducted in 38 states.²⁴ The largest state monitoring program is that performed in California; the California program spends more than \$48 million each year to regulate pesticide use and to analyze food and environmental samples for pesticide residues.³⁵ Results of California's Routine Marketplace Surveillance program for 1995 are given in Figure 7.4. Approximately 64.6% of the 5502 samples analyzed in 1995 showed no detectable residues. In another 33.7% of the samples, residues were detected that were within the established tolerances. Violative residues were detected in 1.64% of the samples and included residues over tolerance (0.31%) and residues detected that were not registered for use on the commodity on which they were found.³⁵

For detectable residues identified in California's Routine Marketplace Surveillance program from 1987, Table 7.3 provides a comparison of the residue levels encountered with the tolerance levels. In general, the majority of samples containing detectable residues showed residue levels at less than 10% of the tolerance level and only a small percentage of samples constituted residues between 50 and 100% of tolerance levels.³⁵

Collectively, results from federal and state monitoring programs indicate that the vast majority of food samples analyzed for pesticide residues do not contain illegal residues and that samples frequently contain no detectable residues. The findings that residue levels rarely approach tolerance levels should not be surprising since tolerances themselves are established to

TABLE 7.3

Comparison of Residue Levels Detected with Tolerance Values, California Routine Marketplace Program, 1987 to 1995

Year	Total Samples	Percentages of Samples with Detectable Residues	Percentage of Samples at 0–10% of Tolerance	Percentage of Samples at 50–100% of Tolerance
1987	7,010	20	12	1.10
1988	9,232	22	13	1.10
1989	9,403	22	13	0.90
1990	8,278	20	12	0.70
1991	7,446	25	16	0.80
1992	7,319	31	21	0.80
1993	6,066	34	23	1.30
1994	5,588	34	23	1.30
1995	5,502	34	24	0.96

represent the maximum residue levels expected following proper handling and application of the pesticide.

Assessment of the Dietary Risks from Pesticide Residues

Pesticide misuse has been implicated historically as a cause for human poisoning following consumption of foodstuffs contaminated with harmful residue levels. Several cases of poisoning have been traced including one that resulted from the illegal application of the carbamate insecticide to watermelons in California in 1985 that resulted in more than 1000 cases of human poisoning in the U.S. and Canada.^{10,36}

Most discussions of the potential risks posed by pesticides in the diet focus on the development and interpretation of risk assessments rather than on documented cases of human illnesses. Frequently, evidence supporting the relative safety of pesticide residues is based on the results of regulatory monitoring programs demonstrating that most food samples analyzed do not contain detectable levels of residues and that violation rates are quite low. An opposite viewpoint may be achieved through a different interpretation of the same residue monitoring results; many argue that a violation rate of 1 to 3% would subject consumers to numerous exposures of violative residues annually and that greater controls are needed. Such an argument implies that violative residues are synonymous with unsafe residues.

Pesticide tolerances, as discussed previously, serve an important role as determinants of the maximum residues expected provided that pesticide use regulations are followed. Violative residues, therefore, typically indicate that appropriate use practices were not followed but do not necessarily constitute “unsafe” residue levels.²³

As an example, Winter²³ compared the TMRC values for 35 pesticides that were subject to EPA tolerance decisions from 1988 through 1991 with their corresponding acceptable daily intake (ADI) values. The TMRC values, as discussed previously, represented the maximum “legal” exposure to the pesticides and dramatically overestimate exposures by assuming that all pesticides are always used on all commodities for which they are registered, that all residues are present at the tolerance levels, and that residues are not reduced through a variety of postharvest factors, such as washing, peeling, cooking, transportation, etc. The ADI values are related to the more contemporary RfD values, and represented the maximum acceptable daily level of exposure to the pesticides.

Results of this analysis are shown in [Table 7.4](#). TMRCs accounting for less than 5% of the ADIs existed for 23 of the 35 pesticides. For four pesticides, the TMRCs were between 5 and 10% of the ADIs, while for six other pesticides, the TMRCs were between 10 and 50% of the ADIs.²³

The TMRC for the pesticide fluridone, assuming tolerance level residues on all 50 commodities for which it was registered, represented 8.6% of the ADI. For an exposure of fluridone to exceed the ADI, all residues of fluridone on all 50 commodities would always have to exceed the tolerance by an average of 12 times, a proposition that is statistically absurd. A specific residue of fluridone above tolerance on a single commodity would represent an improper application of the pesticide, but would not be considered as an “unsafe” residue since an enormous difference between actual exposure and the ADI would still exist.

Results of other dietary pesticide risk assessments alleging excessive risks have generated widespread media coverage and public awareness. The NRC, in its efforts to examine the statutory basis for establishing tolerances and to consider the potential impacts of the Delaney Clause, calculated theoretical oncogenic risk estimates using the TMRC approach to estimate exposure.¹¹ While it was explained in the NRC report that the risks calculated should not be considered as “actual” risks due to the exaggerated assumptions used in the report, the results were frequently misinterpreted to indicate excessive cancer risks from pesticide residues in foods. Commonly, the results generated in the NRC report were converted to depictions of “actual” cases of human cancers; examples include “the National Academy of Sciences estimates approximately 1.4 million cancer deaths due to the consumption of pesticide residues in foods”³⁷ and “the potential risks posed by cancer-causing pesticides in our food are over 1 million additional cancer cases in the U.S. population over our lifetimes.”³⁸ A subsequent study by Archibald and Winter using more realistic assumptions of exposure to pesticides indicated that the exposure estimates used in the NRC report often exaggerated exposure by factors of 10,000 times or more.²⁷

Another significant study that significantly increased public awareness concerning pesticide residues in foods was one prepared by the Natural Resources Defense Council that was primarily disseminated through the news media rather than through the conventional scientific channels.¹² This

TABLE 7.4

Comparison of Theoretical Maximum Residue Contributions (TMRCs) with Acceptable Daily Intakes (ADIs) for Selected Pesticides

Pesticide	TMRC ($\mu\text{g}/\text{kg}/\text{day}$)	ADI ($\mu\text{g}/\text{kg}/\text{day}$)	ADI (%)
Avermectin	0.053	13 ^a	13.3
Bifenthrin ^b	0.45	15	3.0
Chlorimuron ethyl	0.033	13	<0.1
Clofentezine ^c	0.59	13	4.5
Clopyralid	8.1	500	16.2
Cyfluthrin	0.26	25	1.0
Cyhalothrin	0.13	5	2.6
Cypermethrin	2.8	10	2.8
Express ^c	0.073	6.3	1.2
Fenoxaprop-ethyl	0.11	2.5	4.4
Fluazifop-butyl	2.1	10	2.1
Fluridone	6.9	80	8.6
Fluvalinate	0.16	10	1.6
Fosetyl Al ^c	1.5	3000	<0.1
Glyphosate	9.9	100	9.9
Hexythiazox ^d	0.037	25	0.1
Imazamethabenz	1.5	62.5	2.4
Imazethapyr	0.042	250	<0.1
Iprodione	47.8	40	119.5
Lactofen ^e	0.018	2 ^f	0.9
Metalaxyl	10.4	60	17.3
Methiocarb	3.6	12.5 ^g	28.8
Metolachlor ^h	1.3	150	0.9
Metsulfuron	0.11	13	0.8
Metsulfuron methyl	0.82	250	0.3
Nicosulfuron	0.033	1250	<0.1
Oxyflufen	0.71	3	23.7
Primisulfuron methyl	0.57	6 ^a	9.5
Propiconazole ⁱ	1.1	13	8.5
Quizalofop-ethyl	0.22	9	2.4
Sethoxydim	32.0	90	35.6
Tefluthrin	0.01	0.75 ^j	1.3
Thiobencarb	1.3	10	1.3
Triadimenol	0.45	38 ^k	1.2
Vinclozolin	13.0	25	52.0

^a Calculation of ADI used 300-fold uncertainty factor.

^b Class C carcinogen at time of ruling; cancer risk at TMRC = 2.4×10^{-5} .

^c Class C carcinogen at time of ruling; quantitative risk assessment not performed.

^d Class C carcinogen at time of ruling; cancer risk at TMC = 1.4×10^{-6} .

^e Class B2 carcinogen at time of ruling; cancer risk at TMRC = 3.2×10^{-6} .

^f Calculation of ADI used 1000-fold uncertainty factor from lowest effect level.

^g Calculation of ADI used 10-fold uncertainty factor.

^h Class C carcinogen at time of ruling; cancer risk at TMRC = 2.6×10^{-6} .

ⁱ Class C carcinogen at time of ruling; cancer risk at TMRC = 8.7×10^{-5} .

^j Provisional ADI; 1000-fold uncertainty factor used.

^k Provisional ADI.

