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Tea and Tea Products

Chemistry and Health-Promoting Properties

Edited by

Chi-Tang Ho

Jen-Kun Lin

Fereidoon Shahidi

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Chemistry and
Health-Promoting Properties

NUTRACEUTICAL SCIENCE AND TECHNOLOGY

Series Editor

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Tea and Tea Products

Chemistry and Health-Promoting Properties

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Contents

Preface

Tea is the second most widely consumed beverage in the world after water. Regular intake of tea is associated with improved antioxidant status *in vivo*, which may contribute to lowering the risk of coronary heart disease, stroke, and certain types of cancer. There are three major categories of tea: the nonfermented green tea, the partially fermented oolong tea, and the fully fermented black tea. Besides these three major teas, other teas, such as white tea and Pu-erh tea, have also been introduced to the Western marketplace in recent years.

Considerable interest has developed in the past decade in unraveling the beneficial health effects of tea, particularly in its polyphenolic components and its antioxidant activity. Catechins, theaflavins, and thearubigins are three important groups of polyphenols present in tea. The formation mechanism of these compounds during tea processing as well as their respective biological activities are of great importance and of scientific and commercial interest.

The market for ready-to-drink tea beverages in the United States has increased considerably in recent years. The total sale of ready-to-drink tea beverages in the United States in 2005 was over $674 million, and this represents a 12.8% growth compared to 2004. This growth has mainly been due to the increased awareness of the health benefits of tea beverages for humans.

In this book, the manufacturing and chemistry of various teas, including green tea, black tea, Pu-erh tea, white tea, and GABA tea, are discussed. We present the analysis, formation mechanism, and bioavailability of tea polyphenols. Various bioactivities of teas, including anticancer, anti-inflammatory, antiobesity, and antidiabetes, are also discussed. The flavor stability of ready-to-drink tea beverages is subsequently reviewed.

We thank all of the contributing authors for their cooperation in preparing this book, which we hope will serve as an excellent reference for those interested in the science and technology of tea as a health-promoting beverage.

Editors

Chi-Tang Ho is a professor II in the Department of Food Science at Rutgers University. Dr. Ho has published over 600 research papers and book chapters, and edited 30 books. Dr. Ho's current research interests focus on the antioxidant and anticancer properties of food phytochemicals. Dr. Ho serves as a senior editor for *Molecular Nutrition and Food Research*, an associate editor for *Journal of Food Science*, and an editorial board member of *Journal of Agricultural and Food Chemistry*, *Biofactors*, *Journal of Food Lipids, Food Reviews International*, and *Critical Reviews in Food Science and Nutrition*. Dr. Ho has received numerous awards, including the 2002 Stephen S. Chang Award in Flavor and Lipid Science from the Institute of Food Technologists, and the 2005 Advancement of Application of Agricultural and Food Chemistry Award from the Agricultural and Food Chemistry Division of the American Chemical Society, and has been elected fellow of the American Chemical Society and Institute of Food Technologists.

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1 Green Tea and Black Tea
Manufacturing and Consumption

Xiaochun Wan, Daxiang Li, and Zhengzhu Zhang

CONTENTS

1.1 INTRODUCTION

Camellia sinensis (L.) Kuntze is a perennial leafy crop. All varieties of tea are produced from the tea plants. Tea plants prefer a warm and humid climate with plenty of rainfall and also like diffused light and weak acidic and well-drained soil. The well-grown tea plants provide high-quality tea shoots, which vary with tea cultivars and the environmental conditions, such as the type of soil and altitude and climate of the tea plant growing area. A Chinese idiom says, "A higher mountain yields higher quality tea," which indicates that the mountain conditions are optimum to tea plant growth, especially the growth of high-quality flush.[1] Moreover, tea quality is also determined by the processing techniques employed. For instance, the same fresh tea leaves can be processed to black tea, oolong tea, and green tea by fermentation, semifermentation, and nonfermentation, respectively. Those basic three types of tea have different quality characteristics, including color, aroma, taste, and appearance.

The fresh tea leaves are usually used for tea manufacturing and are harvested by hand plucking or mechanical plucking. Compared to mechanical plucking, hand plucking is more labor intensive and time consuming and less efficient, but with higher uniformity. The well-known high-quality green teas are mostly produced from hand-plucking fresh tea leaves in China. Fresh tea leaves could be harvested during different seasons in a year, which changes with the climate of tea growing area. In China, the leaves are plucked in spring, summer, and autumn; in winter the

tea bush is dormant. After being plucked, the fresh tea leaves are sent immediately to tea factories for manufacturing.

According to the different ways of processing, especially the extent of fermentation, tea is usually divided into three basic types: green tea (nonfermented), oolong tea (semifermented) and black tea (fully fermented). Alternatively, with the combination of the ways of processing and the characteristic quality of manufactured tea, tea is classified into six types: green tea, yellow tea, dark tea (containing brick tea and pu-erh tea), white tea, oolong tea, and black tea (figure 1.1).[2] The so-called fermentation in tea processing is not the anaerobic breakdown of an energy-rich compound (as a carbohydrate to carbon dioxide and alcohol or to an organic acid), but in essence is mainly the oxidative polymerization and condensation of catechins catalyzed by endogenous polyphenol oxidase and peroxidase. The oxidation products such as theaflavins and thearubigins contribute to tea color and the taste of black tea.[3]

Unlike either black tea or green tea, oolong tea has an excellent characteristic with the combination of the freshness of green tea and the fragrance of black tea. It is produced by a special process called green leaf shaking (yaoqing) and green leaf cooling (liangqing). In this process, the moderately withering green tea leaves are bruised at the edges by hand or mechanical shaking and vibrating. The leaf appearance of oolong tea is featured with the reddish edges and green centers. Oolong tea is produced in China, particularly in Fujian, Guangdong, and Taiwan, and is currently popular in China and Southeastern Asia. It has a good function in helping body building and dieting.[2]

1.2 GREEN TEA MANUFACTURING

Although different green teas may be produced by different processing techniques, the general green tea processing is achieved as follows: fresh tea leaves plucked →

Green tea: Fresh tea leaves ⟶ Fixed ⟶ Rolled ⟶ Dried
(steamed or pan-fried to inactivate oxidase)

Yellow tea: Fresh tea leaves ⟶ Fixed ⟶ Rolled ⟶ Yellowed ⟶ Dried
(piled and smothered to auto-oxidize)

Dark tea: Fresh tea leaves ⟶ Fixed ⟶ Rolled ⟶ Pile fermented ⟶ Dried
(oxidized by the oxidases of resident microbes)

White tea: Fresh tea leaves ⟶ Withered ⟶ Air dried or baked ⟶ Re-fired
(with more fine hairs/flosses)

Oolong tea: Fresh tea leaves ⟶ Withered ⟶ Bruised (shaken) ⟶
⟶ Partially fermented ⟶ Fixed ⟶ Rolled ⟶ Dried

Black tea: Fresh tea leaves → Withered → Rolled → Fully fermented → Dried
(orthodox rolling or CTC)

FIGURE 1.1 Schematic of primary tea processing.

fixed (inactivating enzyme, in Chinese called shaqing) → rolled → dried (or fired). It is worthy to note that different kinds of green tea have different requirements of plucked young tea shoots. The common young tea shoots required are one bud and two or three leaves, while those for famous high-quality green tea are usually one bud and one leaf, even just one bud.[2] In Japan, some high-quality green teas such as gyokuro and matcha are produced from the fresh tea leaves grown under 90% shading for 2 weeks and 40–50% shading for 1–2 weeks before being plucked, respectively.[4] The quality of fresh tea leaves plays a key role in the characteristics of green tea.

In the harvesting season, the tender tea shoots are plucked by hand or a mechanical tea plucker and delivered to tea factories immediately. In order to prevent fermentation (the oxidation of catechins) and formation of the characteristic "green leaf, green liquor" of green tea, the leaves are immediately fixed to inactivate the endogenous enzymes by steam or heat with pan frying, roasting, or baking. However, on the heavy harvesting days, the leaves are thinly spread out indoors in a shallow basket in the racks, or some ventilating beds like bamboo mesh mats, trays, troughs, or spreading machines, and wait for fixation. The waiting time should be as short as possible to avoid oxidation, which turns the green leaf and stem to red. The tea fixation apparatus include a tea steaming machine, wood-fired pan, electrical pan, rotary heated drum, microwave, and far infrared. When fixed leaves become soft and flaccid, they are conveyed to be rolled, and then the rolled tea masses are loosened by a roll breaker or ball breaker and moderately cooled. Subsequently, the leaves are fired (dried) by charcoal-fired baking baskets, electrical heaters, coal heaters, liquid petroleum gas heaters, or natural gas heaters. The moisture content of the final product (crude tea) should be less than 6%.[2]

According to the fixation ways, green tea is subdivided into steamed green tea and pan-fried green tea. Steamed green tea is mainly produced and consumed in Japan, while pan-fried green tea is mainly produced in China and exported to the world.

Crude green teas are usually refined by the wholesale tea dealers. The refining process generally includes sifting, cutting, grading, refiring, polishing, blending, and packing. Through refining, the stalks, dust, and impurities are removed. The final tea has a uniform size and standardized appearance, such as Chunmee, Sowmee, and Gunpowder. Chunmee and Sowmee are renowned for their curve shape, like the human eyebrow, while Gunpowder is featured with tiny, tight, round balls like gunpowder.[2]

1.3 BLACK TEA MANUFACTURING

Unlike the enzyme inactivation process of green tea, the enzyme activities are completely utilized in black tea processing to form the pigments (theaflavin and thearubigin). Although the processing methods vary with the producing regions, the general black tea processing is: fresh tea leaves plucked → withered → rolled → fermented → dried.[2] Among those four steps, the fermentation process is crucial to the quality of the final black tea product, which is predominated with the oxidation of catechins and production of oxidation reaction products.

The typical plucked tea flush for black tea processing is one bud and two leaves. On arrival at the factory, they are spread out on large trays, racks or mats, troughs,

or a machine and are left to wither by natural air current under sunshine or indoor controlled ventilation/aeration with the aid of warm-air fans. The moisture in the leaves evaporates and the leaves become limp and flaccid. Subsequently, the leaves are processed by orthodox roller or rotorvane, or CTC (crushing, tearing, and curling) machine, or LTP (Lawrie tea processor) machine. Most of the black tea in India, Sri Lanka, and Kenya is manufactured using the CTC process, while that in China is processed principally by traditional orthodox rollers. The objective of the rolling is to break the leaf cells and release the oxidases, including polyphenol oxidase and peroxidase, and initiate the process of catechin oxidation with oxygen in the air. Importantly, CTC can be used to handle efficiently large volumes of tea leaves, rapidly rupturing withered tea leaves to small particles and forcing out most of cell sap, which leads to sufficient fermentation.[5]

After rolling, the broken tea leaves are transferred to the fermentation room and laid out thinly on trays, in troughs, or on the floor at a little warm (25–35°C), high humidity (>95%) atmosphere for fermentation. The fermentation time ranges from a half hour to 3 hours, depending on the variety of tea plants, the age of tea leaves, the particle size of broken tea leaves, and the fermentation condition. Among these factors, the rupturing technique plays a key role. Generally, tea leaves macerated by CTC machine need a short time, from 30–60 minutes, while tea leaves ruptured by orthodox roller take a long time, from 2–3 hours. In this process, the broken tea leaves set to fully oxidize, which starts during rolling. Due to the oxidation, green leaves gently turn to golden russet color and the greenish leaf note turns to a fresh or floral aroma.[2]

As the optimum fermentation is achieved, the leaf mass is dried or fired to inactivate the enzymes and halt the fermentation. Continuous driers are usually used, in which hot air is generated by electrical heater or coal furnace. In this process, the leaf turns dark brown or black, the aroma changes to floral, and the moisture is reduced to less than 6%.

The crude black teas produced in the world are mainly congou (Gongfu) black tea and CTC black tea, which are processed by orthodox rolling and CTC machine, respectively. Apart from those two, there is still a minor productivity Souchong black tea produced in the Wuyi (Bohea) mountain area in China. It is said that souchong black tea was created in the middle of the fifteenth century, and lapsang souchong (Zhengshan Xiaozhong) is known to be the origin of black tea. The processing of lapsang souchong is similar to that of congou black tea, except that the fermented dhool (refers to the tea leaf during fermentation, noted for its coppery color) is fired at 200°C for several minutes and rerolled before final drying. In addition, souchong black tea can be further processed by the absorbance of the scents released from the burning pine branches to smoked souchong black tea, which is known for its smoked flavor.[2]

Similar to crude green tea refining, crude black teas are refined through sifting, cutting, grading, blending, refiring, and packing. Through the refining, the stalks, fibers, and impurities in crude tea are removed. The fine teas are graded to four varieties as whole leaf grades, brokens, fannings, and dusts. Moreover, each variety is subdivided into several categories as summarized in the following list.[6]

Whole leaf grade: flowery orange pekoe (FOP), golden flowery orange pekoe (GFOP), tippy golden flowery orange pekoe (TGFOP), finest tippy golden flowery orange pekoe (FTGFOP), special finest tippy golden flowery orange pekoe (SFTGFOP), orange pekoe (OP), pekoe (P), flowery pekoe (FP), pekoe souchong (PS), souchong (S)

Brokens: flowery broken orange pekoe (FBOP), golden flowery broken orange pekoe (GFBOP), golden broken orange pekoe (GBOP), tippy golden broken orange pekoe (TGBOP), tippy golden flowery broken orange pekoe (TGFBOP), broken orange pekoe (BOP), broken pekoe (BP), broken pekoe souchong (BPS)

Fannings: orange fannings (OF), broken orange pekoe fannings (BOPF), pekoe fannings (PF), broken mixed fannings (BMF)

Dusts: pekoe dust (PD), red dust (RD), fine dust (FD), golden dust (GD), super red dust (SRD), super fine dust (SFD)

1.4 TEA CONSUMPTION

Tea is a popular nonalcoholic healthy beverage around the world. Its discovery dates back to an ancient Chinese legend that Chinese emperor Shen Nung in 2737 b.c. was boiling water under a wild tree and a few tea leaves fell into the water, which gave a refreshing, revitalizing brewing. From that time, tea was used as an herbal medicine and chronically evolved to a healthy beverage. Prior to the middle of the nineteenth century, all teas consumed in the West were produced in China. At the end of the nineteenth century, black tea overcame green tea and became dominant in the world tea market. In the 1970s, tea bags and CTC tea replaced loose tea and orthodox tea to become the principal tea products.[7] There are diverse tea consumption habits; for example, developed countries prefer mainly tea bags, while Chinese like hot green tea drinks and Japanese like canned or bottled tea drinks. Particularly, ready-to-drink tea products are favorites of youth in the world.