Source: Winter, C. K., *Reg. Toxicol. Pharmacol.*, 15, 137, 1992. With permission.

report claimed that “between 5500 and 6200 of the current population of American preschoolers may eventually get cancer solely as a result of their exposure before 6 years of age to eight pesticides or metabolites commonly found in fruits and vegetables.” The report also claimed that at least 17% of the U.S. preschool population is exposed to potentially dangerous levels of food residues of organophosphate insecticides. Such findings were based upon the use of controversial estimates of food consumption, reliance on worst-case exposures, and no correction for postharvest effects on residue levels.¹² One review of the report indicated that the “risks to preschoolers are based on a series of worst-case assumptions that have little in common with the real world” and that “the results are exacerbated by seriously flawed methodology, mathematical errors, and scientifically unsound speculation.”³⁹

It is clear that neither recitation of the results of regulatory monitoring programs nor the development of risk assessments relying upon unreasonable assumptions of exposure provides appropriate means to address the magnitude of pesticide risks in the diet. A more realistic approach involves consideration of the results of the FDA’s Total Diet Study, a marketbasket survey designed to most appropriately determine exposure to pesticide residues and other food contaminants on food products at the time of consumption. The Total Diet Study has been performed annually since 1961 and involves collection of marketbaskets from each of four geographical areas in the U.S. that each contain 234 food items chosen on the basis of national food consumption surveys to represent approximately 5000 specific foods.²³ The foods are prepared for consumption in institutional kitchens using standard recipes involving washing, peeling, mixing, and cooking into table-ready forms. The foods are analyzed for residues using multiresidue techniques and modifying analytical methods to allow the sensitive detection of residues at levels 5 to 10 times lower than those normally achieved in regulatory monitoring programs.²³

While FDA performs the Total Diet Study annually, it has not published pesticide exposure estimates for the past several years, citing the use of questionable data obtained from food consumption surveys.³³

Table 7.5 compares exposure estimates obtained from the 1991 Total Diet Study (the most recent compilation of such estimates) with EPA’s RfDs and the analogous ADIs established by the WHO.⁴⁰ For most pesticides in all three population subgroups studied, exposure estimates represent only a small fraction (frequently less than 1%) of the RfDs and ADIs. To put such exposures in perspective, consider that the RfDs or ADIs are obtained by identifying the highest level of exposure that does not cause a noticeable effect in the most sensitive animal species tested and then typically applying a 100-fold uncertainty factor to yield the RfD or ADI. Thus, a RfD constitutes a level 100 times lower than a level that does not show any demonstrated toxicity in an animal study; exposure at a level of 1% of the RfD represents an exposure 10,000 times lower than the level that does not produce noticeable effects in animals.²⁸

TABLE 7.5

Pesticide Intakes Estimated from FDA's Total Diet Study, 1991

Pesticide	Estimated 6–11 Mos.	Exposure 14–16 yr. Male	($\mu\text{g}/\text{kg}/\text{d}$) 60–65 yr. Female	ADI or FAO/WHO ADI ^a	RfD ($\mu\text{g}/\text{kg}/\text{d}$) EPA RfD ^a
Acephate	0.0089	0.0113	0.0165	30	4
Azinphos-methyl	0.0039	0.0033	0.0029	5	— ^b
BHC, alpha	0.0002	0.0004	0.0002	— ^b	—
BHC, gamma (lindane)	0.0004	0.0008	0.0003	8	0.3
Captan	0.0478	0.0209	0.0595	100	130
Carbaryl	0.1801	0.09	0.0811	10	—
Carbofuran, total	0.0002	0.0001	0.0004	10 ^c	5 ^c
Chlordane, total	0.0001	0.0003	0.0002	0.5	0.06
Chlorpyrifos	0.0082	0.0034	0.0024	10	3
Chlorpyrifos-methyl	0.0104	0.0126	0.0066	1	—
DCPA	0.0002	0.0001	0.0002	—	500
DDT, total	0.0095	0.0056	0.0043	20	0.5 ^d
DEF	<0.0001	0.0001	<0.0001	—	—
Demeton	0.0005	0.00003	0.0006	—	0.04
Diazinon	0.0049	0.0022	0.0022	2	—
Dichlorvos	<0.0001	<0.0001	0.0001	4	0.5
Dicloran, total	0.1926	0.044	0.1175	30 ^d	—
Dicofol, total	0.0218	0.007	0.0235	25	—
Dieldrin	0.0027	0.0021	0.0021	0.1 ^c	0.05
Dimethoate	0.034	0.0022	0.0035	10	0.2
Endosulfan, total	0.0173	0.0158	0.0242	6 ^c	0.05 ^d
Endrin	<0.0001	<0.0001	<0.0001	0.2	0.3
Ethion	0.0128	0.0034	0.0035	2	0.5
Fenitrothion	<0.0001	<0.0001	<0.0001	5	—
Fenuron	0.0004	0.0002	0.0003	—	—
Fonofos	<0.0001	<0.0001	<0.0001	—	0.2
Heptachlor, total	0.0005	0.0005	0.0003	0.1 ^c	0.5 ^d
Hexachlorobenzene	0.0003	0.0004	0.0002	—	0.8
Iprodione, total	0.0026	0.0008	0.0019	300 ^d	40 ^d
Linuron	0.0021	0.0008	0.001	—	2
Malathion	0.0779	0.0487	0.0275	20	20
Methamidophos	0.012	0.0102	0.019	4	0.05
Methomyl	0.0053	0.0037	0.0068	30	25
Methoxychlor, p, p ¹	0.0006	0.0007	0.0002	100	50
Metobromuron	— ^e	<0.0001	0.0001	—	—
Mevinphos, total	0.0066	0.0026	0.0081	1.5 ^c	—
Neburon	<0.0001	<0.0001	<0.0001	—	—
Omethoate	0.0144	0.0013	0.0019	0.3	—
Parathion	0.0097	0.0016	0.0042	5	—
Parathion-methyl	0.0007	0.0001	0.0001	20	—
Pentachlorophenol	0.0016	0.0004	0.0008	—	30
Permethrin, total	0.0251	0.0338	0.0495	50	50
Phosalone	0.0073	<0.0001	<0.0001	5	—
Phosmet	0.0043	0.0009	0.0027	20 ^c	20
Pirimiphos-methyl	0.0007	0.001	0.0006	10	10
Propargite	0.0991	0.0495	0.0491	150	20
Quintozene, total	0.0004	0.0009	0.0003	7 ^c	3 ^d

TABLE 7.5 (continued)

Pesticide Intakes Estimated from FDA's Total Diet Study, 1991

Pesticide	Estimated 6–11 Mos.	Exposure 14–16 yr. Male	($\mu\text{g}/\text{kg}/\text{d}$) 60–65 yr. Female	ADI or FAO/WHO ADI ^a	RfD ($\mu\text{g}/\text{kg}/\text{d}$) EPA RfD ^a
Thiabendazole	0.3950	0.2992	0.3062	300	—
Toxaphene	0.0033	0.0059	0.0024	—	—
Vinclozolin	0.0052	0.0018	0.0061	70	25

^a ADIs and RfDs are usually expressed as mg/kg body wt/day but are expressed here as $\mu\text{g}/\text{kg}$ body wt/day for ease of comparison. The ADIs cited here reflect revisions made in 1991. The RfDs cited here reflect May 27, 1992 revisions.

^b ADI or RfD not established.

^c Includes other (related) chemicals.

^d Parent chemical only.

^e On the basis of phenylurea analysis of 14 selected foods in 1 market basket and 20 foods in each of the other 3 market baskets.

^f No consumption of a food item containing this residue in this age/sex group.

Source: Adapted from General Accounting Office, International Food Safety: Comparison of U.S. and Codex Pesticide Standards, GAO/PEMD-91, Washington, D.C., 1991.

Due to the relatively low exposure of consumers to pesticide residues in foods, it is the opinion of the majority of health professionals involved in food safety that the risks of pesticide residues are far lower than risks from issues such as microbiological contamination, nutritional imbalance, environmental contaminants, and naturally occurring toxins.²⁸ Still, the risks from pesticides in the diet are not zero; examples of consumer poisoning from misapplication of pesticides have been documented, while pesticides may still pose theoretical risks from long-term exposure to consumers due to the scientific impossibility of proving otherwise.²⁵

It also is important to understand that pesticides may provide health benefits as well. Pesticide use allows greater productivity that may translate into greater availability and lower consumer costs. Epidemiological evidence indicates that diets rich in fruits, vegetables, and grains may significantly decrease one's risk of heart disease and certain types of cancer.⁴¹ The NRC concluded that the theoretical increased risks from pesticides presented through consumption of increased amounts of fruits, vegetables, and grains are greatly outweighed by the health benefits provided by eating more of those foods.⁴¹

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8

Food Additives

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Introduction

Food additives have been used for centuries in food processing practices such as smoking and salting meat. Prior to the advent of refrigeration, food grown in the summer had to be preserved for the winter; salt, sugar, and vinegar were commonly used preservatives. The pursuits of explorers such as Marco Polo were often for food additives. Additives serve many roles and common uses include maintaining product consistency and palatability, providing leavening or control pH, enhancing flavor, and imparting color.

A food additive can be defined in many ways. The Codex Alimentarius Commission, which develops international regulatory guidelines for food additives, provides the following definition of a food additive:

Any substance not normally consumed as a food by itself, and not normally used as a typical ingredient of the food, whether or not it has nutritive value, the intentional addition of which to food for a technological (including organoleptic) purpose in the manufacture, processing, preparation, treatment, packing, packaging, transport or holding of such food results, or may reasonably be expected to result, directly or indirectly, in it or its by-products becoming a component of or otherwise affecting the characteristics of such food. The term does not include contaminants or substances added to food for maintaining or improving nutritional qualities.¹

Food Additive Functionality

The functions of food additives and the mechanisms by which they work are innumerable. Over 2800 food additives are approved for use in the U.S. [Table 8.1](#) lists properties and functions of several food additives.

Food Additive Regulations

Just as there are numerous ways to define food additives, there are also many ways to classify them. Additives which are “generally recognized as safe” (GRAS) need not be regulated. Other additives are subject to restricted use status and some fall under the provisions of the zero-tolerance Delaney Clause. The presence of unintentional additives also is permitted under certain conditions.

TABLE 8.1

The Properties and Functionalities of Selected Food Additives

Property	Function	Additive
Anticaking and free flow agents	Tie up moisture in dry ingredients to keep product free flowing during storage and use	Salt, powdered sugar, ground spice blends
Antioxidants	Prevent oxidation, which results in rancidity (off flavors and aromas)	Butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT)
Antibrowning agents	Slow-browning reactions	Citric acid, sulfites
Antimicrobial agents	Chemical preservatives used to control microbial growth	Sodium benzoate, calcium propionate, sorbic acid
Coloring agents	Enhance product appearance	Natural and synthetic dyes, such as erythrosine
Curing agents	Fixing meat color	Nitrites
Dough conditioners and strengtheners	Improve dough properties	Phosphates, sulfates, enzymes
Fat replacers	Replace fat and reduce caloric value of food	Olestra
Flavor enhancers	Intensify flavors	Monosodium glutamate
Humectants	Prevent drying out of semi-moist foods	Propylene glycol
Nonnutritive sweeteners	Replace sugar and reduce caloric value of food	Saccharin, aspartame
Sequestrants	Tie up trace minerals that cause color changes	Ethylenediaminetetraacetic acid

Source: Adapted from Maga, J. A., *Food Additive Toxicology*, Marcel Dekker, New York, 1995, 1.