Nowadays, tea is the second largest consumed beverage, only next to water, in the world. According to statistics from the Food and Agricultural Organization (FAO) of the United Nations, production and consumption of tea are steadily increasing.[8] The worldwide production of tea in 2005 reached up to 3.50 million tons (table 1.1). The main tea-producing countries are China, India, Kenya, Sri Lanka, Turkey, Indonesia, and Vietnam, which accounted for 26.68, 26.49, 9.38, 9.05, 5.87, 4.73, and 2.97%, respectively, of the 2005 output of total global tea production.

The worldwide tea consumption in 2005 reached up to 3.36 million metric tons (table 1.2). From 2001 to 2005, world consumption increased 12.59%. Over the past decade, world tea consumption has increased by 2.25% annually. India is the largest tea consuming country, accounting for 22.52% of the total 2005 world consumption, followed by China at 20.09%. Of the total 2005 world consumption, the Russian Federation, Japan, Pakistan, the United Kingdom, and the United States accounted for 5.36, 4.47, 3.99, 3.81, and 2.98%, respectively.

TABLE 1.1
World tea production (thousand tons)

Location	2001	2002	2003	2004	2005
World	3046.0	3173.7	3249.3	3387.9	3503.7
China	722.0	765.7	791.0	856.2	934.9
India	856.0	883.0	907.0	893.0	928.0
Kenya	295.0	287.1	293.7	324.6	328.5
Sri Lanka	296.0	310.6	303.2	308.2	317.2
Turkey	143.0	150.0	155.0	205.6	205.6
Indonesia	173.0	172.8	167.5	169.8	165.8
Vietnam	81.7[a]	93.0	94.5	97.0	104.0

Source: FAO. 2006. Current market situation and medium term outlook. Paper presented at the Seventeenth Session of the Intergovernmental Group on Tea, November 29–December 1.

[a] Average of 2000–2002.

TABLE 1.2
World tea consumption (thousand tons)

Location	1996–2000	2001	2002	2003	2004	2005
World	2833.4	2985.4	3092.6	3199.1	3227.2	3361.6
India	635.4	671.3	693.0	714.0	735.0	757.0
China	482.0	496.2	537.8	555.3	603.7	675.3
Russian Federation	145.6	156.0	166.1	168.6	169.1	180.3
Japan	138.1	149.1	134.9	138.2	156.0	150.2
Pakistan	108.6	106.8	99.4	118.3	120.0	134.1
United Kingdom	142.2	136.7	134.2	119.3	127.8	128.2
United States	91.0	96.7	93.5	94.1	99.5	100.1

Source: FAO. 2006. Current market situation and medium term outlook. Paper presented at the Seventeenth Session of the Intergovernmental Group on Tea, November 29–December 1.

1.4.1 Green Tea Consumption

In 2005, global green tea production increased to 883.9 thousand tons, which accounted for 25.23% of global tea production. FAO[8] projected that world green tea production would grow at a faster rate than black tea by 2.0% annually, to reach 1097.7 thousand tons by 2016. Apart from being the largest tea producer, surpassing India in 2005, China is also the largest green tea producer and exporter, contributing 78.18% of global green tea production and 88.78% of global green tea exports in 2005. China is expected to continue to produce green tea at an annual 3.8% growth rate, to reach 877.5 thousand tons, while exporting green tea at an annual 4.6% growth rate, to reach 368.0 thousand tons by 2016.[8]

China is the largest green tea consumer, with 484.9 thousand tons of green tea consumed in 2005 (table 1.3), which accounts for 70.17 and 54.86% of its total green

TABLE 1.3
Green tea consumption (thousand tons)

Location	2000	2001	2002	2003	2004	2005
World	681.0	781.6	784.4	756.1	832.0	883.9
China (Mainland)	342.7	350.0	375.7	388.1	417.5	484.9
Japan	108.6	106.6	94.0	100.7	115.7	113.5
Vietnam	16.0	15.5	18.4	17.0	16.5	18.6
Indonesia	30.2	33.3	34.6	37.4	36.9	31.3

Source: International Tea Committee (ITC). 2006. Annual bulletin of statistics 2006.

tea production and 54.86% of world green tea consumption in 2005 (691.0 thousand tons). From 2000 to 2005, its green tea consumption increased 41.49%. However, its per capita annual tea consumption was only 0.52 kg in 2005; thus, there is remarkable potential for domestic consumption in the near future, accompanied by a rapid expansion of the middle-income class. Japan is the second largest green tea consumer with 113.5 thousand tons, including 13.5 thousand tons of imports in 2005, which dominates 12.84% of world green tea consumption. Green tea consumption fluctuated from 2000 to 2005, in Japan as well as in Vietnam and Indonesia. The green tea consumption in Vietnam and Indonesia was 18.6 and 31.3 thousand tons, respectively, in 2005, which together account for 5.65% of world green tea consumption.[9]

1.4.2 Black Tea Consumption

In 2005, black tea production was 2455.9 thousand tons, which accounted for 70.09% of global tea production. According to the medium outlook of FAO,[8] world black tea consumption is projected to expand to reach 2.69 million tons by 2016, an annual growth rate of 1.3%. The Commonwealth of Independent States (CIS) is the largest potential consumer of black tea, with consumption expected to reach 342.4 thousand tons in 2016, and with an annual 3% growth rate. In the European Community,[8] Italy, Germany, and Ireland are projected to have an increase, with an annual growth rate greater than 1.4%, but consumption in the United Kingdom and France may decline, with 1.6 to 1.8% annually.

India is the largest black tea consumer. Moreover, India is the largest black tea producer by 919.4 thousand tons, contributing 37.44% to world production in 2005.[8] Almost 85% of total Indian households drink tea. In 2005, 694.5 thousand tons of black tea was consumed, which accounted for 29.65% of world black tea consumption (table 1.4) and 74.84% of its own tea production (928.0 thousand tons).[9] India's per capita annual tea consumption has increased from 0.61 kg in 1991 to 0.64 kg in 1998, and to an estimated 0.69 kg in 2005. Unlike the international market, where iced tea is prevalent, tea in India is generally consumed as a hot beverage infused either separately or as a mixture of milk and sugar. CTC is generally preferred all over India.

TABLE 1.4
Black tea consumption (thousand tons)

	2000	2001	2002	2003	2004	2005
World	2214.0	2224.4	2232.6	2279.7	2368.0	2342.3
India	617.0	668.1	634.9	684.0	693.1	694.5
CIS	206.1	202.0	219.8	222.6	228.5	234.0
Pakistan	109.0	105.4	95.6	116.2	118.7	137.6
United Kingdom	134.0	162.3	164.2	154.3	152.8	150.9
China (Mainland)	37.0	25.0	41.0	28.0	44.0	48.8
United States	81.0	86.5	84.2	82.1	87.7	85.9
Indonesia	33.0	56.8	56.8	48.0	56.4	67.9

Source: International Tea Committee (ITC). 2006. Annual bulletin of statistics.

1.5 CONCLUSION

In the past several years, the global tea production has been greater than tea consumption. The world tea market remains in oversupply. As FAO[8] mentioned, the increase of consumption should be done by exploration of both the traditional export markets and domestic markets of producing countries. Particularly, the producing countries' market has remarkable potential to increase their per capita tea consumption levels. For example, per capita consumption levels in India, China, and Kenya are only 0.69, 0.52, and 0.40 kg, respectively, while per capita consumption levels in the Russian Federation and the United Kingdom are 1.26 and 2.20 kg, respectively.

FAO[8] also suggested that the results from the scientific researches on tea and health should be used more extensively in promoting consumption in the world. Moreover, the higher value-added tea products such as organic tea, ultra-fine tea powder, tea beverage, and tea extracts, including polyphenols, pigments, theanine, and theasaponin, are to be exploited extensively to enhance tea consumption.

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2 Pu-erh Tea
Its Manufacturing and Health Benefits

Huai-Yuan Chen, Shoei-Yn Lin-Shiau, and Jen-Kun Lin

CONTENTS

2.1 INTRODUCTION

Pu-erh tea, a well-known traditional Chinese tea, has been categorized as sun-dried green tea, and its pressed products from large tea leaves (*Camellia sinensis* O. kuntze var. assamica Kitamura) are manufactured mainly in Yunnan, China. In the markets, three kinds of pu-erh teas have been sold: loose pu-erh tea, pressed pu-erh tea, and pu-erh tea bags.

Pu-erh tea is a popular tea with a long history, which originated from the districts of Xi-shuang-ban-na, Si-mao, and Lan-chuang-jiang valley, Yunnan Province, China. Pu-erh tea is of interest to consumers because of its quality and health care function and is distinct from other kinds of tea. Because of its health care function, especially when aged, pu-erh tea is loved by many tea drinkers.[1] The collection and drinking of pu-erh tea has become a fashion and a unique pu-erh tea culture. It is generally believed that the older the pu-erh tea, the better. However, the major challenge for a pu-erh tea purchaser is to identify the exact age of a pu-erh tea product.

Many tea consumers have the ability to evaluate the quality of oolong, black, and green teas, while few are able to do so with pu-erh.

Tea experts and scientists have paid more attention to the special quality and health care function of pu-erh tea and performed a great deal of research using modern scientific methods.[2–5] The consumption of pu-erh tea is increasing steadily because of consumers' recognition of its health care function and its drinking merits.

The export of Yunnan pu-erh tea is also increasing; according to statistics, in 2003, its consumption in Guangdong, Hong Kong, France, Japan, Korea, Taiwan, and Malaysia reached 20,000 tons. In 2004, the sale increased again globally, but the production of Yunnan pu-erh tea was less than 10,000 tons. Another half of the market share is supplied by Guangdong, Guizou, Sicun, and Vietnam manufacturers.[1] The quality control of pu-erh tea from different manufacturers is quite different. It appears that a strictly formal set of standards should be developed and controlled by a national authority in the near future.

2.2 MANUFACTURING OF PU-ERH TEA

The good quality of pu-erh tea comes from large tea leaves from *Camellia sinensis* O. kuntze var. assamica Kitamura. Some pu-erh teas are also made from other species with small leaves, such as *Camellia sinenesis* Linn. According to the shapes of pu-erh tea, most products in the market can be classified into two categories: loose tea and pressed tea. The loose tea is the dried product of pu-erh tea in its original curled strip shape, while pressed tea is made by pressing the loose tea into a bowl shape, brick shape, or ring cake shape. All pu-erh tea products with different shapes have to be broken into pieces before brewing with boiling water.

According to the degree of fermentation, pu-erh tea products can be classified into green tea-like pu-erh tea, fermented pu-erh tea, or postprocessed pu-erh tea. The green tea-like pu-erh is processed without fermentation or storage under high humidity. The products in this category consist of Yunnan green loose tea, green cake tea, raw cake tea, green tou-tea, and raw tou-tea. Fermented pu-erh tea is produced by microbial fermentation of piled fresh loose tea or green pressed tea, and then stored at room temperature for an extended and appropriate period of time, and may be called Yunnan pu-erh loose tea, pu-erh tou-tea, or pu-erh ring cake tea.

There are two manufacturing procedures that have been widely employed for production of pu-erh tea: the classical pressing method and the wet-piling method. The former dates back to the Tan Dynasty (864–1278 A.C.), while the latter has been developed recently (1938 A.C. to present).[6]

2.2.1 Classical Pressing Method

The classical pressing method includes seven steps as described below:

Step 1. Plucking of fresh tea leaves. The best fresh tea leaves are plucked in the early spring (March). The tea leaves plucked in the middle of spring (April) and late spring (May) are still good for making pu-erh tea.

Step 2. Blanching. The fresh tea leaves harvested in step 1 should be subjected to a blanching process as soon as possible by baking on a hot pan with constant manual mixing. As soon as tea leaves become very soft and liberate a unique grass fragrance, the blanching process should be stopped at once. At this stage, presumably the enzyme reactions in the tea leaves are also terminated.

Step 3. Rolling. The purpose of rolling is to liberate the juice of the tea leaves to the outside of the leaf surface. Thus, the flavor of the tea leaves will be released easily when brewing with hot water. In the classical pressing method, the rolling process is done manually. Therefore, approximately 5 kg of this tea product may be manufactured by a skillful tea maker on a daily basis.

Step 4. Sun drying. The rolled tea leaves are dried under sunlight with constant mixing. The heat and infrared rays from sunlight penetrate the tea leaves and remove the moisture as well as promote chemical reactions in the leaf matrix. This process takes two working days to accomplish. The moisture content of the final products should reach 12–15%. The product obtained is called dried raw tea.

Step 5. Storage in a dry place. The dried raw tea is packed in a large, clean gunny bag and stored in a temperature- and humidity-controlled stockroom.

Step 6. Pressing into different shapes.

(a) Picking. The dried raw teas are subjected to selection and classification on the basis of quality and homogeneity. Some dried raw teas may be contaminated with stems and foreign bodies, and these should be removed.

(b) Weighing. Generally, a cake of pu-erh tea weighs 357–400 g.

(c) Steaming. The steaming step is critical for making good-quality pu-erh tea. In most cases, the dried raw tea is packed in a cloth bag and a bronze cylinder is used for steaming. The steaming time should be kept within 40–50 seconds. If the time is too long, the tea becomes too ripe and yellow coloration develops. The quality of water used for steaming is very important, and in most cases, mountain-stream water is used.

(d) Pressing. The steamed soft tea in the cloth bag is pressed between two stone templates. A tea maker stands on top of the stone template and twists his or her body to distribute the body weight while pressing on the cloth bag in order to make the cake of pu-erh tea.

Step 7. Long-term incubation for ripening. This step is essential for making good-quality pu-erh tea. It is a general rule that the longer the storage, the better the quality of pu-erh tea. In the same batch of manufactured products, a 50-year-old pu-erh tea should be better than a 5-year-old one for drinking and health care function; the prices of these products will differ accordingly. The incubation is carried out in a temperature- and moisture-controlled room. However, in ancient times, this incubation process was done on horseback during the transportation.

2.2.2 Wet-Piling Method

The wet-piling method has recently been developed; the initial four steps are similar to those described in the classical pressing method:

Step 1. Plucking. As described in step 1 above.
Step 2. Blanching. As described in step 2 above.
Step 3. Rolling. As described in step 3 above.
Step 4. Sun drying. As described in step 4 above.
Step 5. Fermentation by wet piling. In the process of pu-erh tea manufacturing, both enzymatic and nonenzymatic oxidations play a very important role in the transformation of tea components. These oxidation reactions take place very slowly and persistently. Furthermore, oxidation is assisted by environmental microbes and oxygen. The sources of microbes are poorly understood and may come from inside (endogenous) and outside (exogenous) of tea leaves. The origin and proportion of both endogenous and exogenous microbes that are actually involved in the fermentation process remain to be investigated.

The water content of most sun-dried tea products is in the range of 9–12% and should be increased by showering with water to an appropriate moisture of 20% or higher (as judged by the experience of the manufacturer). The tea mass is mixed thoroughly during the showering process. The water should be clean and free from harmful microbial contamination. High mountain streams are the water of choice and are generally used in this process. The resultant wet tea mass is piled up as high as 1.0–1.5 m. Each pile contains approximately 10 tons of tea mass and is covered with wet gunnysack (or wet gunny cloth) to maintain its moisture and temperature during fermentation. The temperature of the pile may reach 50–60°C and should not exceed 65°C. These piles are kept in a ventilated cleanroom for fermentation.

Step 6. Repiling and mixing. The temperature and moisture of the piles are essential for determining the degree of fermentation and are important factors affecting the quality of pu-erh tea. The temperature and moisture of the incubation room and tea piles are strictly controlled. The tea piles should be broken down, mixed, and piled up again every 2 days. More water should be showered during the repiling and mixing process. It takes five to eight repetitions of repiling and mixing to accomplish the fermentation process. The finishing point should be determined by an expert by judging the color of the fermented tea mass (should be reddish brown) and taste of the tea extract with water. The tea extract should be "friendly" to the mouth, free from astringent taste, deep red brown in color, and persistent with old tea flavor. Once the piled tea mass fulfills these criteria, the fermentation process can be stopped.
Step 7. Air drying. The fermented piled tea products are broken down and separated into several valleys for air drying (not sun drying). The water content of piled tea products is around 20%, but is reduced to 14–20% upon

air drying. The tea mass is then broken down and separated into several new valleys again every 3–5 days. The air-drying process is repeated again and again until the moisture of the tea product reaches 14%.

Step 8. Selection and grouping. The air-dried tea products are subjected to strict selection and grouping based on their color, size, fragrance, and appearance.

Step 9. Packaging and pressing. The selected air-dried tea products are packed in a gunnysack and stored in a dried stock room. The air-dried tea products could also be pressed into different shapes, such as cubic, brick, bowl, and ring cake, and then stored in a stock room.

2.3 HEALTH BENEFITS OF PU-ERH TEA

Studies on the health benefits of pu-erh tea are quite scarce. Recently, a few reports describing the hypolipidemic and antiobesity effects of pu-erh tea have appeared.[3,5] Meanwhile, Chinese folk medicine has described several health benefits for this beverage, but the experimental and clinical evidence for these benefit effects is incomplete. Additional investigations should be carried out in order to confirm these claims.

2.3.1 Health Benefits as Described in Folk Medicine

The following health benefits have been ascribed to pu-erh tea:[7]

1. Promotes imagination
2. Promotes eye health
3. Promotes mental activity and alertness
4. Promotes digestion and nutritional conditions
5. Has an antiobesity effect
6. Detoxifies certain toxicants
7. Acts as an antidiarrheal
8. Promotes bone formation
9. Promotes longevity and well-living.

As mentioned above, the evidence for these health benefits is limited and incomplete. Therefore, it is necessary to provide adequate experimental and clinical data to support these interesting claims.

2.3.2 The Hypolipidemic and Antiobesity Effects of Pu-erh Tea

Recently, research on the biomedical activity of pu-erh tea has focused on its antibacterial, antioxidative, lipid-lowering, and antiobesity effects. Many *in vitro* studies have shown that pu-erh tea has antioxidative activity. Lin et al.[8] reported that the water extract of pu-erh tea (100 μg/ml) can protect the plasmid DNA from strand breakage induced by the Fenton reaction as well as the control, regardless of total catechin content. Duh et al.[4] reported that pu-erh tea water extract with less catechins (8.01 mg/ml), compared to green tea water extract with more catechins (79.1 mg/ml), could still chelate metal ions, scavenge DPPH radicals, and decrease nitric oxide production in lipopolysaccharide-activated RAW 264.7 macrophages.

Sano et al.[9] and Kuo et al.[3] demonstrated lipid-lowering effects of pu-erh tea in Wistar rats in a 16-week feeding study and in Sprague-Dawley rats in a 30-week feeding trial.

The suppression of lipogenesis by oolong, black, green, and pu-erh tea leaves in rats has been demonstrated by suppression of plasma triacylglycerols (TAG), cholesterol, and low-density lipoprotein (LDL) cholesterol in the experimental Sprague-Dawley rats.[3] The results indicated that pu-erh tea and oolong tea could lower the levels of plasma TAG more significantly than those of green tea and black tea. Meanwhile, pu-erh tea and green tea were more efficient than oolong tea and black tea in lowering the levels of total cholesterol.

2.3.3 Inhibition of Plasma Lipid through Suppressing Hepatic Fatty Acid Synthase

Fatty acid synthase (FAS) is a key enzyme of lipogenesis. Overexpression of FAS is dominant in cancer cells and proliferative tissues. The expression of FAS in livers of rats fed pu-erh tea leaves was significantly suppressed. The gains in body weight, levels of TAG, and total cholesterol were also suppressed in the tea-treated rats.[10]

FAS expression in hepatoma HepG2 cells was suppressed by the extracts of pu-erh tea at both the protein and mRNA levels. FAS expression in HepG2 cells was strongly inhibited by PI3K inhibitor LY294002 and JNK inhibitor II, and slightly inhibited by p38 inhibitor SB20358 and MEK inhibitor PD98059, separately. Based on these findings, it is suggested that the suppression of FAS in the livers of rats fed pu-erh tea leaves may occur through downregulation of the PI3K/Akt and JNK signaling pathways.[10] The active principles and molecules responsible for hypolipidemic effects of pu-erh tea deserve further investigation.

2.3.4 Suppression of Plasma Uric Acid by Inhibiting Xanthine Oxidase

Several pu-erh tea consumers have claimed that drinking pu-erh tea, especially aged pu-erh tea, can attenuate the pain of gout at the bone joint. The mechanism of this interesting clinical effect of pu-erh tea may come from the inhibitory effects of tea polyphenols on the uric acid–generating enzyme xanthine oxidase (XO). The inhibitory effects of six tea polyphenols, namely, theaflavin (TF1), theaflavin-3-gallate (TF2), theaflavin-3,3'-digallate (TF3), epigallocatechin gallate (EGCG), gallic acid, and propyl gallate, on XO have been investigated.[11] These six antioxidant compounds reduce oxidative stress in HL-60 cells. Theaflavins and EGCG inhibit XO to produce uric acid and also act as scavengers of superoxide. TF3 acts as a competitive inhibitor and is the most potent inhibitor of XO among these compounds. It has been shown that pu-erh tea contains different levels of theaflavins, thearubigins, and higher polymerized catechins. Therefore, pu-erh tea is expected to inhibit the XO activity through its polyphenol constituents.

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3 White Tea
Its Manufacture, Chemistry, and Health Effects

He-Yuan Jiang

CONTENTS

3.1 INTRODUCTION

There is much recent awareness about white tea in the United States and Europe. White tea is considered a new tea having a wide range of health benefits, such as antioxidant, antimicrobial, and anticancer effects. However, white tea was first discovered during the Tang Dynasty, about the sixth century, and soon became the choice of the royal courts. White tea has undergone much change since 1885, when specific varieties of tea bushes were selected to make Silver Needle and other specialty white teas. Modern white tea appeared after that. Chinese export of these fine white teas began in 1891.[1]

White tea is named from its unique characteristics, covered with white hair. The unique quality is obtained as a result of a special processing procedure of "no frying and no crushing." The annual production of white tea is relatively small, just

2,000 tons, about 0.1% of that of black tea. White tea is mostly produced in Fuding, Zhenghe, Jianyang, and Songxi of Fujian Province in China, and this accounts for more than 90% of the annual world production of white tea.[1]

Although white tea is only produced in a very small quantity in China, it is consumed in many countries. Today, Chinese white tea is mainly exported to countries and areas such as Germany, France, the Netherlands, Switzerland, Indonesia, Singapore, Malaysia, Japan, Hong Kong, and Macao.[1]

The unique quality and flavor of white tea are derived from a special growth place, special breed, and special processing method. Dried white tea appears natural and elegant outside, and tastes sweet and refreshing. There are many grades of white tea, depending on the different plucking criteria; these are Silver Needle with White Hair, White Peony, Gongmei, and Shoumei. Silver Needle is made of the pure bud, while White Peony is made of the bud and one or two leaves of Dabaicha or Shuixian varieties. Gongmei is made from the Qingzhong variety, and Shoumei is manufactured from fresh leaves without the bud.[1]

White Peony is the most dominant grade of white tea. White Peony can be further classified into many grades, according to the tea tree variety. For example, Dabai, meaning big white tea, is made from the variety of Fuding Dabaicha; Shuixianbai is made from the Shuixian variety; and Xiaobai, meaning small white tea, is made from the Qingzhong variety.[1]

3.2 MANUFACTURING OF WHITE TEA

The difference among all tea categories is due to their varied manufacturing technology. Green tea, from nonfermented leaves, is processed as steamed or fired prior to being rolled. Black tea is brewed from fully fermented leaves, oolong tea is partially fermented, and pu-erh tea is doubly fermented. Unlike the high-temperature treatment of green tea or the all-fermentation treatment of black tea, white tea is manufactured with minimal treatment, and it is rapidly steamed and dried, leaving the leaves fresh.

White tea is harvested when the buds on the trees are still covered with fine white hair and the leaves have not yet fully opened. After plucked, fresh tea leaves are sunshine withered and dried, and the leaves are kept relatively fresher than those of green or black tea. With a lighter and sweeter taste after processing, white tea is closer to fresh tea leaves than green tea, and is far different from the other varieties of tea.

Usually, white tea is processed with only two procedures, withering and drying. Sometimes, the same processing method, such as sunshine drying, can be looked upon as both withering and drying. The earliest manufacturing method is sun drying for white tea. Modern diverse white tea can be manufactured by three methods of sun drying, airing, and basket frying.[1] White tea can be manufactured in the spring, summer, and autumn, but not the winter. Usually, spring white tea has premium quality, autumn white tea is next, and summer white tea is the lowest grade.

The details of the processing technologies for white tea manufacture are introduced below according to their grades.

3.2.1 Silver Needle with White Hair

Silver Needle with White Hair is named after the color quality and shape of this type of tea. Its color is white like silver, and its shape is long and like small needles. The material for Silver Needle is new bulky bud. Some are made from the separated bud from the aired material, which have the bud and one or two leaves plucked from Dabaicha and Shuixianzhong. There are two dominant processing methods for this grade of white tea originating from Fuding and Zhenghe.

The processing method in Fuding is more prevalent. The first step for Silver Needle processing is sunshine drying. The buds are spread evenly on sieves or withering mats right after they are plucked from tea trees, and insolated directly under the sun. Keeping for one whole day under the strong sunshine in spring is sufficient, upon which the white tea might be nearly dry. The nearly dried white tea can be baked on slow fire until fully dried. It will take about 30 minutes for 250 g of tea buds to dry at 40–50°C in a baking basket.[1,2]

In the case of hot and wet days, insolating for 2 days is needed. After the tea buds are nearly dried, they can then be baked on slow fire. For rainy or foggy days, direct baking with slow fire is necessary.

The processing method in Zhenghe is a little different from that in Fuding. Airing under the shadow or mild sunshine prior to direct sunshine drying is practiced. Mostly dried tea buds are moved under strong sunshine to dry completely. The duration for the whole process is often 2–3 days.

Dried white tea, covered with white hair and silver shining, has a bulky shape and vivid color and luster. The quality of white tea produced in different areas is different due to the existing differences in the processing methods practiced. Fuding Silver Needle appears silver white and tastes clear and delicious, whereas Zhenghe Silver Needle appears silver gray and tastes fresh, brisk, and luscious.[1]

3.2.2 White Peony

White Peony is made from the bud and one or two leaves. Although Gongmei and Shoumei are the lower grades and made from different materials, their processing techniques are similar to White Peony, as described here.