Generally Recognized as Safe (GRAS)

This list of food additives was established in 1958 under the Food Additives Amendment to the U.S. Federal Food, Drug, and Cosmetic Act (FFDCA). According to this act, GRAS substances are

... generally recognized, among experts qualified by scientific training and experience to evaluate its safety, as having been adequately shown through scientific procedures (or in the case of a substance used in food prior to January 1, 1958, through either scientific procedures or experience based on common use in food) to be safe under the conditions of its intended use.³

GRAS additives have been classified as such through either scientific procedures or their historical use in the food supply. Additives not classified as GRAS have regulated food additive status. Substances not used in food prior to the Food Additives Amendment must undergo toxicity testing to prove their safety, then must be classified as either GRAS or approved by the Food and Drug Administration (FDA) for regulated food additive status.

The Delaney Clause

According to the Delaney Clause of the 1958 amendments to the FFDCAs, any food additive found to induce cancer in humans or in animals would be banned in the U.S., regardless of the level of the additive or the magnitude of the theoretical cancer risk. Many food and chemical manufacturers have pushed for a revision of the clause as it has been argued that the general terms of the FFDCAs sufficiently controlled the use of hazardous additives. Furthermore, the clause could technically prohibit the addition of essential nutrients to foods, as they could cause cancer in massive doses.⁴ Some argued on the grounds that a zero tolerance law is scientifically impossible. Substances causing cancer in animals, but not proven to be harmful to humans, also would have to be banned.⁵ These arguments were refuted by the Food Protection Committee of the National Academy of Sciences, who stated that “no effect” levels could be carcinogenic as the effects may be too weak to be demonstrated in feasible numbers of animals for testing, whereas carcinogenic effects may be evident in the large human population potentially exposed to additives. The committee also recognized the possibility of synergistic effects between diet and a person’s susceptibility to carcinogens, although these factors had not been adequately explored at the time.⁴

Prior to 1996, pesticides that were found to concentrate as a result of processing from raw to processed food commodities or those directly added to processed foods were also considered to be food additives and, therefore, were subject to the Delaney Clause.^{6,7} Subsequent legislation passed in 1996 eliminated the classification of pesticides as food additives.

Unintentional Additives

The remainder of additives not classified as GRAS or regulated through intentional additive use are unintentional additives. These additives are found in foods after production, processing, storage, or packaging, and include plant growth regulators and minute quantities of packaging substances.⁵ These indirect additives are permitted in foods by law provided the processor takes every precaution to maintain good manufacturing processes and only if the quantity of the additive remains at an insignificant level.

Assessment of Food Safety

The safety of a food additive is determined through extensive testing in animal models before FDA approves the additive. Although the regulations for animal testing are well outlined, there are no regulatory requirements for human testing. The FDA Redbook II, otherwise known as the FDA Draft Toxicological Principles for the Safety Assessment of Direct Food Additives and

Color Additives Used in Food, includes such guidelines for conducting human testing of food additives for safety assessment.⁸

For an additive to be approved, animal toxicity and metabolism studies of the additive must supply substantial information covering the following areas:⁹

- Identification of hazards posed by the additive
- Indication of the dose-toxicity relationship for those hazards
- Estimation of the probable human consumption of such additives

U.S. federal regulations outline the requirements for the FDA safety assessment. A determination of the NOEL (no observed effect level) or the NOAEL (no observed adverse effect level) from animal toxicity studies is essential. These are determined through chronic toxicity or lifetime exposure studies to the additive. The NOEL or NOAEL, given in terms of the weight of the additive per kg body weight per day, will be used to determine the ADI (acceptable daily intake) for humans. The ADI is intended to reflect the amount ingested over an entire lifetime; it is commonly set at 1% of the NOEL or NOAEL which presumably allows for consideration of possible greater toxicity in humans relative to experimental animals and for increased susceptibility to specific members of the human population.¹⁰

Specific Food Additives Under Scrutiny

Saccharin

Saccharin is a nondigestible sugar substitute that is 300 times sweeter than sugar.¹¹ Diabetics and persons requiring a low caloric intake may benefit from the use of sugar substitutes. Saccharin is used in the U.S. in products such as soft drinks, tabletop sweeteners, and cosmetic products. It is available commercially as an acid salt, sodium salt, or calcium salt. In long-term feeding studies of 5.0 and 7.5% saccharin in the diet, rats showed an increase in urinary bladder tumors.¹² However, more than 20 studies have failed to demonstrate an affiliation between saccharin consumption and cancer in humans.

The controversy surrounding saccharin has been debated for decades. In 1907, the chief of the USDA's Bureau of Chemistry, Dr. Harvey Wiley, voiced his concern regarding the safety of saccharin. President Theodore Roosevelt, a diabetic, retorted by saying, "My doctor gives it to me everyday. Anybody who says saccharin is injurious to health is an idiot."⁵ Saccharin was banned for a short time until its use was reinstated due to the sugar shortage during World War I. In 1958, saccharin was given GRAS status due to long-term animal studies performed throughout the 1950s.⁵ The GRAS status was removed

in 1972 due to a possible association found between bladder cancer in rats and saccharin. In 1977, the FDA proposed to ban the sweetener and a moratorium was placed on the ban pending additional toxicity studies. In addition, any saccharin-containing products required labels stating its potential to cause cancer in laboratory animals. The World Health Organization/United Nations Food and Agricultural Organization Joint Expert Committee on Food Additives (JECFA) estimated the ADI of saccharin to be 2.5 mg/kg body weight.¹² This level was determined using large amounts of epidemiological and mechanistic data so as to incorporate a large safety factor due to the potential severity of toxicity.¹³ Rat data models extrapolated from animal studies to predict theoretical human risks indicate that drinking 2.3 12-oz cans of a saccharin-sweetened beverage poses a human risk of cancer of much less than 1 in 1,000,000.¹¹

Although the risk to humans may be minimal, extensive studies have shown a definite link between saccharin consumption and cancer in rats. The threshold dose causing bladder cancer in rats is 3% saccharin in the diet.¹⁴ This NOAEL was based on a two-generation rat bioassay, one of the largest ever undertaken. The studies reviewed by Meister agree with these results; no increases in tumors were noted with 1% saccharin diets in rats.¹¹ Sodium saccharin has been shown to promote cancer with subcarcinogenic doses of known bladder cancer agents. Saccharin's carcinogenic effect also may be species-specific, as 5.0% saccharin in the diets of mice does not indicate any significant increase in bladder cancer.

Because saccharin is not metabolized, it cannot be activated and is not able to form adducts with DNA. Renwick describes the effects of saccharin on DNA as structural disturbances that are paralleled by similar doses of sodium chloride.¹⁴ The carcinogenic effect is suspected to be cation-specific, as sodium saccharin is the most prominent tumor promoter compared with calcium saccharin and acid saccharin. Researchers have hypothesized a physical effect of saccharin that may cause the increased cancer incidence. Very high doses of saccharin may produce crystals that physically damage the inner walls of the bladder. The rat responds to this insult by producing large numbers of bladder wall cells. This increased production of cells may be the cause for increased tumor incidence.¹¹ Saccharin, a nongenotoxic agent, can be carcinogenic by causing inflammation and chronic mitogenesis.¹⁵ This dose response would likely fit a threshold level. The species specificity of saccharin carcinogenicity is due to the unique chemistry of rat urine.

Crystals containing silicate were discovered in the urine of male rats who were fed large quantities of saccharin.¹⁴ An increase in sodium ions, which subsequently causes an increase in pH, increases the formation of silicate crystals. The presence of protein in the urine amplifies crystal formation. Small proteins may enter the kidney and find their way into the urine. The suspected mechanism for crystal formation is the complexing of saccharin anions with urinary proteins and subsequent enhancement of precipitation and crystallization. However, the binding of the protein is likely to be of limited importance in comparison with an increase in pH and sodium concentration due to

the low specificity of saccharin anions for the urinary proteins. Yet under certain conditions, the crystal formation theory could explain the species specificity; the suspected protein involved in the formation of silicate crystals, alpha-2-microglobulin, is more prominent in rats than in mice or humans.^{11,14} Until silicate crystal formation can be positively linked with bladder tumors, species specificity cannot be assumed.

Epidemiological studies examining bladder cancer incidence in diabetics consuming saccharin and saccharin consumption by bladder cancer patients do not implicate saccharin as a human carcinogen. Due to the frequent use of saccharin in Denmark between 1941 and 1945, it was thought that this population may demonstrate an increase in bladder cancer rates, although no association between saccharin consumption and bladder cancer was found.¹¹ In 1981, saccharin was added to the National Institute of Health's (NIH) list of substances that can be "reasonably anticipated" to cause cancer in humans. Most recently, a panel from the NIH met to vote on the possible delisting of saccharin, due to 2 decades' worth of studies, which failed to associate saccharin with cancer in humans. By a narrow margin, the panel voted to keep saccharin on the NIH carcinogen list; some panelists preferred to err on the side of caution considering the controversy.

Aspartame

Aspartame is a dipeptide formed from the amino acids phenylalanine and aspartic acid. Quoted to be 180 times sweeter than sucrose without a bitter aftertaste, its sweetness varies with pH and temperature conditions.⁵ It has also been shown to enhance fruit flavors and is heat unstable. Initially, due to its composition of two essential amino acids, it was thought to be very safe if hydrolyzed by the digestive system.