White Peony can be processed with the two simple steps of withering and basket drying. Withering is the key step that determines the final quality of white tea. There are usually three withering methods for White Peony: sunshine withering, airing, and low-temperature withering.

Airing is the most used method for White Peony. The requirements for airing in spring are 18–25°C, and relative humidity (RH) of 67–80%. Usually, a temperature of 30–32°C and an RH of 60–75% are more acceptable for summer and autumn airing. The airing time is often 52–60 hours.[1]

Low-temperature withering could be used instead of airing during rainy days. The withering room is heated by the pipeline, keeping at 20–32°C and RH of 65–70%. A temperature of 29–30°C is most suitable for withering. The withering time for airing is often more than 36 hours. When tea bud and leaves are half dried, they can be basket dried twice.

Sunshine withering could be used on sunny spring days, too. Fresh leaves can be insolated for 25–30 minutes under slight sunshine during morning or at dusk, at 25°C and RH of 63%. Tea leaves should be moved into a prewarmed withering room. Ordinarily, two to four times of sunshine withering is best for processing of White Peony. However, sunshine withering is not favorable in the strong sunshine in summer.

After withering, basket drying is the last processing step for White Peony. Timely drying can keep the color and taste good, prevent variation, and enhance the aroma of tea. The basket drying of White Peony can be done over 15–20 minutes at fire temperature of 70–80°C. If tea leaves are only half dried, basket drying should be done twice, with the first drying at 100°C and the second at 80°C. A baking machine could also be used instead of baskets, with operating parameters similar to those of basket drying.[1]

3.3 MAIN CONSTITUENTS OF WHITE TEA

Tea polyphenols, amino acids, and water are the key components in tea. They affect the flavor and taste of tea liquor and determine intrinsic quality of white tea. Tea quality is dependent on its processing. The key processing step for white tea production is withering, which induces much of the metabolic reactions of water, enzymes, polyphenols, and other chemical constituents.

3.3.1 Water

Water content in white tea is very important, because it can affect the activities of enzymes and chemical reactions involved. During withering, water vaporizes, and the rate of water evaporation is related to the humidity and temperature. Table 3.1 shows that 50% of water can be lost after 30 hours of withering, while only 10% water is lost after 10 hours of withering.[3]

3.3.2 Enzymes

Polyphenol oxidase (PPO) and peroxidase (POD) are the two important enzymes for the processing of tea. During the manufacturing of green tea, these enzymes are undesirable, because they can catalyze the oxidation of catechins, which can negatively affect the quality of green tea. However, these enzymes are desirable during the manufacturing of black tea, and can catalyze the transformation of catechins to theaflavins and thearubigins, responsible for the characteristic flavor and color

TABLE 3.1
The content of water in white tea during withering

Withering time (h)	0	10	20	30	40
Water content in tea leaves (%)	69	62	42	32	16
The loss of water (%)	0	10.1	39.1	53.6	76.8

Source: Anhui Agricultural University. 1979. *Tea manufacturing science*, 269–77. Beijing: Agriculture Press. With permission.

of black tea. Like the manufacturing of black tea, white tea encounters the same withering treatment. Unlike black tea making, however, white tea does not need any special shape-making and fermentation treatment.[3]

PPO and POD activities are influenced by many factors, including moisture, temperature, withering time, and stacking height of tea leaves. During the withering process, PPO and POD undergo various changes. At the initial phase, PPO activity increases more quickly than POD activity. After 4 hours, the PPO activity is inhibited by quinones, which are formed by the oxidation of catechins, but POD activity is not affected. After 8 hours, PPO activity increases again, and stops after 20 hours. The activity of POD reaches its maximum at 12 hours. Although the activities of PPO and POD decrease later due to dehydration, their activities are still higher than the initial values (table 3.2).[4] During drying, PPO and POD, together with other enzymes, are denatured by the heating treatment at 80°C or higher. Their activities are almost zero after drying.

Withering is the key process for the formation of white tea color, and PPO and POD are needed for color development of white tea. However, the best condition for white tea is the slight and slow oxidation of polyphenols under a temperature of less than 30°C.

3.3.3 Polyphenols

Like all other teas, polyphenols are the most important constituents in white tea. Polyphenols are responsible for the bitterness and astringency of tea liquor. During the manufacturing of white tea, the content of polyphenols increases slightly at the beginning of withering, but decreases right after (table 3.3).[5] Although the trend for the changes of polyphenols appears similar in different varieties, the absolute contents of polyphenols are different among them. For example, Fuyunzhong No. 6 has a higher content of polyphenol than Fuding Dabaicha.

Catechins are the most abundant polyphenols in white tea and account for about 70% of total polyphenols. Catechins in tea consist of (–)-epigallocatechin (–)-EGC), (–)-epicatechin (–)-EC), (–)-epigallocatechin-3-gallate (–)-GCG), (–)-epicatechin-3-gallate (–)-ECG), (+)-gallocatechin (+)-GC), (–)-gallocatechin (–)-GC), (+)-catechin (+)-C), and (–)-catechin (–)-C). (–)-EGCG is the most abundant catechin among them. The content of catechins was decreased by more than 50% during withering (table 3.4).[6]

TABLE 3.2
PPO and POD activities in white tea during withering

Withering time (h)	0	4	8	12	16	20	24	28	32	Dried
Temperature (°C)	27.9	28.8	32.8	31.7	29.2	27.0	27.5	29.1	31.1	110.0
Moisture (%)	76.2	74.1	68.2	63.4	57.9	52.8	49.3	42.5	31.7	2.2
PPO	100.0	334.4	190.0	251.0	373.0	283.3	140.5	184.3	155.0	0
POD	100.0	146.6	208.9	438.1	240.1	93.8	193.5	187.4	143.8	0

Source: Cheng, Z. 1984. Brief introduction of the catalyzation of enzymes during white tea manufacturing. *Brief Rep. Tea Sci.* 3:9–10. With permission.

TABLE 3.3
The content of polyphenols during white tea withering

Variety	Time (hours)			
	0	**24**	**48**	**66**
No. 6 Fuyunzhong	29.55	30.50	22.62	18.69
Fuding Dabaicha	22.98	25.06	19.78	17.78

Source: Liu, Y., Guo, Y., and Zheng, Z. 2003. Investigations on the variation of main chemical substances and the formation of white tea during manufacturing. *J. Fujian Tea*, 13–14. With permission.

TABLE 3.4
The variation of catechins of white tea during processing

Catechins	Fresh leaves	Withering 8 hours	Withering 16 hours	Withering 32 hours	Drying raw tea
EGC	36.70	24.54	19.62	8.61	1.83
GC	23.74	16.56	11.42	4.91	0.76
EC + C	24.32	20.76	15.20	10.51	7.59
EGCG	122.56	90.08	77.02	55.49	31.13
ECG	40.62	31.89	26.31	20.21	14.77
Total	247.94	183.83	149.57	109.73	56.08

Source: Chen, C. 1984. *Theory in tea processing*, 51–52, 205–17. Shanghai: Shanghai Science and Technology Press. With permission.

The content of catechins is mostly influenced by PPO and POD. The oxidation of catechins under the action of PPO and POD results in the decrease of catechins during the withering stage. The amount of catechins remains steady during terminal withering stage, while PPO and POD activities are inhibited.[6]

3.3.4 Amino Acids

Amino acids are important constituents of tea and account for the freshness and briskness of the tea liquor.[7] Tea leaves lose water easily, and the enzymatic reactions, such as hydroxylation of proteins, are induced during withering. The content of amino acids is gradually increased during withering, particularly during the primary stage. After 24 hours of withering, the content of amino acids is almost doubled compared to that of fresh tea leaves (table 3.5). As for the variety, the content of amino acids in Fuding Dabaicha is higher than that in Fuyunzhong No. 6 (table 3.5).[5]

3.3.5 Carbohydrates

There are significant changes in the amount of carbohydrates in white tea during processing. Heating is the primary factor for the changes of carbohydrates in white tea, and the enzymatic hydrolysis is the secondary factor. Carbohydrates can also

TABLE 3.5
The content of amino acids during white tea withering

Variety	Time (hours)			
	0	24	48	66
No. 6 Fuyunzhong	2.41	4.13	4.73	4.79
Fuding Dabaicha	3.12	4.14	5.00	5.21

Source: Liu, Y., Guo, Y., and Zheng, Z. 2003. Investigations on the variation of main chemical substances and the formation of white tea during manufacturing. *J. Fujian Tea*, 13–14. With permission.

react with other compounds, such as amino acids, to form products that might contribute to the color and aroma of tea.

Carbohydrates in white tea vary along with withering time. At the early withering stage, the content of carbohydrates decreases. Both reducing and nonreducing sugars follow the same trend as the total carbohydrates (table 3.6). However, their contents increase under the enzymatic hydrolysis of polysaccharides at the later withering stage, and decrease again during heating at the drying stage.[6] In addition, different processing methods have different effects on the changes of carbohydrates. Basket drying decreases the total carbohydrates more than sunshine drying and airing due to the combined impact of both heat and light.[6]

3.3.6 Other Chemicals

Other chemical components in white tea also change during processing. Chlorophyll is one of the most important components, which is the key factor for the color of white tea, just like green tea. During the withering and drying, the content of chlorophyll decreases steadily (table 3.7). Like the change of carbohydrates, basket drying has a stronger influence on chlorophyll than sunshine drying and airing[5] (table 3.7).

3.3.7 Chemicals in White Hair

White hair is the symbol for Silver Needle and is also important for its color and taste quality. Results showed that white hair contains significant amounts of amino

TABLE 3.6
The content of carbohydrates during white tea withering

Carbohydrates	Time (hours)					
	0	6	12	18	24	Dried
Reducing sugar	0.49	0.41	0.38	0.44	0.66	0.37
Nonreducing sugar	2.14	1.47	1.06	0.99	1.11	0.95
Total	2.63	1.88	1.44	1.43	1.77	1.32

Source: Chen, C. 1984. *Theory in tea processing*, 51–52, 205–17. Shanghai: Shanghai Science and Technology Press. With permission.

TABLE 3.7
The variation of main chemical substances of white tea during processing

Chemical substances		Fresh leaves	Withering 12 hours	Withering 24 hours	Airing tea 47 hours	Sunshine dried	Withering 22 hours + baking	Withering 27 hours + baking	Withering 27 hours + sunshine + baking
	Moisture	73.27	57.86	33.11	11.98	7.93	3.96	3.66	5.70
	pH	5.30	5.00	4.90	5.23	5.01	5.09	5.10	5.20
Water soluble	Reducing sugar	0.69	0.34	0.66	0.38	0.53	0.54	0.37	—
	Nonreducing sugar	2.14	1.06	1.11	—	0.99	1.24	0.59	1.92
	Total	2.62	1.44	1.77	—	1.51	1.77	0.97	—
Starch	3.78	1.62	0.92	1.76	1.27	2.03	1.44	1.50	
Free amino nitrogen	0.68	0.53	0.42	0.71	0.74	0.49	0.86	0.54	
Chlorophyll	Total (mg/g)	6.63	—	6.75	5.41	5.27	6.36	5.05	5.21
	Chlorophyll a	4.34	—	4.05	3.21	3.19	4.26	3.06	3.10
	Chlorophyll b	2.20	—	2.70	2.20	1.96	2.10	1.96	2.10
Polyphenol oxidase	100.00	16.90	150.00	4.60	0.00	23.60	0.00	0.00	
Peroxidase	100.00	53.40	23.40	0.00	0.00	55.10	0.00	0.00	
Polyphenol substances (%)	Water soluble	30.87	24.03	30.05	26.75	26.98	26.81	28.12	28.30
	Alkaline soluble	6.79	4.92	6.84	3.33	2.88	2.43	3.26	3.86
	Total	37.66	28.94	36.87	30.08	29.86	29.24	31.38	32.16

Note: The unit for chlorophyll is mg/g, and the units for polyphenol oxidase and peroxidase are relative values.

Source: Chen, C. 1984. *Theory in tea processing*, 51–52, 205–17. Shanghai: Shanghai Science and Technology Press. With permission.

acids, polyphenols, and caffeine. There are more amino acids in white hair than the tea itself[8] (table 3.8).

3.4 HEALTH BENEFITS OF WHITE TEA

Without high temperature and fierce kneading or rolling in the processing, white tea retains more beneficial components than other teas. White tea is traditionally consumed to cure diseases in many places of Fujian. Nowadays, white tea is gaining popularity in the United States and Europe. New studies suggest that white tea also offers the benefits of green tea, and may be the most potent among teas in human health promotion.

3.4.1 Health-Beneficial Components

Generally, teas are considered to be healthful because they contain catechins, other polyphenols, vitamins, minerals, alkaloids, amino acids, and volatile oils. Although more rare and expensive than green tea and black tea, white tea is being used due to its possible medicinal and health implications. White and green teas are considered especially beneficial due to the presence of higher amounts of epigallocatechin-3-gallate (EGCG), a biologically active polyphenol.[9,10] White tea is thought to have a higher proportion of polyphenols, which may render a better health benefit than other types of tea.[11]

White tea also contains the highest amounts of flavones, 14.2- to 21.4-fold higher than other teas. Flavones have the strongest antioxidative activities. Another benefit of drinking white tea over other teas is its lower level of caffeine. There is only 15 mg of caffeine per serving for white tea, considerably less than the 40 mg in black tea and 20 mg in green tea.[12]

Liu et al.[13] systematically measured the stable free radicals in processed Chinese teas with electron spin resonance (ESR) spectrometry. They found a large number of stable free radicals in teas. The contents of stable free radicals in processed teas varied along with different processing steps. Among all tested Chinese teas, the amount of stable free radicals in white tea was the lowest, while green tea was the second. The other teas contained more free radicals, 1.6- to 143-fold higher than Sil-

TABLE 3.8
The comparison of constituents in white hair and tea itself

White tea		Water extracts	Amino acids	Polyphenols	Caffeine	Percentage
1	White hair	28.9	3.3	25.0	5.5	13.5
	Tea removed hair	49.2	2.7	32.1	5.9	86.5
2	White hair	28.0	3.2	23.9	5.3	11.8
	Tea removed hair	47.9	2.5	29.6	5.9	88.2

Source: Shi, Z. 1997. *Tea processing science*. Beijing: China Agriculture Press. With permission.

ver Needle white tea. The lower the content of free radicals in white tea might mean the healthier the product compared to other teas.