Hydrolytic products include L-aspartic acid, L-phenylalanine, aspartylphenylalanine, phenylalanine methyl ester, and methanol.⁹ In certain food and beverage matrices, aspartylphenylalanine diketopiperazine (DKP), beta-aspartylphenylalanine methyl ester, and its free acid may be present. The FDA set the ADI for aspartame at 50 mg/kg body weight/day from the NOEL value of 2000 mg/kg body weight/day based on clinical studies.¹⁶

G.D. Searle submitted a petition for the approval of aspartame in 1973. It included metabolism and toxicity tests which demonstrated that methanol was produced during aspartame degradation.¹¹ However, blood levels of methanol obtained after aspartame consumption were considered to be too low to have an adverse effect.⁵ In 1974, the FDA approved the use of aspartame. Subsequent objections were made based on allegations that aspartame might cause brain damage. Searle suspended the marketing of aspartame until the safety issues were resolved.

The safety issues surrounding aspartame included increased concentrations of amino acids and methanol. Aspartame is hydrolyzed by peptidases and esterases; its constituent amino acids and methanol can then enter portal

circulation.¹⁷ Individual safety concerns regarding aspartic acid, phenylalanine, methanol, and DKP are discussed below.

Hydrolysis Products of Aspartame

Aspartic Acid

This essential amino acid constitutes approximately 40% of aspartame by weight.¹⁸ It was speculated that ingestion of monosodium glutamate (MSG) in combination of aspartame-derived aspartic acid (closely related to glutamic acid) would increase plasma concentrations of aspartate and glutamate to a level that may induce brain damage. Tests in neonatal mice failed to show a significant increase in plasma aspartic acid concentrations until a level of 100 mg aspartame/kg body weight was exceeded. This is equivalent to ingestion of 12 l of an aspartame-sweetened beverage by a 60 kg person. Acute administration of 200 mg aspartame/kg body weight resulted in a peak aspartic acid concentration of $7.6 \pm 5.7 \mu\text{mol/l}$ in plasma, far below neurotoxic levels in animals. Studies of aspartame and MSG given simultaneously in doses of 34 mg/kg body weight in humans failed to elevate either aspartate or glutamate plasma to levels similar to those achieved after ingestion of a high protein meal. A serving of milk contributes 13 times more aspartic acid to the diet than a serving of an aspartame-sweetened beverage.¹⁹

Phenylalanine

Phenylalanine comprises about 50% of aspartame by weight.¹⁸ The concern for phenylalanine toxicity stems from persons with phenylketonuria (PKU) who are unable to metabolize phenylalanine normally. Neurotoxicity, including mental deficiencies in children with PKU, results from sustained extreme elevations of phenylalanine plasma levels in the order of $\geq 1200 \mu\text{mol/l}$. However, these levels cannot be achieved by aspartame consumption, regardless of being heterozygous for PKU. Acute aspartame doses of 200 mg/kg in normal humans and 100 mg/kg in humans heterozygous for PKU result in phenylalanine plasma levels far below the threshold for neurotoxicity. Milk contains six times more phenylalanine than an aspartame-sweetened beverage.¹⁹

Methanol

Methanol makes up approximately 10% of aspartame by weight.¹⁸ Methanol is metabolized in the liver to make formic acid, which is ultimately broken down to carbon dioxide and water. Methanol toxicity, due to the accumulation of formate, results in metabolic acidosis and ocular damage. To attain toxic levels (200 to 500 mg/kg) of formate in the body, a 60 kg person would have to ingest 240 to 600 l of an aspartame-sweetened beverage. Administration of a 240 mg aspartame/kg body weight dose in humans, equivalent to 24 l of an aspartame-sweetened beverage, does not appreciably raise the blood methanol concentration (25.8 mg/l, far below toxic levels). This dose

does not cause a significant increase in blood formic acid concentration. A 500 mg dose of aspartame, equivalent to 1 l beverage, caused no distinct change in serum methanol concentration. Chronic tolerance studies of ingestion of 75 mg/kg body weight for 6 months in healthy adults did not increase either methanol or formate levels in the blood. Five to six times more methanol is consumed by ingestion of a serving of tomato juice than an equivalent amount of an aspartame-sweetened beverage.¹⁹

Diketopiperazine

DKP is a cyclization product formed by breakdown of aspartame in certain pH or temperature conditions, particularly in liquid systems.¹⁸ This causes a loss of sweetness but it does not affect the safety of an aspartame-sweetened beverage.¹⁸ The NOEL for DKP established by the FDA through animal studies was 3000 mg/kg body weight. Should all the aspartame in a normal serving of an aspartame-sweetened beverage be cyclized to produce DKP, the DKP level consumed would still be well below the ADI level determined by the FDA.

Marketing of Aspartame

Consumer concerns regarding the safety of aspartame frequently have been raised. The number of complaints regarding anecdotal health effects following aspartame ingestion increased during its initial marketing. The FDA prompted the U.S. Centers for Disease Control and Prevention (CDC) to evaluate these complaints to determine the need for further study. The results could not pinpoint any specific subpopulation that was susceptible to these health effects, nor could any group of symptoms be clearly related to aspartame.²⁰ The CDC stated, "Despite great variety overall, the majority of frequently reported symptoms were mild and are symptoms that are common in the general populace."²⁰ As reported by the CDC, the most commonly reported symptoms anecdotally associated with aspartame from 1987 to 1993 were headache, dizziness, and gastrointestinal distress.

A postmarketing surveillance system for aspartame was developed voluntarily by the Nutrasweet Company. There was an initial surge of complaints regarding aspartame during its first years of being marketed (between 1983 and 1986); however, the frequency of complaints declined from 1987 to 1993, each year yielding approximately 300 complaints. Meanwhile, the products available increased over time.

A 6-month tolerance study of aspartame demonstrated no significant difference in frequency of anecdotal symptoms between aspartame consumption and a placebo consumption.¹⁸ The randomized, double-blind, placebo-controlled parallel group design study used a 75 mg/kg body weight dose per day, a dose 25 times the current 90th percentile of aspartame consumption. Eighty-three percent of participants (n = 108) reported 72 different complaints, ranging in severity from mild to moderate. The most common symptoms were headache, upper respiratory tract symptoms, and abdominal

discomfort. There was no significant difference found between the treatment and the control (placebo) group.¹⁸

Some food intolerance may exist for aspartame, and it may be a source of hives (urticaria) in some hypersensitive individuals.⁵ There is apparently no link between aspartame and seizures in adults and children, nor is there a risk to fetuses as aspartame does not cross the placenta.⁵

Erythrosine (FD&C Red #3)

Erythrosine, known also as FD&C Red #3, is a xanthene dye containing four iodine atoms. Synthesized by iodination of fluorescein, this brown powder turns red with slight fluorescence in 95% alcohol.²¹ It was approved for use in 1907. The possible carcinogenic and oncogenic effects of erythrosine are caused by secondary effects on the thyroid and pituitary glands.²² The ADI for erythrosine was determined by the JEFCA to be 0.1 mg/kg body weight based on erythrosine's NOEL for thyroid and pituitary effects in humans.

Thyrotropin (TSH) produced in the pituitary gland regulates thyroid structure and function, and stimulates thyroid growth.²² Tumors can be caused by hyperstimulation of the thyroid. TSH stimulates the synthesis and secretion of thyroxine (T_4), which can then be monoiodinated to the biologically active form of 3,3',5-triiodothyronine (T_3). Rats fed 4.0% erythrosine in a lifetime study showed inhibition of the T_4 to T_3 conversion, resulting in a long-term increased stimulation of the thyroid through TSH.²² Increased incidence of thyroid follicular cell hyperplasia, adenomas and carcinomas were found in male rats receiving this 2464 mg/kg body weight/day dose, equivalent to 4.0% of the diet, for 30 months following *in utero* exposure. The NOEL was established at 0.5% of the diet, or 251 mg/kg body weight/day.

Studies of absorption, distribution, metabolism, and excretion determined that less than 5% of an erythrosine dose is absorbed.²³ Nearly all the color is excreted unchanged in the feces.⁵ After ingestion, the compound is relatively stable. That which is absorbed is rapidly excreted through the bile.⁵ Erythrosine is partially deiodinated in the gut to lower-iodinated fluoresceins. An elevation in protein-bound iodine was observed, although this had no effect on the thyroid. In subchronic feeding studies, erythrosine was shown to inhibit the conversion of thyroxine to triiodothyronine.²¹ This results in increased secretion of thyrotropin by the pituitary gland, which causes increased stimulation of the thyroid. While *in vitro* studies show that erythrosine may inhibit neurotransmitters,⁵ *in vivo* implications have not been determined. Human studies failed to identify any adverse effects.²¹

Due to the indirect mechanism by which massive doses of erythrosine cause thyroid tumors, most scientists believe erythrosine genotoxicity in humans does not constitute a major health threat.²² The FDA determined that "the Delaney Clause does not apply to substances that act secondarily or indirectly or to those which no-effect levels can be reasonably established," so erythrosine use is still allowed.⁵

Olestra

Olestra consists of hexa-, hepta-, and octaesters of sucrose formed from long-chain fatty acids of edible oils. Olestra is a nonabsorbable, energy-free fat substitute approved by the FDA in 1996 to replace cooking oil used to make savory snacks, such as potato chips and crackers.

Anecdotal Reports of Health Effects Due to Olestra

As in the case of other food additives or processing methods, there has been much publicity regarding the safety characteristics of olestra. Anecdotal reports of adverse gastrointestinal (GI) effects prompted further research in the possible health effects of olestra. A study by Cheskin et al. concluded that consumption of olestra-containing chips *at libitum* does not cause increased frequency of GI events as compared to regular (triglyceride) chips.²⁴

A randomized, double-blind parallel placebo-controlled study was performed where participants were invited to a movie screening while given a 13 oz (369 g) bag of either regular triglyceride chips or chips made with olestra. They were permitted to consume as much or as little of the chips as they wished during the film. Forty hours after the movie, the participants were interviewed regarding any symptoms they may have experienced. There was no significant difference found between the occurrence of GI symptoms between olestra and triglyceride chips. The mean consumption of olestra chips was larger than a typical 2 oz bag of chips; thus, enough olestra was consumed to evaluate its potential GI effects. The participants that consumed more than 4 oz (113 g) chips had no difference in the severity or frequency of reported GI symptoms between groups. Furthermore, there was “no indication of a dose-response relationship of increasing symptoms with higher consumption levels.”²⁴

These findings do not suggest that olestra causes loose stools or cramping, as stated by the information label on olestra products. Since GI events are frequent in the general population (up to 69% of individuals report one or more symptoms in a 3-month period), this may be an alternative explanation to the symptoms experienced by the participants of the study and consumers of olestra.²⁴ A “nocebo” effect may result in increased reports of GI events; the participants’ informed consent mentioned that GI symptoms might be experienced. Cheskin et al. report that typical consumption of olestra does not cause increased frequency or severity of adverse GI events.²⁴

Clinical studies did not report any medically significant health-related conditions due to olestra ingestion.²⁵ Studies collecting information on common GI symptoms have reported that similar symptoms occurred in both olestra and placebo groups.²⁶⁻²⁸ There was no dose-response relationship between olestra intake and severity of symptoms. Further studies indicate that subjects eating >8 g olestra/day from savory snacks reported no symptoms on 90% or more of the days that olestra was consumed.

Ingesting large amounts of a lipophilic substance can cause loose or soft stools. Thus, it is not surprising that numerous GI symptoms reported related to a change in stool consistency, which may be interpreted as diarrhea. However, the diarrhea reported by the subjects tested by Koonsvitsky et al. was not pathological diarrhea, but rather represented stool softening.²⁸ A loss of water soluble nutrients due to malabsorption caused by pathological diarrhea would not be experienced by a diet containing olestra.^{26,27} No evidence of significant fluid loss has been found due to olestra consumption. The symptoms experienced are not unlike those associated with a large intake of dietary fiber. Furthermore, severity of symptoms is not evident due to olestra ingestion in individuals with diseased GI tracts.

Effects of Olestra on Nutrient Absorption

The possible ingestion of large amounts of olestra by humans has stimulated research investigating the interference of olestra with absorption of lipophilic nutrients such as fat-soluble vitamins and essential fatty acids. A partitioning between lipophilic constituents and olestra may occur in the GI tract.²⁹ The factors which control the partitioning mechanism between olestra and fat-soluble nutrients include:

- Lipophilicity of the nutrient; increasing lipophilicity increases nutrient partitioning into olestra
- Relative amounts of olestra and the nutrient; as the amount of olestra increases, the partitioning of the nutrient into olestra increases
- Time between the consumption of olestra and the nutrient; a longer contact period between olestra and the nutrient in the GI tract increases the nutrient partitioning into olestra

Peters et al. summarize various studies in pigs and humans regarding the potential effects of olestra on the absorbance of various fat- and water-soluble compounds. Subjects were fed daily amounts of olestra in the diet, up to 10 times the estimated mean intake from savory snacks. It was determined that olestra will not deplete the body of nutrients, although it may affect the absorption of fat-soluble nutrients eaten simultaneously with the fat substitute.²⁵

Vitamin A

In pig studies, liver vitamin A content was decreased by 45% in pigs fed 0.25% olestra in the diet.³⁰ This dose represents a level similar to the 90th percentile chronic human intake from savory snacks, 3.7 to 10.0 g/day.³¹ However, if the pigs ate olestra in potato chips, the liver vitamin A content decreased by only 15%. Cooper et al. found that 93 µg retinyl palmitate/g olestra restored liver vitamin A content to the norm.³⁰

Vitamin E

Pigs fed 0.25% or 0.5% olestra experienced a decrease in liver vitamin E content by 24 or 31%, respectively.³⁰ Serum vitamin E levels decreased by 26 or 49% from the 0.25 or 0.5% doses, respectively. These levels parallel the 90th percentile chronic human intake.³¹ If olestra was consumed as potato chips, the serum vitamin E content would be reduced by 12 or 25%. Restoration of liver vitamin E requires a supplement of 2.1 mg tocopheryl acetate/g olestra.³²

Vitamin D

Serum concentration of vitamin D decreased by 20 to 25% in humans depending on the dose of olestra.^{26,27} This effect was achieved even with a supplementation of vitamin D to the diet, resulting in a diet contribution of 68% of total vitamin D. Without supplementation, the dietary contribution of vitamin D was approximately 20%. Less than 20% of vitamin D is received from the diet.²⁵ In extreme climate conditions, such as a Canadian winter, less than 50% of vitamin D is received from the diet. The overall change in vitamin D absorption is not significantly affected by olestra consumption in pig and human studies.²⁸

Vitamin K

Overall, vitamin K absorption is not significantly affected by olestra consumption.^{26,27} Serum concentration of phylloquinone in humans decreased by 36 to 47% depending on the dose of olestra. However serum phylloquinone reflects mainly short-term intake of vitamin K.²⁵ Supplementation of 3.3 µg vitamin K/g olestra was found to offset the decrease in serum phylloquinone from olestra consumption.^{26,27}

Under the extreme conditions of olestra intake in these studies, the absorption of vitamins D and K were not significantly affected. The decrease in absorption of vitamins A and E are not likely to be nutritionally significant for most people eating olestra in savory snacks.²⁵ However, due to possible ingestion of large quantities of olestra, the FDA determined that supplementation of all vitamins in olestra-containing products is necessary.

Triglycerides

The effects of olestra on the absorption of triglycerides is minimal.³³ The absorption of ¹⁴C-triolein in male humans from meals containing 8, 20, or 32 g olestra was compared to the absorption from a meal without olestra. A 32 g dose of olestra caused a 1.2% reduction in triolein absorption.³³ This dose, like those in the pig and human studies in vitamin absorption, is exaggerated compared to typical olestra consumption. Although this reduction in absorbance is a statistically significant difference, it will not be nutritionally significant as the 32 g olestra dose is 25% greater than the estimated 90th percentile single-day intake of olestra by the subgroup of heaviest eaters, 13- to 17-year-old adolescents.³¹ To put things in perspective, common dietary components

such as fiber impose a much larger decrease in fat absorption than that achieved by olestra.³³ A 1.2% reduction of fat absorbance relates to a reduction of only 7 kcal in a typical 2000 kcal/day. Absorption of essential fatty acids linoleic and alpha-linoleic acid will be less affected by olestra due to their physical properties. The efficiency of absorption increases as the melting point decreases.³³ Triolein melts at -32°C , while trilinolein melts at -43°C , therefore trilinolein would have increased absorbance over triolein. Triolein and trilinolein prove to be good models for olive oil and other vegetable oils.

Dietary Phytochemicals

Dietary phytochemicals, such as phytosterols and carotenoids, are hypothesized to reduce the risk of cancer and other chronic diseases; they are found in fruits and vegetables.²⁹ Due to their lipophilic nature, there is some concern regarding their interaction with olestra in the diet. Olestra has been shown to affect the bioavailability of those compounds whose log octanol/water partition coefficients are ≥ 7.5 .²⁹ The bioavailability of phytosterols would be decreased by less than 10% if olestra was consumed at every meal.²⁹ Phytosterols may possibly reduce cholesterol absorption; however, olestra may have the same quality and, thus, the change in phytosterol absorbance is not likely a concern. A 5.9% decrease in bioavailability of beta-carotene would be observed if olestra was consumed with carotenoid-containing foods and all snacks eaten contained olestra.²⁹ Similar data was obtained by Koonsvitsky et al. and Schlagheck et al.^{27,28} A high-fiber diet decreased beta-carotene absorption by 50%.²⁹ Cooper et al. conclude that the reduction of beta-carotene absorption from olestra ingestion will have no significant effects over time.²⁹

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9

Analysis of Chemical Toxicants and Contaminants in Foods

James N. Seiber

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Introduction

Food is a complex chemical mixture, consisting of primary constituents such as fat, protein, carbohydrates, fiber, moisture, and minerals, and what might be termed “secondary” or “minor” constituents that include natural chemicals as well as those added which may influence the flavor, stability, longevity, mechanical handling, and other properties of foods. Many of these secondary or minor constituents are intentionally added to foods, and thus are regulated in terms of what and how much may be added. The legal definition of a food additive includes any chemical that is present in a food in (normally) minor amounts at any time, either intentionally to produce a functional or technical effect or unintentionally as a consequence of the production, processing, storage, or packaging of a food item.¹ This includes any source of radiation, as well as those products (such as pesticide residues, and

drugs and feed additives for food-producing animals) that are washed off or removed in some way and do not appear, or cannot be detected, in the final product as a result of processing.²

Contemporary concern over the safety of foods and, particularly, the addition either intentionally or unintentionally of chemicals which might be toxic to the consumer, has given rise to an extensive array of analytical tests, many of which are mandated by laws, regulations, or guidelines. The methods are standardized by such organizations as the U.S. Food and Drug Administration (FDA), U.S. Department of Agriculture (USDA), U.S. Environmental Protection Agency (EPA), Association of Official Analytical Chemists (AOAC), Institute of Food Technologists (IFT), National Food Processors Association, and the departments of agriculture of states such as California, Florida, and Texas. For many intentional food additives, unintentional additives (such as pesticides), and some inorganics and natural toxicants, analyses are done routinely on sizeable percentages of shipments and lots — a monitoring activity conducted by federal and state agencies, and by the food industry and its trade organizations.

In this chapter the primary focus will be on chemical contaminant residues in foods, with examples included primarily from among pesticides, but with some reference to animal drugs, food additives, and some natural toxicants. Most of the techniques used for pesticide residue analysis also are used for animal drugs, natural toxicants, and the intentional food additives such as antioxidant and antimicrobial preservatives. These classes of chemicals have in common their predominately organic chemical structures, their presence in foods at relatively low, often sub-ppm levels, and their tendency to coexist with derived breakdown products which in many cases also must be included in the analysis.