3.4.2 Anticarcinogenicity

Tea is one of the most widely consumed beverages, with potential health benefits against such chronic diseases as cardiovascular disease and cancer. Green tea is more effective in its anticarcinogenic benefits than black tea, because green tea has a higher level of catechins and other polyphenols. White tea has high levels of polyphenols, even more than green tea, and might have the strongest potential of all teas for fighting cancer.[14]

Heterocyclic amines are procarcinogens generated from sugars, amino acids, and creatinine during the cooking of foods.[15] They occur widely in meats and fish. Certain heterocyclic amines can induce the multiplication of tumors in the small intestine and colon in experimental animals.[15] Recent studies have shown that four grades of white tea have marked antimutagenic activity in the *Salmonella* assay against heterocyclic amines, particularly in the presence of S9 mix (microsome fraction of rat liver).[16] The most active of these teas, Exotica China white tea, was significantly more effective than the premium green tea (Dragonwell special grade) against 2-amino-3-methylimidazo[4,5-f] quinoline (IQ) and four other heterocyclic amines.[16] These results also suggest that the greater inhibitory potency of white tea in the *Salmonella* assay might be related to catechins and caffeine, perhaps acting synergistically with other minor constituents to inhibit mutagen activation as well as scavenging the reactive intermediates. The mixture prepared from the combination of major constituents of white tea or green tea, consisting of catechins and caffeine according to their relative levels in teas, exhibited less antimutagenic and anticarcinogenic potency than with white and green teas.[16]

Orner et al.[17,18] examined the relative effectiveness of white and green teas in suppressing heterocyclic amine–initiated intestinal tumorigenesis in Apc(min) mice. After 12 weeks of treatment, mice given white tea, green tea, or sulindac, a nonsteroidal anti-inflammatory drug known to be highly effective in Apc(min) mice, had significantly fewer tumors than the controls ($p < 0.05$). The protection provided by 1.5% green or white tea was comparable to that provided by 80 ppm sulindac. Mice treated with a combination of white tea and sulindac had significantly fewer tumors than either treatment alone. This research provided evidence that teas, particularly when administered in combination with sulindac, were highly effective in inhibiting intestinal neoplasia in mice via direct or indirect effects on the beta-catenin–APC pathway, and are also effective against intermediate and late stages of colon cancer, via effects on the beta-catenin–Tcf signaling pathway. Consistent with the findings from *in vivo* studies, *in vitro* cell model studies also suggested the suppression of intestinal polyps by white tea via an apparent downregulation of beta-catenin and Wnt target genes.[19]

In another study, Santana-Rios et al.[20] found 5.65 ± 0.81 and 1.31 ± 0.27 aberrant crypt foci (ACF) per colon in groups of rats given 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) and PhIP + white tea, respectively, during 8 weeks of study. White tea also inhibited cell proliferation and suppressed early lesions in the colon.[21]

Although no changes were detected in *N*-acetyltransferase or arylsulfotransferase activities compared with controls, there was marked induction of ethoxyresorufin *O*-deethylase, methoxyresorufin *O*-demethylase, and UDP-glucuronosyltransferase after treatment with white tea. Results from PCR assays showed that the inhibition of white tea in PhIP-induced ACF might be due to the altering of the expression of carcinogen-metabolizing enzymes. These data provide additional support for the possible chemopreventive role of white tea against cancer of the large bowel.

Although there were many studies on white tea as a potential anticarcinogen, these data are highly preliminary and cannot be extrapolated to human cancer prevention or treatment. Further studies with white tea in animal models and in human trials are needed.

3.4.3 Antioxidant Activity

White tea has good antioxidative and antiaging functions. The oxidative stress in skin cells leads to immune system damage, and can promote skin cancer and photo damage, such as wrinkling or mottled pigmentation. The white tea extract could limit and prevent the DNA damage in cells after exposure to sunlight.[22] Therefore, white tea extract is believed to be an effective antioxidative agent, and may provide antiaging benefits.[23]

In a model of oxidative damage of human red blood cells initiated by cumene hydroperoxide, Gawlik and Czajka[11] showed that white tea extracts at a level of 4 g/150 ml water significantly decreased the formation of the oxidation product, malondialdehyde. White tea extract also showed a superior reducing activity compared to other plant extracts, such as wheat sprouts, *Morinda citrifolia*, and fermented papaya.[24]

3.4.4 Antimicrobial Activity

A recent study showed that white tea has better antimicrobial function than green tea. White tea extract (WTE) may have prophylactic applications in retarding growth of bacteria that cause *Staphylococcus* infections, *Streptococcus* infections, pneumonia, and dental caries.[25] White tea is very effective in inactivating bacterial viruses. Results obtained with a bacterial virus suggest that WTE may have an antiviral effect on human pathogenic viruses. Studies have also indicated that WTE has an antifungal effect on *Penicillium chrysogenum* and *Saccharomyces cerevisiae*. In the presence of WTE, *Penicillium* spores and *Saccharomyces cerevisiae* yeast cells were totally inactivated.[26] In the commodity market in China and the United States, the white tea extract has now been added into several toothpastes to enhance antiviral and antibacterial effects.

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4 Biological Functions and Manufacturing of GABA Tea

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CONTENTS

4.1 INTRODUCTION

GABA tea, or Gabaron tea as known in Japan, is a new type of specialized tea with a high content of γ-aminobutyric acid (GABA). GABA tea contains greater than 150 mg of GABA per 100 g of tea. Besides its health benefits, related to antioxidation, anticancer, and blood lipid-lowering effects, GABA tea also has special biological functions due to GABA. Since GABA tea was first developed, in 1987, many techniques have been used for increasing its GABA content. In addition, many research works concerning the health benefits of GABA tea have been reported. Besides the biological functions of GABA, this chapter introduces the manufacturing process and health benefits of GABA tea.

4.2 INVENTION OF GABA TEA

In 1987, when Tsushida et al.[1] were searching for a good method for extending the storage time of fresh tea leaves for making green tea, they treated the fresh tea leaves anaerobically, just as it is done for fresh vegetables. After examining the amount of free amino acids in green tea product, they found that GABA and alanine content in the tea were increased by 8.9- and 5.2-fold, respectively, whereas 79% of glutamic acid and 91% of aspartic acid had disappeared.

In the 1950s, GABA was already reported as an important chemical transmitter for the brain nerves. Many researchers also reported that GABA could reduce the blood pressure in experimental animals[2] and humans.[3–5]

Stanton[6] further demonstrated that the amount of GABA needed to reduce blood pressure by 20% in different animals was 11 μg/kg for dog, 19 μg/kg for rabbit, 174 μg/kg for pig, and 96 mg/kg for cat. These results motivated Tsushida et al.[1] to continue working on the manufacturing techniques for GABA tea, and cooperate with Omori in Otsuma Women's University to perform animal studies on GABA tea.[7] After clearly confirming that GABA tea could reduce the blood pressure of the spontaneously hypertensive rat (SHR), GABA tea was then mass produced and commercialized in Japan.

4.3 BIOLOGICAL FUNCTIONS OF GABA

GABA is found in a number of biological species, including (1) bacteria, such as *Escherichia coli*, *Streptococcus pneumoniae*, *Neurospora crassa*, and *Lactobacillus brevis*; (2) insects, such as cockroach, grasshopper, moth, honeybee, and fly; (3) plants, such as GABA tea, tomato, soybean, mulberry leaf, germinated brown rice, and petunia; and (4) animals, such as human, rat, rabbit, cat, and dog, among others,[8] and is widely distributed in living cells of the cerebral cortex and hypothalamus.[9] It is decomposed from glutamate by glutamate decarboxylase (GAD) in the substantia nigra, pallidum, and striatum of the basal ganglia.[2,10]

GABA is found in high amounts in cerebrospinal fluid (CSF), blood, and the central nervous system (CNS) in humans. GABA can modulate monoamines and growth hormones.[11,12] GABA in neurons plays an important role in cardiovascular function.[13] Higher concentrations of GABA will enhance GABA receptors, to open

more chloride channels in the tract of the cell membrane on the neuron axon, and increase the chloride penetration of the neuron membrane. Low GABA in plasma is reasonably specific to Parkinson's disease, epilepsy, alcoholism, depression, and menopause.[14] Therefore, GABA is an important inhibitory neurotransmitter in the brain and CNS[15] for being able to decrease the excitation of nerves. GABA-mediated neurotransmission regulates many physiological and psychological functions.

Several studies, discussed below, further report that GABA improves hypertension, depression, sleeplessness, alcoholism, release of growth hormones, diabetes, and nervous regression.

4.3.1 GABA and Hypertension

It has been proposed that GABA plays an important role in the modulation of cardiovascular function[16] by acting in not only the CNS but also peripheral tissues.[17,18] Indeed, GABA has been reported to reduce blood pressure in experimental animals[2] and humans[3] following its systemic or central administration, and it has been suggested that the depressor effect induced by systemic administration of GABA[6,19] is due to the blockade of sympathetic ganglia.

Studies have evidenced that GABA plays an important role in the antihypertension effect induced by systemic or central disease, and improves the hypertension occurring in the spontaneously hypertensive rat (SHR).[20–23] The results show that GABA may have a relationship with increased norepinephrine.[24,25] High blood pressure is caused by a high-salt diet in animals, but consumption of GABA can reduce blood pressure effectively.[7,26] Therefore, dietary GABA should improve blood pressure.

4.3.2 GABA and Depression

Depression is one of the most common conditions in the UK, affecting one in five people at some stage in their life. The World Health Organization (WHO) estimates that by 2020 depression will be the biggest global health concern after chronic heart disease. Depression occurs in children, adolescents, women in menopause, the elderly, or those with mental disorder, especially in menopause.[27,28] The symptom of depression in menopause will cause mood obstacles about 10 years later.[28,29]

Evidence from preclinical and clinical data suggests that GABA plays a role both in the pathophysiology of depression and bipolar disorder and in the mechanisms of action of antidepressant agents.[30,31] Low GABA concentrations and receptor activity in the brain cause depression, palpitation, insomnia, learning disorders, and memory failure.[29,32–34] GABA concentration in the CSF is inversely correlated with the severity of depression.[35]

Murphy et al.[36] found that during the treatment process for menopause disorder by estrogen replacement (such as estradiol), expression of the GABA receptor, GAD of the hippocampus neuron, and synthesis of GABA decreased in the rat brain. Even for alcoholism, the GABA content in plasma decreased and the symptoms of neurosis (anorexia, sense of pain) and mental disorder (depression, mania, anxiety) were significant.[37] Therefore, supply of GABA and enhancement of activity of the GABA receptor improve depression.[30,31]

4.3.3 GABA and Sleep

GABA is a good therapy medicine for sleeplessness. Sleeplessness caused by stress and strain is a common sickness in modern society. In addition, aging may decrease the quality of sleep and lead to sleep deprivation. Sleeplessness is associated with changing the sleep electroencephalogram (EEG) and endocrine activity. The effects of sleeplessness are spending more time awake, having less slow-wave sleep (SWS) and rapid eye movement (REM) during sleep,[38] and having low growth hormone secretion[39] and hypothalamo-pituitary-adrenocortical (HPA) system activity.[40]

Using seven young male and seven elderly subjects, the effects of 40 hr sleep deprivation on sleep EEG and the secretion of growth hormone, cortisol, and prolactin were studied.[41] The results indicated that SWS was less in the elderly than in the young. Total sleep deprivation (TSD) decreased sleep onset latency, REM density, and, by trend, REM latency in the elderly. The use of GABA-benzodiazepine can improve sleeplessness and nighttime hormone secretion for TSD patients. The GABA receptor is one of the calcium receptors induced from tranquilizers, and it inhibits nerve activity by GABA. Therefore, GABA may improve sleeplessness, including discontinuing sleep, cycling sleep, and HPA system activity.[42–44]

4.3.4 GABA and Alcoholism

Alcoholism is a major social, economic, and public health problem. Social need and abuse of alcohol induced the development of tolerance and dependence. A literature survey on ethanol and alcoholism indicates that GABA is an important target of ethanol in the CNS. Drinking ethanol alters the activity of GABA. Thus, it is speculated that the initial excitatory effect of ethanol may be due to the inhibition of GABAergic activity and decrease of GABA secretion in the brain.[45]

Some studies (human, animal, and *in vitro* test) have found an inverse correlation between GABA level in plasma and alcoholism.[46–52] Gomez and Barros[14] reported a low GABA concentration in the plasma of alcohol abusers and alcoholics. Drinking alcohol alters the activity of the GABA-containing system in several ways, and may have a bearing on the clinical syndromes associated with alcohol abuse.[53,54] Plasma GABA was lower in alcoholics than in nonalcoholic drinkers. Others have reported a low GABA concentration in the plasma of alcoholics[55] and their sons.[56] Therefore, drinking alcohol for a long time will alter synthesis, release, and metabolism of GABA, which will change GABA concentration in plasma and the brain. Furthermore, through use of GABA-mimetic agents in animals it was found that these agents could inhibit seizures and stop pain.[45] The higher activity of the GABA inhibitor in the brain could increase alcohol intake.[57,58]

4.3.5 GABA and Growth Hormone

Some studies have found that the amount of growth hormone in newborn animals (2 ~ 3 weeks) was higher than that in adults. This is due to the thyrotropin-releasing hormone (THR), GABA, and growth-hormone releasing hormone (GHRH), which stimulate the secretion of growth hormone. For GABA, it is because GABA may strengthen mobility of calcium ion and increase calcium ion in cells to elevate the

secreting amount of growth hormone. Mergl et al.[59] have reported that GABA started its action right after the animal was born and was about 2 days old.