Who Performs Food Analysis and Why

Ultimately, all food analyses are conducted to safeguard the consumer, but there are several more proximate reasons for doing so which have regulatory and marketing imperatives. Methods are selected and used based upon the specific needs of companies and agencies within the larger framework of consumer protection and food quality/safety needs.³

Registration

Companies that develop pesticides, animal drugs, and food additives must develop methods capable of determining their potential product in or on the crops, animals, and food-based products of intended use, and in the environment. The development of such methods may involve several iterations

because the method must account for the parent and all toxicologically important breakdown products in all products/environments in which the chemical might ultimately be found as a residue. The breakdown products and affected products/environments may not be completely known in the development phase, so that an initial method may require several modifications. Feeding trials with experimental animals or dosing trials with crops generally use radiolabelled parent chemicals, and analyses are based upon radioassay of the parent and products in various tissues and excreta. These studies are important for understanding conversion pathways, target organ specificity, and clearance and accumulation pathways, but the methods are not applicable to the subsequent needs for routine analytical methods for ensuring the proper use and ultimate safety of the product when in large-scale environmental testing or, eventually, in commercial use. Thus, methods must be developed by the registrant for detecting unlabelled parent/conversion products which can be submitted to EPA (pesticide) or FDA (animal drug or food additive) at the time the registration packet is submitted, so that the appropriate regulatory agency can detect the product in the treated agricultural commodities and any food items prepared or processed from them. These methods, after checking and validation, may ultimately find their way into one or more compendia of analytical methods, or other appropriate references, such as the following:

*Official Methods of Analysis of the Association of Official Analytical Chemists.*⁴ This compendium contains detailed methods for the analysis of drugs, pesticides, metals, vitamins, food additives, natural poisons, and other chemical and microbial contaminants in food and feed.

Journal of the Association of Official Analytical Chemists. Same coverage as the compendium of the AOAC, but including the results of validation testing and new methods not yet incorporated in the compendium.

*Pesticide Analytical Manual.*⁵ Volumes II and III contain detailed methods for all registered pesticides, applicable to the food and feed items included in the pesticides' label. Volume I contains multiresidue methods for use in screening and enforcement analysis, as well as general directions for extraction, cleanup, and gas and high performance liquid chromatographic (HPLC) determination.

Journal of Agricultural and Food Chemistry. Published by the American Chemical Society and includes research articles on new methods for crop and animal protection agents, flavors and aromas, additives, and contaminants. Many papers in this journal describe breakdown pathways indicating what secondary products of the parent pesticides, drug, or food additive may need to be included in analysis.

Enforcement

Regulatory agencies generally need to analyze foodstuffs for all toxic residues (e.g., all pesticides, all animal drugs, all toxic metals), and not just one or two specific products. For this purpose, the single residue methods (SRMs) developed by the registrant and described briefly in the previous section are not appropriate. The regulatory methods are mostly “multiresidue methods” (MRMs) capable of detecting and determining many chemicals in several types of food products, and doing so in the somewhat routine, high volume, rapid turn-around atmosphere of a large monitoring laboratory.^{6,7} A partial listing of agencies and other entities that perform such monitoring activities is in [Table 9.1](#).

The volume of samples analyzed just for pesticide residues can be gauged from the summary of FDA surveillance data cited in NRC³ and reproduced in [Table 9.2](#). The overall incidence of positives was small, averaging less than 5%, and most of these were nonviolative; that is, within established tolerances.⁸ Of the low incidence violations that do occur for pesticides, less than 1% are for over-tolerance violations while 5 to 10% are for the presence of residues in a food for which no tolerance has been established. This situation can arise from carry-over of soil residue to a nontarget crop grown in a subsequent season or year, or from uptake of residue from the air or irrigation water of the nontarget crop.

To summarize, SRMs are generally chosen when the sample is known or suspected to contain a residue of a specific chemical. They are used when there is some special concern over a given chemical in foods, such as occurred during the contamination of watermelons by the pesticide aldicarb in the 1980s; the suspected contamination of flour, cake mixes, etc. with the fumigant ethylene dibromide in the 1980s; and for such natural toxicants as the aflatoxins and potato alkaloids in contaminated foods — a continuing concern. MRMs are chosen when the residue history of the sample is unknown, and the question is, “Are pesticides present and, if so, how much of each?” MRMs will provide information on a much broader range of chemicals than SRMs for a similar investment of time and energy.⁹ The FDA and other agencies often use simplified versions of SRMs to screen samples for potential violations before proceeding to quantitation with a more elaborate MRM or SRM.

Analytical Approach

Whether a method is single or multiresidue in scope, it will include a series of discrete steps or unit processes whose ultimate goal is to detect and quantify specific chemicals at levels of interest, in a relatively complex food matrix. The matrix may contain hundreds or even thousands of natural and man-made chemicals which can potentially interfere with the analyte(s) of

TABLE 9.1**Agencies and Other Organizations that Conduct Monitoring Analysis of Foods**

Name	Purview
<i>Federal</i>	
Environmental Protection Agency	Reviews and checks out analytical methods for pesticides submitted by registrants
Food and Drug Administration	Monitors residues in imported and domestic food, including processed food
Food Safety and Inspection Service	Monitors residues in meat and poultry
Agricultural Marketing Service	Monitors residues in raw egg products
Fish and Wildlife Service	Monitors pesticides in fish and wildlife
<i>State</i>	
California Department of Food and Agriculture	Monitors pesticides and other contaminants in, primarily, fruits and vegetables
Florida Department of Agriculture	Monitors pesticides and other contaminants in raw and processed foods
Texas, New York, Oregon, Washington, Massachusetts and other states	Monitor foodstuffs of specific interest to those states
<i>Universities</i>	
Cornell University, University of California, Davis, University of Florida, Michigan State University, and various satellite university laboratories.	Conduct analyses for pesticides in minor crops as part of the USDA IR-4 Minor Use registration program
<i>Industry</i>	
National Food Processors Association	Monitor pesticide residues, other additives/contaminants in fresh and processed commodities
General Mills, DelMonte, Campbell, and other food companies	Monitor pesticides and other chemical contaminants for their company's products
DowElanco, DuPont, Zeneca, Monsanto, and other chemical companies	Conduct analytical support for their own products in food and environmental media
<i>Private Laboratories</i>	
Commercial analytical laboratories	Conduct analyses for pesticides and other toxicants (metals, solvents, additives) in foods, soil, water, and wastes, under contract with companies, agencies, and food producers/processors

interest, often at concentrations many-fold higher than those of the analytes. It is a proverbial "needle in the haystack" undertaking. Thus, methods are designed to take advantage of unique physical properties, such as polarity, volatility, and optical properties, and chemical properties (reactivity, complex formation, combustion characteristics) which allow the analyte to stand out

TABLE 9.2

Total of Samples and Positive Detections in FDA Residue Data

Chemical	Samples (Total No. Sampled)	No. Positive	Percent (%) Positive
Bromophos-ethyl	113	1	0.9
Dichlorvos	763	1	0.1
Prothiofos	1	1	100.0
Trichlorfon	1	1	100.0
Cyanophos	912	2	0.2
Ethoprop	1927	2	0.1
Atrazine	669	4	0.6
Fonofos	290	4	1.4
Fenthion	267	6	2.2
Penthoate	2328	10	0.4
Carbophenothion	7332	11	0.1
O-Ethyl-O- <i>p</i> -nitrophenyl phenyl- phosphorothioate	6912	12	0.2
Mecarbam	16	14	87.5
Dicrotophos	15	15	100.0
Ethylene Thiourea (ETU)	22	15	68.2
Fenitrothion	5171	30	0.6
Quinalphos	40	30	75.0
Methoxychlor	5643	36	0.6
Phorate	40	36	90.0
Phosphamidon	3499	63	1.8
Chlorfenvinphos	9299	66	0.7
Methomyl	2706	69	2.5
Aldicarb	1141	76	6.7
Phosalone	11,857	82	0.7
Profenofos	9689	105	1.1
Disulfoton	15,121	117	0.8
Daminozide	514	125	24.3
Primiphos-methyl	4449	176	3.9
Monocrotophos	18,617	191	1.0
Dicofol	12,430	216	1.7
Parathion-methyl	30,361	240	0.8
Benomyl	1023	292	28.5
Ethylenebisdithiocarbamate	2539	296	11.6
Phosmet	15,604	335	2.1
Methidathion	15,948	437	2.7
Azinphos-methyl	15,320	474	3.1
Parathion	40,029	591	1.5
Carbaryl	11,212	632	5.6
Diazinon	35,896	648	1.8
Ethion	30,588	699	2.3
Malathion	39,226	1161	2.9
Mevinphos	25,639	1320	5.1
Dimethoate	40,496	1418	3.5
Captan	30,108	1499	5.0
Chlorpyrifos	45,418	2180	4.8
Acephate	39,940	3845	9.6

Source: Based on unpublished FDA surveillance data, 1988 to 1989.

from the forest of matrix-derived interferences. This theme is found in all of the steps in analysis:^{10,11}

- *Extraction* — Remove the analyte from the matrix, leaving the bulk of the matrix behind as a filterable or nonvolatile mass. This is most frequently accomplished by extraction with an organic solvent, but, increasingly, “solventless” or solvent-minimizing methods are being substituted.
- *Cleanup* — Remove unwanted coextractives by such operations as column chromatography, liquid-liquid partitioning, volatilization, or chemical degradation. The cleanup procedure also may result in the fractionation of target analytes into subgroups, or fractions, for further processing. This is particularly important in multiresidue analysis.
- *Modification* — Convert the target analyte to a derivative which is more readily separated, detected, or quantitatively determined than the parent. This is an optional step, reflecting the needs of specific analytes and analyte classes. Modification may be done pre- or post-cleanup, or after the resolution step in operations such as post-column derivitization.
- *Resolution* — Separate the analyte from remaining interferences, usually by some form of refined chromatography, such as gas chromatography (GC), high performance liquid chromatography (HPLC), or ion chromatography (IC).
- *Detection* — Obtain a response related to the amount of analyte present. Chromatographic detectors, spectrophotometers, and mass spectrometers are the mainstays for achieving this objective, although immunosorbent-based methods are coming into more common use.
- *Measurement* — Relate the response of the analyte to some known standard, of the analyte itself or a surrogate with similar properties, for calculating the concentration in the original matrix. Integrating recorders and computers are generally used for routine calculations.
- *Confirmation* — Provide assurance that the primary method gives correct (i.e., accurate and precise) results, by use of a second, independent method. This has become much more important in recent years due to the emphasis on quality assurance/quality control (QA/QC) in the analytical laboratory.