The growth hormone (GH) in the human body is produced from the liver and other organs (kidney, heart, skeleton, skin, and brain). It secretes insulin-like growth factor-1 (IGF-1) to stimulate the pituitary gland. The amount of secreted human growth hormone is related to age, drug (amphetamine, heroin), disease (alcoholism, cranial nerve degradation), and mood stress (such as melancholia, anxiety, and insomnia). As the ratio of GH and IGF-1 changes, the lean body mass (LBM) may shrink, and the adipose tissue increases and the bone density is reduced.[60] Therefore, normal secretion of growth hormone is a good way to keep young.

4.3.6 GABA and Diabetes

GABA can inhibit the secretion of glucagons by α-cells[61] and stimulate the expression of glutamate decarboxylase(65) (GAD_{65}). GAD_{65} will stimulate β-cells to produce GAD. Then GAD catalyzes the decarboxylation of glutamate to synthesize GABA. This mechanism has the feedback function to inhibit the activity of GAD.[8]

Pancreatic GAD_{65} was identified as a target antigen for autoantibody found in blood circulation of patients with insulin-dependent diabetes mellitus (IDDM). Other studies have shown that streptozotocin-induced diabetes brought about an imbalance in GABA metabolite concentration in the ventromedial hypothalamus.[62] Pancreatic islet binding sites for GABA and benzodiazepines have been shown to be of functional significance in the secretion of insulin in rodents and humans. A decrease in GABA concentration in pancreatic islet cells was associated with the decline of insulin synthesis and release levels present in diabetes.[14] The functions of GABA and GAD in pancreatic β-cells are still unclear, but GABA is considered to have a primary role as a signaling molecule in the pancreatic islets.

4.3.7 GABA and Parkinson's Disease

Ericson et al.[63] have studied the relation of GABA and cranial nerve disease (such as Parkinson's disease). GABA and GAD concentrations were reduced in Parkinson's disease patients' brains and plasma. After gene therapy (to plant into GAD_{65} or GAD_{67}), the GAD activation and GABA release were increased. In addition, nitric oxide (NO) is an intra- or interneuronal messenger that modulates neurotransmitter release in the mammalian brain. Nitric oxide synthase (NOS), including neuronal isoform (nNOS) and endothelial isoform (eNOS), was related with neurotransmitters (glutamate and GABA).[64–66] It caused neuron depolarization and calcium ion flow into cell acceptors and excited the chain reaction of toxic free radicals.[67] Furthermore, it enlarged nerve damaging and cell death and resulted in inducing neurodegenerative diseases.[68]

Many studies have recently been conducted in the area of gene therapy. GABA may change the neuron excitability. GABA content in the brains and blood of people with brain neurodegenerative disease was lower than that of individuals without disease.[69] In addition, researchers have used the adeno-associated virus (AAV) vector to deliver GAD into the brain cells of animals in order to adjust the synthesis of GABA and increase the stability of the athletic control system.[70–73] These results show that

Parkinson's disease patients should keep a stable supply of GABA so that their athletic control system can properly be improved.

4.3.8 GABA and Epilepsy

Epilepsy is a congenital or acquired chronic brain disease. The disease is induced from abnormal discharge in brain cell by factors including (1) incomplete brain development; (2) injured brain (such as traumatic brain injury [TBI] or brain injury during birth); (3) meningitis, encephalitis, and phrenitis; (4) stroke (from brain injury or CNS injury), cerebral atrophy, or metabolic brain disease; and (5) brain tumor or other diseases. Furthermore, patients may experience seizure when they suddenly stop taking drugs, do not sleep enough, are fatigued, overdrink, are alcoholics, suffer stress from sound or light, have a hormone effect (periods or menses), or are uncomfortable (cold and fever), among others.[74]

Vigabatrin (γ-vinyl GABA, Sabril®) is a specific, irreversible inhibitor of GABA-transaminase (GABA-T), inhibiting the degradation of the inhibitory neurotransmitter GABA, thereby increasing the GABA concentration in the plasma, brain, and CSF.[74–78] Petroff et al.[79] reported that taking vigabatrin in doses of up to 3 g/day could increase brain GABA levels, but doubling the dose failed to increase brain GABA any further.[79] The authors[80] also pointed out that there was a significant association between low GABA levels and recent seizures. Patients with complex partial seizures had lower GABA levels than did subjects without epilepsy. Poor seizure control was found to be associated with low brain GABA levels.

4.4 PATHWAY FOR THE FORMATION OF GABA IN TEA

The content of GABA in plant tissues is usually around 0.3–32.5 μmol/g. Much research on the mechanism for the formation of GABA in botanic food materials has been reported.[81] In the early 1970s, Streeter and Thompson[82] reported that GABA and alanine were accumulated in radish leaves (*Rapnanus sativus* L.) under anaerobic conditions. They further investigated the metabolic pathway of GABA in radish leaves both *in vivo* and *in vitro*. By using ^{14}C and ^{3}H labeling, they proved that GABA was formed by the glutamate decarboxylation via glutamate decarboxylase in radish leaves.[83]

For tea, Tsushida and Murai[84] also demonstrated that GABA was transformed from glutamate by using ^{15}N-glutamic acid as the starting material. They found that GABA and alanine increased about 17.7- and 6.8-fold, respectively, after 3 hr of anaerobic incubation of tea leaves. Changes in the ^{15}N atom percent and amounts of ^{15}N of the eight main amino acids in tea leaves fed with ^{15}N-glutamate during incubation aerobically or anaerobically for various periods are shown in table 4.1. ^{15}N from ^{15}N-glutamate was incorporated in all amino acids during aerobic or anaerobic incubation of tea leaves. The decreases in glutamate and aspartate levels were more rapid during anaerobic incubation. Approximately 95% of the ^{15}N in glutamate and 90% of the ^{15}N in aspartate disappeared in the first 3 hr of incubation. On the contrary, the amounts of ^{15}N incorporated in alanine and GABA increased markedly during anaerobic incubation of tea leaves up to 6 hr. This suggests that the ^{15}N in accumulated GABA was derived from glutamate.

TABLE 4.1
Changes in percent of ^{15}N and amounts of ^{15}N in amino acids in tea leaves during aerobic or anaerobic incubation after feeding ^{15}N-glutamic acid for 6 hours

Incubation time (hr)	Asp	Asn	Glu	Gln	Ser	Ala	GABA	Theanine
				Aerobic incubation				
0 Atom %	17.71	7.74	47.07	12.17	11.54	13.57	—	0.48
0 ^{15}N(μmol/g)	1.80	0.21	9.48	0.67	0.65	0.46	—	0.14
3 Atom %	16.24	10.07	36.15	13.93	—	12.05	—	0.51
3 ^{15}N(μmol/g)	1.79	0.39	7.11	0.83	—	0.42	—	0.14
6 Atom %	9.64	7.35	31.32	12.68	5.75	5.74	—	0.44
6 ^{15}N(μmol/g)	1.02	0.59	6.20	0.95	0.58	0.24	—	0.12
12 Atom %	12.90	6.43	24.56	5.39	4.89	2.27	—	0.43
12 ^{15}N(μmol/g)	0.68	1.37	4.52	0.47	0.78	0.21	—	0.11
				Anaerobic incubation				
3 Atom %	6.57	8.53	28.06	10.24	9.08	11.46	9.91	—
3 ^{15}N(μmol/g)	0.03	0.22	0.97	0.26	0.55	2.42	1.50	—
6 Atom %	5.95	9.06	29.89	8.20	11.61	18.87	18.48	0.82
6 ^{15}N(μmol/g)	0.02	0.27	0.76	0.21	0.63	3.64	3.49	0.28
12 Atom %	1.67	2.77	6.15	2.94	2.87	5.11	13.22	0.33
12 ^{15}N(μmol/g)	0.04	0.10	0.60	0.13	0.14	0.86	4.53	0.09

Source: Tsushida, T. and Murai, T. 1987. Conversion of glutamic acid to γ-aminobutyric acid in tea leaves under anaerobic conditions. *Agric. Biol. Chem.* 51:2865–71. With permission.

Note: —, not determined.

However, from the results of enzyme activity analysis, it was interestingly found that although glutamate decarboxylase and alanine: α-ketoglutarate transaminase seemed to be important for GABA and alanine accumulation, their activities did not increase under anaerobic conditions. In fact, GABA accumulation caused by anaerobic incubation is just because the activity of succinic semialdehyde dehydrogenase decreases under anaerobic conditions, so that the succinic semialdehyde can not be further metabolized to succinate[81–84] (figure 4.1).

4.5 THE MANUFACTURING OF GABA TEA

Concerning the quality of GABA tea, the content of GABA in GABA tea should be greater than 150 mg/100 g of product. The higher the amount of GABA, the better the quality of the GABA tea will be. Therefore, how to increase the amount of GABA in tea is an essential and important task.

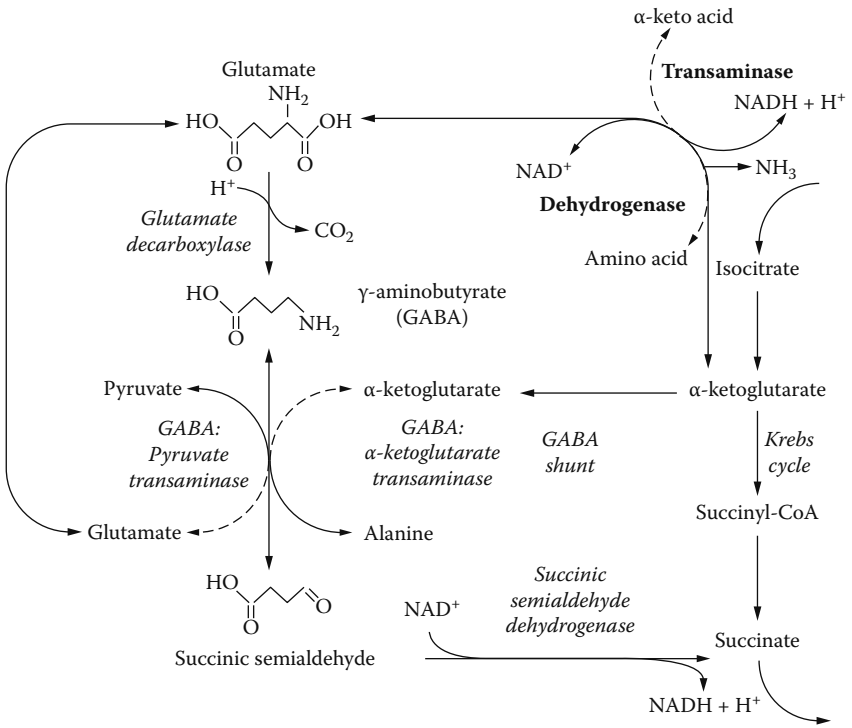


FIGURE 4.1 The pathway for the formation of GABA in tea. (From Shelp, B. J., McLean, M. D., and Bown, A. W. 1999. Metabolism and functions of γ-aminobutyric acid. *Trends Plant Sci*. 4:446–52. With permission.)

4.5.1 GABA Accumulation

GABA accumulated in plants usually results from the stress condition of extreme outside temperature or mechanical force.[81,86–88] Many researchers have pointed out that GABA could be accumulated in the cells when the plant is under stress, like CO_2 treated soybean sprouts and chilling treated tomatoes. In Japan, germinated brown rice with high GABA was successfully developed,[89] and its GABA content was found to be two and seven times more than that of normal brown rice and white rice, respectively. These are all examples that GABA accumulation is brought about by stress conditions.[90,91] Therefore, stress conditions such as lack of oxygen, loss of water, mechanical damage, lower pH, and changes of cytosolic calcium/calmodulin concentration, among others, could be carried out to enhance GABA accumulation.[86]

Researchers have reported that under anaerobic conditions, the oxidation of phosphoric acid in the mitochondria of plants became weakened and reduction potential increased. That means the ratio of NADH and NAD+ increased and ADP transformed to ATP. This caused the dehydrogenation activity of succinic semialdehyde dehydrogenase to decrease, and resulted in weakening the reaction of succinic semialdehyde dehydrogenase to form succinate; this is beneficial to the accumulation of GABA in tea leaves. Sawai et al.[92] further reported that the amount of

glutamate significantly decreased while GABA content increased as the tea leaves were treated anaerobically by using nitrogen or carbon dioxide.

During tea manufacture, the plucked fresh tea leaves are first subjected to withering. The water permeability of the cell wall and membrane increases as the tea leaves keep losing water with concurrent increase of hydrolysis activity. The amount of theanine and glutamine is reduced due to hydrolysis, so that the amount of glutamate is greatly increased and the GAD activity is induced. Therefore, further anaerobic treatment results in the accumulation of GABA in tea leaves.[84]

The optimal pH values for GAD and GABA-transaminase activities are around 5.8 and 8.9. During withering (a kind of stress), the pH of cell cytoplast in tea leaves was decreased. Therefore, it favored the GAD but not GABA-transaminase activity. It resulted in the block of GABA transamination and further GABA accumulation.

Damaging of the cell membrane may cause increased formation of GABA. Therefore, using machinery force to damage the cell membrane is a good and effective way to produce GABA.

4.5.2 The Basic Procedure of Manufacturing GABA Tea

Anaerobic treatment is the key step for manufacturing GABA tea. The basic procedures for manufacturing GABA tea with different degrees of fermentation are shown in figure 4.2.[93]

To prevent the loss of GABA produced by anaerobic fermentation, the processes of pan blanching, rolling, and drying have to be carried out right after anaerobic fermentation in order to fix the content of GABA in the resultant tea. For fermented-type GABA tea such as oolong and black teas, anaerobic fermentation must occur after solar withering and indoor withering. Otherwise, GABA would be easily lost. Table 4.2 shows the contents of some amino acids in oolong-tea-type GABA tea and black-tea-type GABA tea made by withering before and after anaerobic fermentation. The GABA contents of both tea samples were much higher in those with anaerobic fermentation occurring after withering than in those with fermentation before withering.

4.5.3 The Effect of Different Gas Treatments on the Content of GABA in Tea

Anaerobic fermentation is the key step of manufacturing GABA tea. However, it is important to know what kind of gas should replace oxygen. Tsushida et al.[1] have

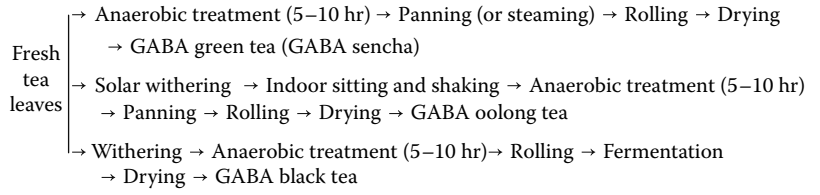


FIGURE 4.2 Basic procedures for manufacturing GABA tea with different degrees of fermentation.

TABLE 4.2
Contents of amino acids in oolong tea and black tea made from tea leaves incubated in nitrogen gas

GABA tea	Amino acid (mg/100 g)				
	Asp	Glu	Ala	Theanine	GABA
Oolong 1	85.7	47.1	63.3	491.1	135.9
Oolong 2	39.1	46.2	58.5	451.1	245.9
Black 1	95.4	176.1	31.4	411.2	81.2
Black 2	29.5	22.4	88.7	377.0	176.5

Source: Tsushida, T., Murai, T., Omori, M., and Okamoto, J. 1987. Production of a new type tea containing a high level of γ-aminobutyric acid. *Nippon Nogeikagaku Kaishi* 6:817–22. With permission.

Note: Oolong 1 and black 1 were made from the tea leaves incubated in nitrogen gas before withering, and oolong 2 and black 2 were made from those tea leaves after withering.

examined the amount of amino acids in GABA tea samples treated with different gases during anaerobic fermentation. The GABA content in GABA tea treated with CO_2 was higher than that in tea treated with N_2 (table 4.3), but N_2 is still recommended for use because the GABA tea treated with N_2 also contains a high amount of GABA and N_2 is cheaper and safer than CO_2.

4.5.4 The Effect of Different Sources of Fresh Tea Leaves on the Content of GABA in Tea

Glutamate is the precursor of GABA. Choosing fresh tea leaves high in glutamate can produce high GABA in GABA tea. Basically, any variety of fresh tea leaves can be used to manufacture GABA tea. Up to now, the highest GABA content of 663 mg% was found by using Assam tea variety.[87] Takeuchi et al.[94] reported that less mature fresh tea leaves produced a higher content of GABA in manufactured GABA tea. The high content of glutamate in less mature tea leaves causes higher GAD activity, and therefore the resultant tea has a higher GABA content.

Generally the content of amino acids, including glutamate in fresh tea leaves, is in the decreasing order of stem, bud, second leaf, third leaf, and old leaves. Therefore, the content of GABA in GABA tea made with tea stem has the highest, followed by tea bud, second leaf, third leaf, and old leaves. Sawai et al.[95] suggested that when manufacturing GABA tea, stems should also be used. In terms of production season, the GABA content of GABA tea made in the spring is the highest, followed by the summer and fall seasons.[1,96]

Previous reports concerning the effect of fresh tea leaves on the GABA content are mostly based on green tea. Taiwan is famous in oolong tea; therefore, recently tea farmers in Taiwan made GABA tea by combining anaerobic fermentation and the oolong tea process. It has been found that varieties such as TTES 12 (Taiwan Tea Experimental Station 12), TTES 13 (Taiwan Tea Experimental Station 13), CS Oolong (Chin-Shin Oolong), and SJChue (Shy-Jih Chue) are suitable for making a partial fermentation type of tea to make GABA tea. The amount of GABA in

TABLE 4.3
Contents of amino acids, caffeine, and tannin in tea leaves during incubation in various gases

Amino acid	Amino acids (mg%)							
	Air		Oxygen		Nitrogen		Carbon dioxide	
	5 hr	10 hr	5 hr	10 hr	5 hr	10 hr	5 hr	10 hr
Asp	149.6	176.7	113.6	237.6	11.7	2.0	3.7	8.6
Glu	133.6	236.3	140.4	148.0	4.8	8.0	4.3	5.7
Ala	14.6	40.1	13.7	44.2	165.1	123.1	67.8	58.6
Theanine	309.8	327.2	343.6	334.4	389.9	334.4	290.6	320.1
GABA	12.7	28.5	4.0	12.7	173.9	233.9	180.2	290.9
Caffeine	2.7	3.0	2.7	2.7	2.5	2.7	2.7	2.5
Tannin	14.3	15.0	13.9	14.0	14.5	14.5	15.0	14.2

Source: Tsushida, T., Murai, T., Omori, M., and Okamoto, J. 1987. Production of a new type tea containing a high level of γ-aminobutyric acid. *Nippon Nogeikagaku Kaishi* 6:817–22. With permission.

GABA tea made using these four varieties with an anaerobic fermentation–withering repeating process was greater than 150 mg%,[96] for partial fermentation type of tea also reaches the commercial level of GABA.

Comparing the difference in tea varieties, the GABA content in GABA tea made with TTES 12 was found to be the highest, followed by CS Oolong, SJChue, and TTES 13. The results also pointed out that the GABA tea made in spring had a higher GABA content than that made in summer because the content of amino acids in spring tea was higher than that in the summer tea. This is consistent with the results of Tsushida et al.[1] For production area, it was found that the GABA content of GABA tea made with fresh tea leaves at higher elevation levels (above 1,000 m) was on average 100 mg% more than that made with leaves at lower elevation levels (200–500 m). As learned from Japan, shading may enhance the increase of amino acids and nitrogen-containing matters and the decrease of catechins.[97] Ou et al.[96] also evaluated the effectiveness of shading on GABA accumulation. The results show that the GABA content in GABA tea made by tea leaves from tea bushes with 14-day shading was 20–80 mg% higher than that made from leaves without shading. This indicates that shading can increase the GABA content in GABA tea, but it may increase the cost of production.

4.5.5 The Effects of N_2 Treatment Temperature and Duration on the Content of GABA in Tea

GAD is the key enzyme for GABA formation from glutamate. The optimal activity temperature of 40°C and pH of 5.8 were reported by Streeter and Thompson in 1972.[82] In Japan, Takeuchi et al.[94] reported that higher N_2 treatment temperature could result in the increase of GABA content. The incubation temperatures of 4, 25, and 37°C did not affect the changes in the amino acids of GABA tea, except that the

increased amount of GABA at 4°C was less than that at 25 or 37°C. In the first 3 hr, it seemed that the amounts of GABA and alanine increased and those of glutamic and aspartic acids decreased. However, later on those of the amino acids remained almost constant. In fact, in the first hour of the anaerobic condition, those of the amino acids already started to change. Tsai et al.[98] reported that the GABA content in GABA tea treated with nitrogen at 20°C significantly increased after 4 hr, while only after 2 hr did the GABA content increase greatly if the leaves were treated with nitrogen at 30 and 40°C. The GABA content reached the highest amount at the 40°C treatment temperature (figure 4.3). Although most reports in the literature indicate that more than 90% of GABA in manufactured tea could be formed after 4 hr incubation at 40°C, and increased little after prolonging incubation time, it is recommended that 6 hr incubation at 40°C or 8 hr at room temperature be used in order to be sure the required GABA content is reached. However, for the green tea type of GABA tea it is recommended the time should not exceed 6 hr; otherwise, the appearance of the resultant GABA tea and the color of infusion are significantly deteriorated.[99]

4.5.6 The New Techniques for Increasing the Content of GABA in Tea

Since the higher the GABA content in GABA tea, the higher the price of the GABA tea, many new techniques for enhancement of GABA content in GABA tea have recently been developed.[81,87,88,94,95,100–110] Some of them are discussed below.

4.5.6.1 Anaerobic–Aerobic Fermentation

Sawai et al.[109] have pointed out that to take anaerobic and aerobic treatments in turn could increase the GABA content in tea significantly. During the first 1–3 hr of anaerobic treatment, GABA content significantly increased, and then the rate of increase gradually decreased. When the tea was treated aerobically, the GABA content decreased slightly but glutamate content increased significantly, even right after the first hour of aerobic treatment. Therefore, after 3 hr of anaerobic treatment, the

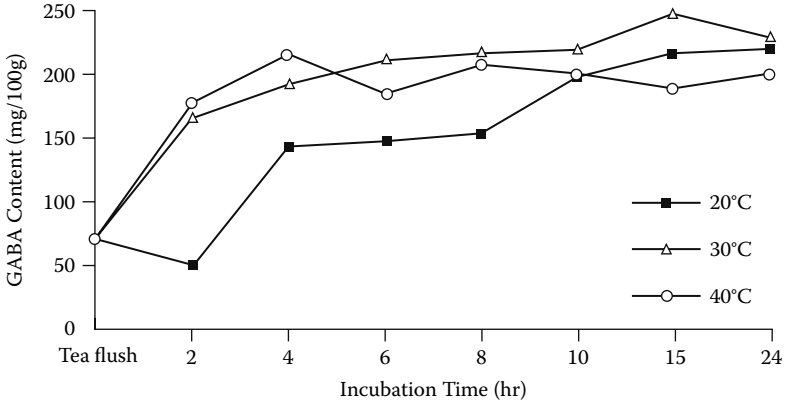


FIGURE 4.3 Effects of temperature and duration on changes of GABA in tea leaves under anaerobic conditions.

glutamate was transformed to GABA. By using this method, the GABA content in spring tea could be increased 1.5-fold. In addition, using three cycles of anaerobic treatment of 3 hr with 1 hr of aerobic treatment after each, followed by one more time of anaerobic treatment, the content of GABA in tea in the summer and fall seasons could increase 2.33-fold, compared to the tea with a single time of anaerobic treatment. However, Sawai et al. also reported that using three cycles of anaerobic-aerobic treatment could cause the color of tea leaves and infusion to become red as well as create an unpleasant odor. Therefore, they suggested that the treatment should be limited to three times of anaerobic treatment with two times of aerobic treatment.

Ou et al.[96] have reported that during GABA tea manufacture with three cycles of anaerobic and two cycles of aerobic treatment the total amino acids decreased from 1.84% in tea leaves at the beginning of anaerobic treatment to 1.14% in the GABA tea. The GABA content was increased from 23.56 to 127.28 mg/100 g after the first 8 hr of anaerobic treatment. After the following 3 hr of aerobic and 8 hr of anaerobic treatment, the GABA contents became 90.72 and 116.72 mg/100 g, respectively. The final content of GABA in the tea after three cycles of anaerobic and two cycles of aerobic treatment was 154.99 mg/100 g, which was above the standard amount in commercial GABA tea. Thus, anaerobic and aerobic treatment in turn for making GABA tea is a good way for oolong-type tea but not for green-type GABA tea.

4.5.6.2 Microwave Irradiation

Shiraki[107] investigated the accumulation of GABA in fresh tea leaves by means of microwave and infrared irradiation. It was found that the amount of GABA in the semifermented tea with fresh tea leaves irradiated both by microwave and infrared was higher than that in the ordinary green tea. Microwave irradiation caused the amount (167–202 mg/100 g) of GABA and alanine to increase, while the amount of aspartic and glutamic acids decreased in the resultant tea. There was a tendency for the amount of GABA and alanine to increase by microwave irradiation. In addition, the amount of theaflavins also increased at the levels of 3.57–11.8 mg/100 g, but the theanine did not differ from the amount in green tea. The study pointed out that the optimum generating power of microwave irradiation was 0.3 kw, and the optimum time for microwave irradiation was 20 min. The results indicate that it is possible to increase the amount of GABA in tea by microwave irradiation without using anaerobic treatment. Shiraki pointed out that the increase of amount of GABA was due to the rise of glutamate decarboxylase activity.

4.5.6.3 Infrared Irradiation

Shiraki[108] also examined the accumulation of GABA in fresh tea leaves by means of infrared ray irradiation with agitation. It was a positive result for increasing the amount of GABA by this method. The GABA contents of a first-picked crop of green leaves increased from 1.73 to 2.70 mg/g, and that of the second-picked crop increased from 0.39 to 1.44 mg/g. The content of theanine in this fermented tea was almost the same as that in green tea. However, that of aspartic acid and glutamic acid decreased. Shiraki (1998)[107] reported that the optimum time for infrared ray irradiation (6500 to ~ 2500 nm) with agitation was between 30 and 50 min.

4.5.6.4 Addition of Sodium Glutamate Solution

Nesumi[105] reported a method to increase the GABA content in manufactured tea by soaking fresh tea leaves in sodium glutamate solution combined with infrared irradiation. The GABA content could increase to 2.89 mg/g and 2.81 mg/g for spring and summer teas, respectively, after soaking in water for 3 hr and treating with infrared irradiation, as compared with the increases of 1.6 mg/g and 1.43 mg/g for spring and summer teas, respectively, after soaking in water for 3 hr but with no irradiation. Using sodium glutamate solution to replace water, the GABA content in GABA tea treated with sodium glutamate and infrared irradiation became 1.7- to 2.7-fold higher than that with sodium glutamate but without irradiation. This indicates that treatment with sodium glutamate solution did enhance the increase of GABA content in the manufactured GABA tea, especially together with infrared irradiation.