Quality Parameters

There are several parameters by which one may judge the suitability of a given method. Accuracy, or the agreement between the measured and true value, is generally assessed by running a series of blanks spiked with known

amounts of the target analyte(s), determining the end result of percent recovery (i.e., the amount recovered \div by the amount added \times 100) or relative error (the percent lost, or $100 -$ the percent recovered). Precision, or the reproducibility of the method, is generally assessed by running replicates of the spiked samples or of actual samples containing incurred residues. The relative standard deviation, or some other statistical parameter, is used.¹² The total error of the method is the sum of the accuracy (relative error) and precision (twice the relative standard deviation) contributions.¹³ For food contaminants which are relatively easy to determine with high accuracy and precision, such as metals, the total error should be fairly small, on the order of 25% or less. For some animal drugs, pesticides, natural toxicants, and metabolites, total error may run well above 50%, but still be considered acceptable.¹³

Another important parameter is the limit of detection (LOD) which is defined as the lowest concentration level of the analyte that can be determined to be different, with a high degree of confidence, from the blank or background.^{14,15} The LOD is assessed by running several portions of the blank or background matrix, i.e., substrate which lacks the analyte of interest, through the method to be used to determine the analyte. If the substrate has a high background of interfering material, which produce elevated absorbance readings at ultraviolet/visible measuring wavelengths, or spurious peaks at retention times to be used in the determination of the analyte, the LOD may be too high to permit analysis of the target analyte at levels of regulatory or toxicological interest. The limit of quantitation (LOQ) is a related parameter that is selected as a cutoff point for the reporting laboratory; a residue may be detected, that is, be above the LOD, but still produce such a small and sporadic signal that there can be little confidence in the concentration level calculated from the signal. The LOQ is typically several times higher than the LOD, moving responses to an area of greater confidence so that the results truly represent, with high confidence, the concentration of target analyte in the matrix under investigation.¹⁵

Because analytical data is increasingly being used for risk assessment or for making regulatory or economic decisions that can affect the availability of chemicals or the safety of the food supply, it has become much more important that analytical chemists pay closer attention to the end data — its quality and meaning — with less emphasis on simply running samples in order to process the workload or inventory. The subjects of good laboratory practices (GLP) and QA/QC are now much more familiar in the analytical laboratory than just 10 years ago, partly because of the need to impose a mentality which emphasizes quality and meaning in addition to speed and throughput.¹⁶

Common Techniques and Methods

Analytical chemistry has undergone an evolution (bordering on a revolution) in methodology over the period dating roughly from the 1940s to the present.

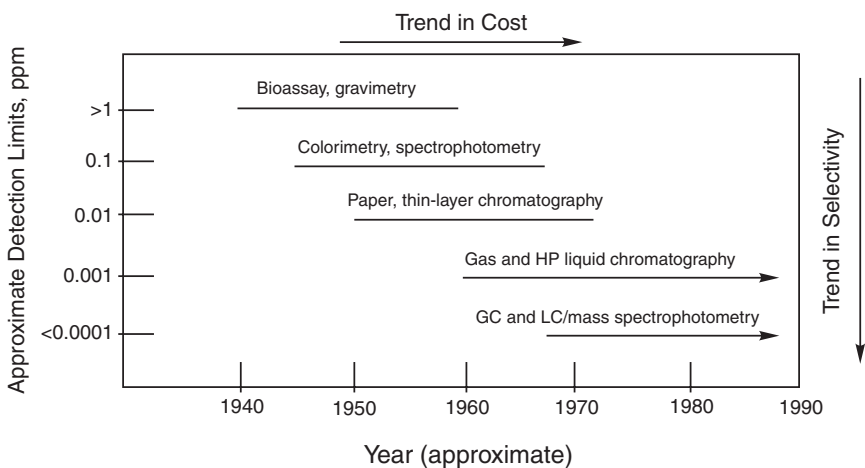


FIGURE 9.1

The evolution of analytical methodology for organic toxicants in food and environmental samples. (Modified from Seiber, J. N., *Regulation of Agrochemicals*, American Chemical Society, Washington, D.C., 1991.)

The methods of today are generally more accurate and precise, more selective, and notably of much lower detection limits than those used in the 1940s and 1950s (Figure 9.1). Primarily, this is due to the development and commercialization of a number of instruments for the detection and measurement of chemicals of interest, and a much improved ability to distinguish between the target analyte and some “mimic” which occurred in the same sample but is of no interest to the analyst.⁷ For example, the only instruments of widespread availability for quantitative analysis in the 1940s and 1950s were the balance (used for gravimetric determination of chemicals that could be precipitated and weighed) and the Beckman DU spectrophotometer and Bausch and Lomb colorimeter (used for determining the absorbance of chemicals which were colored (visible absorbers) or could form colored or strongly UV-absorbing derivatives). The bottom line was that, if it couldn’t be weighed or wasn’t colored either as the parent or after modification, quantitative determination was not possible and the best one could hope for was a qualitative determination based upon a bioassay endpoint. Detection limits were high and selective and, thus, confidence in the results were low. The advent of chromatography, starting with paper and thin-layer chromatography in the 1950s and eventually evolving to gas and HPLC from, roughly, the 1960s to the present, substantially lowered detection limits, to sub-ppm and ppb for most chemicals and provided much greater selectivity, and thus confidence, in the results. Selective GC detectors have been particularly important in this regard.¹⁷ Mass spectrometry represents, in many ways, the ultimate among present-day instruments in terms of ability to select for specific targets at very low levels and to ignore the extraneous material of no interest.

Similar developments occurred for metals and other inorganics. For metals, the development of atomic absorption and atomic emission spectrophotometry

in the 1950s and 1960s, and of the electrochemical techniques of polarography and voltammetry in the same period were of critical importance. The advent of inductively coupled plasmas as heat sources for electronic excitation in AAS and AES, and of ICP-mass spectrometry for determination, represent state-of-the-art developments now finding increasing applications in “routine” analyses. Many inorganic anions, such as nitrate, nitrite, cyanide, and selenium anions, are best determined by the relatively new techniques of ion chromatography and ion selective electrodes.¹⁸

Clearly, there has been a tradeoff in terms of investment and cost, such that a modern analytical laboratory must have an array of highly sophisticated and expensive instruments, and of equally sophisticated trained personnel to maintain, run, and interpret the results of the instruments.¹⁹

The evolution of methods for pesticides is illustrative of the field. A general methodology evolved which was heavily slanted toward pesticides of relatively high stability and low-to-medium polarity, and which contained a heteroatom such as chlorine, phosphorus, or sulfur, primarily because these features recurred in the synthetic organic pesticides introduced in the post-World War II era. Common organochlorine (OC) pesticides (such as DDT, lindane, and dieldrin) and organophosphates (OPs) (such as parathion and malathion) were, in fact, relatively nonpolar, so that they could be extracted with an organic solvent, were of relatively high stability so that they could be cleaned and/or fractionated on Florisil or silica gel adsorption columns, and also were stable to common GC temperatures, in the 100 to 250°C range. Additionally, they contained chlorine or bromine, phosphorus, or occasionally sulfur heteroatoms for detection using “element-selective” GC detectors (Table 9.3). Background from the interferences which lacked the heteroatoms, thus, was suppressed, and the analyte signal was enhanced,

TABLE 9.3

Selective GC Detectors Used in Pesticide Residue Analysis

Detector	Basis for Selectivity	Year First Reported (Approx.)
Electron-capture (EC)	Halogen	1959
Microcoulometric (MC)	Cl, Br, N, S	1961
Alkali-flame (thermionic) (AFID)	P, N	1964
NP-thermionic selective detector (NP-TSD)	P, N	1974
Electrolytic conductivity		
Coulson (CECD)	Cl, Br, N, S	1965
Hall (HECD)	Cl, Br, N, S	1974
Flame photometric (FPD)	P, S	1966
Thermal energy analyzer (TEA)	NO	1975
Photoionization (PID)	Halogen, S, aromatics	1978
GC/MS (benchtop)		
Ion trap (ITD)	Diagnostic ions	1983
Mass selective detector (MSD)	Diagnostic ions	1984
Atomic emission detector (AED)	Several elements	1988

resulting in a much improved signal-to-noise ratio and a much improved limit of detection.

Other analytical operations which suppressed background (such as streamlined cleanup methods and the use of ultrapure solvents and reagents) or enhanced the analyte signal (such as improved signal acquisition equipment) were built into the methods to support the lead role of the selective detector. With this technology, detection limits of 0.01 ppm and lower were readily attainable.

As pesticide chemistry changed to newer classes of chemicals, such as N-methyl carbamate insecticides and synthetic pyrethroids that did not always conform to the analytical prerequisites mentioned above, and as the need for analysis of metabolites increased, and as the regulatory trend toward testing at lower levels intensified, new technologies were introduced to help keep pace. Some examples include:

- Capillary columns replaced packed columns in GC, dramatically improving resolution and, through the additional modification of bonded phase capillaries, durability and reproducibility.²⁰ Micro-particulate-packed columns had a similar influence on HPLC resolution, and capillary HPLC columns also are in increasing use.
- Solid phase extraction (SPE) is replacing solvent extraction, at least for liquids, minimizing the use of organic solvents and the problems posed in their evaporation, handling, and disposal.^{21,22}
- Automation of some routine operations, such as gel permeation cleanup, some derivatization steps, and some partitioning and evaporation steps, has replaced wet chemistry and minimized the opportunity for error in some common procedures.⁷
- Mass spectrometry is in increasing routine use as a detection tool, coupled to both GC and HPLC. The advent of low-cost, benchtop instruments usable by virtually any chemist-technician has improved the reliability of results, particularly in trace analyses.

Some of these modifications are apparent in the multiresidue methods of the FDA and those used by the California Department of Food and Agriculture, which annually analyzes nearly as many samples of fruits and vegetables as the FDA.²³ For example, in a current version of the CDFA MRM (Figure 9.2), acetonitrile is used as a universal extracting solvent, and the aqueous acetonitrile is cleaned up via reversed phase SPE cartridge technology.²⁴ The recovered acetonitrile is then exchanged by evaporation to hexane, for analysis of OCs by chlorine-selective GC, or acetone for analysis of OPs and some organonitrogen compounds by FPD or NP-TSD GC. A separate aliquot is exchanged to methanol-methylene chloride, cleaned up on another SPE column, and then exchanged to acetonitrile-water for HPLC with automated post-column derivatization for analysis of carbamates. Even more recent modifications report the use of GC-MS in the selective ion monitoring

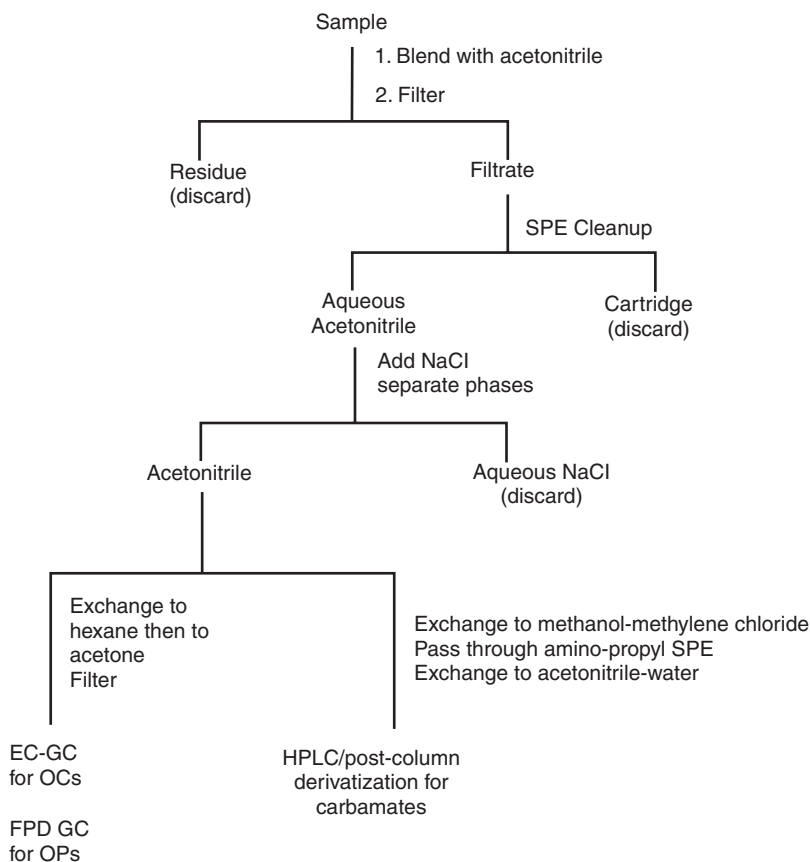


FIGURE 9.2

Multiresidue method used by California Department of Food and Agriculture for fruit and vegetable samples. (Adapted from Seiber, J. N., *Pesticide Residues in Food: Technologies for Detection*, U.S. Congress, Office of Technology Assessment, Washington, D.C., 1988, and Lee, S. M. et al., *Fresenius J. Anal. Chem.*, 399, 376, 1991.)

(SIM) mode as a single instrument replacement for the several element-selective GC detectors needed for prior versions of the MRM.^{25,26} The SIM is programmed to scan for groups of ion masses that represent the common gas chromatographable pesticides of regulatory interest.

More innovations are in the offing. New techniques are under development for extracting organic toxicants from solid matrices, including most foods, which eliminate or greatly minimize the use of organic solvents. One of these is supercritical fluid extraction (SCFE), using as a solvent a common gas such as carbon dioxide kept above its critical pressure-temperature point in a flow-through extraction chamber.^{27,28} SCFE already enjoys some important uses in the food industry, such as in removal of caffeine during decaffeination of coffee, removal of cholesterol from powdered eggs, and defatting of foods for either consumption or analytical endpoints. In the latter case, SCFE can

replace acid hydrolysis, Mojonnier, or Soxhlet extraction for total fat analysis. In pesticide residue analysis, SCFE extraction is quicker, more reproducible, more efficient, and safer when compared with organic solvent-based methods, leading to intensive efforts to utilize it in multiresidue methodologies. An even more recent technique for accomplishing the same purpose as SCFE is ASE — accelerated solvent extraction — in which common solvents such as methanol are used in pressurized extraction chambers at temperatures above their boiling points, resulting in more efficient mass transfer and, thus, better extractabilities with less time and much reduced solvent volumes.²⁹

Headspace sampling, as in headspace gas chromatography (HSGC), eliminates solvent extraction by determining residue in the vapor above the matrix in a sealed, equilibrated vial.^{30,31} Examples in the food area are provided for various aromas and fragrances, and volatile pesticides such as methyl bromide, ethylene oxide, and the dithiocarbamates that degrade to carbon disulfide. HSGC is especially amenable to automation, with modules capable of being loaded with as many as 100 prepared samples.

Immunoassay (IA) represents another promising newcomer to the analytical chemists portfolio. Van Emon et al.³² summarized applications to the analysis of pesticides in foods. IA is widely touted as an alternative to the analytical treadmill of improved analytical capability coupled to high-cost sophisticated instrumentation such as mass spectrometry. Following a rather lengthy and costly development process in which antibodies are recovered from an experimental animal exposed to a suitable derivative of the analyte of interest, and formatting the assay for routine use, IA can provide many analyses at a throughput rate and cost much improved over conventional approaches. An interesting development is the availability of IA-based kits which can be used in the field without any sophisticated instrumentation. The kit approach may allow for screening of suspected contamination by a field inspector who then diverts only the screen-positive samples to the lab for follow-up using GC or GC-MS. This approach has the potential to reduce the volume of samples and, thus, the costs in the laboratory without sacrificing consumer safety.

Many of these same techniques are under intensive examination, or have already been adapted for other toxicant/additive classes in foods. SPE is now in routine use for extracting aflatoxins from milk and juices prior to determination. IA methods are being used for aflatoxin screening and quantitative analysis. Several animal drugs now have IA methods for use in residue regulatory compliance work.³³ Holstege et al.³⁴ reported a new alkaloid multiresidue method which uses SPE and GC-MS for detecting alkaloids in food animals exposed to alkaloid-bearing plants in their feed and forage.

Conclusions

The need for more and better analytical data will continue to stimulate developments in analytical chemistry applied to foods. Better methods are needed

for chemicals of concern now, and new methods will be needed as additional chemicals are added to the long lists of concerns in foodstuffs. The identification of domoic acid as the causative agent in food poisoning associated with consumption of some shellfish has led to the development of an IA-based method for screening seafoods for domoic acid.^{35,36} More examples are sure to follow.

But improvements in LOD will no longer be the primary goal because today's detection limits will suffice for virtually any situation involving potential health effects. In fact, if present-day LODs are lowered even more, inadvertent residues will likely be found frequently in foodstuffs, but at levels well below those of any conceivable biological significance. A more likely driver of analytical advances will be improved data quality at reduced costs, and with greater flexibility in where the analysis can be performed (i.e., in the field if needed) and the training and skill needed for the operator. Better and more comprehensive multiresidue methods also will be needed to include not only parent materials but also toxicologically significant residues. Miniaturization and automation also will be in demand, to replace the wet chemistry, hands-on methods of the past with all of their attendant potential for error, lab personnel exposure, and slowness.

While pesticides, food additives, and animal health drugs have been the stimulators of improved analytical methods because of their intense regulatory scrutiny, there are many other groups of chemicals which have been virtually ignored as foodborne residues but almost certainly exist as residues in foods. Examples are provided by the organophosphorus plasticizers and fuel additives, polynuclear aromatic hydrocarbons, phthalate ester plasticizers, monomers and oligomers in plastic wraps and packaging, and naturally occurring pesticides and other bioactive natural products.³⁷ Analytical methods which can be applied to the whole gambit of chemical contaminants, not just those which are the subjects of specific regulations, need to be developed and placed in the repertoire of the laboratories responsible for analyzing foods for public safety.

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Food Science/Toxicology

New data continually indicate that antitoxicants may contribute to reductions in cancer risks and that chronic consumption of low levels of chemical carcinogens in our diet may contribute to an increased risk of developing specific types of cancers. Research also shows that in America today, the leading causes of death are cancer and heart disease. Considering that diet plays a significant role in the development of both of these diseases, issues of food toxicology become particularly topical.

Food Toxicology provides a comprehensive look at contemporary food toxicology issues. Its nine chapters, all authored by leading American academic experts, concisely address critical chemical food safety and food toxicology issues that emerged in the latter half of the last decade. Specifically, the book discusses food allergens and their diagnoses, biotechnology, estrogenic factors, and antitoxicants. It also addresses more traditional topics such as pesticide residues, food additives, plant toxins, microbial toxins, and food analysis.

Food Toxicology presents a comprehensive treatment of food toxicology topics of scientific, regulatory, and public interest. In essence, therefore, the book represents an up-to-date analysis of important topics that are found both at the cutting edge of science and on the front pages of newspapers.

Features

- Presents important topics in food safety and toxicology
- Evaluates the relative risk associated with consumption of low levels of pesticides
- Addresses cutting-edge developments of critical importance to the food industry and public health
- Debates the merits of risk evaluation, a particularly important issue to all involved in food toxicology
- Discusses the history of the development and commercialization of genetically modified foods, an issue at the forefront of international trade discussions and one which is clearly of significant interest to consumers
- Discusses the potential risks and benefits of dietary estrogens and antiestrogens, concerns which are likely to form the toxicology issue of the early part of this century