4.6 THE BIOLOGICAL FUNCTIONS OF GABA TEA

The most special characteristic of GABA tea is not having the original biological functions of tea, but having the functions of blood pressure moderation.[111] Although the catechins in tea have good benefits for reducing blood pressure, Hara et al. in 1987[112] demonstrated that the mechanism for reducing blood pressure by catechins was due to their inhibition of angiotensin I converting enzyme (ACE) activity.

For GABA tea, the biological function of blood pressure moderation was even more predominant due to the mutual enhancement of GABA and catechins combined.[113,114] Until now, research about the biological functions of GABA tea has concentrated more on its blood pressure moderation. However, several foods rich in GABA, such as germinated brown rice and GABA-enriched fermented milk product, have gained considerable attention due to their biological functions. Besides reducing blood pressure, many other functions were reported, such as reducing anxiety and stress,[115] aiding against onset of Alzheimer's,[116] helping with sleep and mood problems,[116] increasing growth hormone,[59,117,118] helping aging persons feel younger, and relieving postmenopausal depression.[88,101,119,120] In addition, new techniques using microbial fermentation to produce a high content of GABA in foods have been developed.[90,115,116,121,122]

Recently, the effect of the GABA-enriched fermented milk product (FMG) on the blood pressure of spontaneously hypertensive and normotensive Vistar-Kyoto rats was studied.[123] During the chronic administration of experimental diets to SHR/Izm, a significantly slower increase in blood pressure with respect to the control group was observed at 1 or 2 weeks after the start of feeding with the GABA or FMG diet, respectively ($p < 0.005$). This difference was maintained throughout the feeding period. The time profile of blood pressure change due to administration of FMG was similar to that of GABA. FMG did not inhibit ACE. Furthermore, an FMG peptide-containing fraction from reverse-phase chromatography lacked a hypotensive effect in SHR/Izm rats. The results suggest that low-dose GABA has a hypotensive effect in SHR and that the hypotensive effect of FMG is due to GABA.

Another recent report also indicates the effects of germinated brown rice extracts with enhanced levels of GABA on cancer cell proliferation and apoptosis.

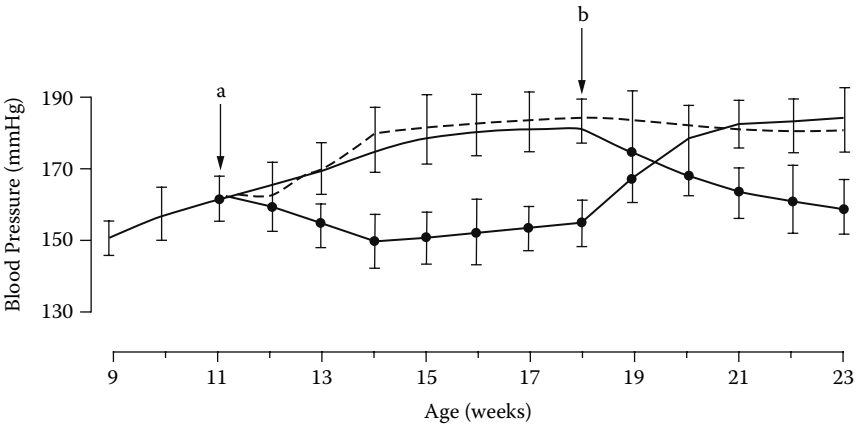
The glutamic acid–germinated brown rice extract (Gex) and chitosan/glutamic acid–germinated brown rice extract (CGex) significantly retarded the proliferation rates of L1210 and Molt4 cells, and the highest retardation rate was with CGex. In addition, the Gex and CGex enhanced significantly the apoptosis of cultured L1210 cells, but no significant apoptosis was seen with the other extracts, which have lower concentrations of GABA than Gex and CGex. The results show that brown rice extracts with enhanced levels of GABA have an inhibitory action on leukemia cell proliferation and have a stimulatory action on the cancer cell apoptosis.[124]

Omori and colleagues[7] were the first group working on the effect of GABA tea on reducing the blood pressure of animals and humans. In 1987, they used 24 spontaneously hypertensive rats (SHR) divided into three groups to feed GABA tea, green tea, and deionized water, respectively. After feeding 2 weeks, the blood pressure of the GABA group SHR was significantly reduced and their urine contained a lot of sodium. Omori implied that drinking GABA tea helped the exclusion of sodium from the body and resulted in reducing blood pressure. Besides, the lack of significant difference in body weight among these SHR in all groups, the blood pressure of SHR fed with GABA tea was 25–30 mmHg lower than that of the other groups of SHR. However, after feeding GABA tea for 9 weeks, once feeding GABA tea was replaced with deionized water, the blood pressure of SHR increased again to a hypertensive level. On the other hand, those SHR fed originally with deionized water for 9 weeks and then changed to GABA tea had significantly reduced blood pressure (figure 4.4).

This experiment clearly indicates that GABA tea indeed has the function of reducing blood pressure. Furthermore, the authors suggested that daily drinking of GABA tea has to be continued in order to lower the blood pressure; otherwise, the blood pressure can increase again once drinking GABA tea stops, just like taking hypertension medicine. The only difference is that GABA tea is a natural product with no side effects. In addition, drinking GABA tea also offers the pleasure of drinking tea.

For further understanding the effect of GABA tea on humans, 13 spontaneously hypertensive patients diagnosed in the hospital were selected for clinical studies. One 3-g bag of GABA tea was provided to each patient per day for 3 months after discontinuing hypertension medicine. It was found that the original blood pressures of 180 and 184 mmHg were down to 165 and 140 mmHg, respectively.[113] In addition, drinking GABA tea had no unpleasant feeling or side effect at all. Patients also described a feeling of comfort and being less tired after drinking GABA tea. Another test using 15 hypertension patients that were not diagnosed by the hospital, also drinking GABA tea for 3 months, resulted in significant blood pressure reduction in 8 of the 15 participants. They all felt relaxed and well. Therefore, Omori et al.[7] suggested that hypertension patients drinking a 3.5-g bag of GABA tea every day for 2 months would reduce their blood pressure.

In 1995, Abe et al.[125] investigated the effects of GABA tea on the blood pressure of 21 Dahl salt-sensitive rats with different ages, 11 months (old) and 5 weeks (young). After feeding a 4% NaCl diet for 3 weeks, 21 old rats were given water (group W), an ordinary tea solution (group T), or a GABA-rich tea solution (group G) for 4 weeks. The average GABA intake was 4.0 mg/rat/day. After 4 weeks of treatment, blood pressure significantly decreased in group G (176 ± 4; $p < 0.01$),



a: SHR are divided into three groups.
b: Feeding stuff was exchanged between the control group (fed on ordinary green tea) and the experimental group.
– – – Control SHR fed by water
—— SHR fed by ordinary green tea
—●— SHR fed by Gabaron tea.
Mean of 8 determinations ± S.D.

FIGURE 4.4 The effect of GABA tea extract on systolic blood pressure of SHR. (From Omori, M., Yano, T., Okamoto, J., Tsushida, T., Murai, T., and Higuchi, M. 1987. Effect of anaerobically treated tea (Gabaron tea) on blood pressure of spontaneously hypertensive rats. *Nippon Nogeikagaku Kaishi* 61:1449–51. With permission.)

compared with group W (207 ± 9) or group T (193 ± 5 mmHg). Plasma GABA levels were more elevated in group G (111 ± 54) than in group W (not detectable) or group T (14 ± 8 ng/ml). For those young rats, the average GABA intake was 1.8 mg/rat/day. The results show that the body weight or chow and beverage consumption did not differ significantly among the three groups. After 4 weeks of treatment, although blood pressure was comparable in groups W and T (165 ± 3 vs. 164 ± 5 mmHg), it was significantly lower in group G (142 ± 3 mmHg) than in the other groups. Plasma aldosterone concentration increased in group G compared to the other groups. Thus, GABA-rich tea seems not only to decrease high blood pressure but also to prevent the development of hypertension in Dahl S rats fed by a high-salt diet. These results indicated that due to the tea drinking habit by people in many countries, the administration of GABA-rich tea might become one of the supportive methods to decrease blood pressure in hypertensive individuals.

In order to understand further the reducing blood pressure function of GABA tea and its effects on kidney metabolism, Lin et al.[26] carried out another experiment. Using three different rats, spontaneously hypertensive (SHR), stroke prone (SHRSP), and Dahl S rats, as testing animals, the blood pressure, kidney functions, and biotests of serum and kidney of these three types of rats were evaluated after feeding a 5% NaCl diet with and without GABA tea. The results showed that for rats fed with GABA tea, blood pressure was reduced significantly (table 4.4). In addition,

TABLE 4.4
Effect of green and Gabaron teas on blood pressure level in SHR and Dahl S rats dosed with saline

		Blood pressure (mmHg)			
		Control rats, basal diet (CE-2)	CE-2 containing 5% saline	CE-2 containing 5% saline with green tea	CE-2 containing 5% saline with Gabaron tea
SHR	Initial (9 weeks old)	167.3 ± 6.4	167.8 ± 7.7	167.4 ± 5.1	168.6 ± 5.4
	4 weeks later	201.9 ± 13.6	240.9 ± 24.2	219.1 ± 10.6	217.1 ± 18.7
	Difference in blood pressure (mmHg/28 days)	34.6 ± 10.0	73.1 ± 16.0	51.7 ± 7.9	48.5 ± 6.7
Dahl S*	Initial (9 weeks old)	153.8 ± 11.8	153.7 ± 8.3	153.7 ± 7.4	153.8 ± 7.6
	6 weeks later	165.4 ± 3.5	191.4 ± 16.0	177.8 ± 3.1	176.8 ± 6.7
	Difference in blood pressure (mmHg/42 days)	11.6 ± 7.7	37.7 ± 7.2	24.1 ± 5.3	23.0 ± 7.2

Source: Lin, Z., Saito, H., Omori, M., Inomata, T., Kato, M., Sawai, Y., Fukatu, S., and Hakamata, K. 2000. Effect of Gabaron tea on the blood pressure and kidney function of rats loaded with saline. *Nippon Kaseigaku Kaishi* 51:265–71. With permission.

Note: Each value is presented as the mean ± SD for six male SHR and seven male Dahl S. $*p < 0.05$ compared with the CE-2 diet containing 5% saline.

SHRSP rats without the GABA tea feeding all died after 14 weeks due to stroke or kidney dysfunction, while those fed with GABA tea were all alive.

The blood pressure level in a group of spontaneously hypertensive rats rose from 168 to about 202 mmHg in the control group, and up to 241 mmHg in the group given saline during a feeding period of 4 weeks. The rise in blood pressure due to additional saline was significantly suppressed to 219 mmHg in the group given green tea, and to 217 mmHg in the group given Gabaron tea. Photomicrographs of kidney tissue showed a swollen and hyalinized renal glomerulus when Dahl S rats were given saline. However, this pathological finding was less notable in the Dahl S rat species when green or Gabaron tea was administered with saline.

In 1992, Chen[126] examined the antihypertensive effect of tea with or without GABA. Oral administration of the aqueous extract of green tea coupled with GABA (GABA/tea) into spontaneously hypertensive rats lowered the blood pressure in a dose-dependent manner. However, similar treatment with green tea without GABA failed to produce any antihypertensive effect. Direct application of GABA, by oral administration, intravenous injection, or both, resulted in a similar hypotensive activity at the concentrations present in tea. Thus, the antihypertensive action produced by GABA/tea is mainly through the action of GABA. The obtained results suggest that the stable bioactive substance can be coupled with food to become a helpful product in human health.

Huang et al.[127] carried out a clinical study in Hunan Province Elderly Hospital for the effect of drinking GABA tea on blood pressure reduction. There were 50 patients total divided into two groups; one was given two 5-g bags of GABA tea each per day (GABA tea group) and the other was given two 5-g bags of green tea each per day (control). After 20 days, the GABA tea group showed a significantly greater blood pressure reduction than the control group with no side effects or adverse effects. Patients all expressed that they felt well and headaches no longer existed, besides the blood pressure reduction.

What is the mechanism for GABA to have the effect of blood pressure reduction on animals and humans? Since ACE activity is highly related to the occurrence of hypertension, inhibiting the ACE activity should reduce the blood pressure. Therefore, Lin and Omori[128] investigated the mechanism for the function of Gabaron tea on hypertension. The results *in vitro* showed that GABA, alanine, theanine, and γ-hydroxybutyric acid had a remarkable inhibiting effect on ACE activity. Among them, GABA showed the best inhibiting ability (table 4.5). Kobayashi et al. in 1996[129] also demonstrated that theanine had a hypotensive effect, so that the reason for GABA tea having such a good hypotensive effect is highly related to the significant amounts of GABA, alanine, theanine, and catechins.

Results, *in vivo*, showed that the rise of ACE activity in the SHR due to additional saline was significantly inhibited by 3% addition of GABA. The results suggested that the inhibition of ACE activity by GABA is one of the major action mechanisms of Gabaron tea. Besides investigating the functional mechanism of Gabaron tea on hypertension, Lin and Omori also investigated the absorption and metabolism of GABA in rats by ^{14}C autoradiography and chemical quantitative analysis. The results showed that after administration with 1 mg/g body weight GABA to the 6- to 7-week-old Wistar rats, GABA in the liver increased to the highest level after 1 hr, and GABA in the plasma and kidney reached the highest level after 3 hr, while GABA in the brain almost remained at the same level as γ-hydroxybutyric acid, which was one of the major metabolic products of GABA in rats.

In 2005, Ou et al.[96] evaluated the effectiveness of the hypertension moderation and bioactive functions of Taiwan GABA tea by component analysis and animal tests. The bioactive components of GABA tea and green tea produced in Taiwan were

TABLE 4.5
Inhibition of ACE activity by the main components of Gabaron tea *in vitro*

Components	Concentration (mM)	ACE activity (%)	Inhibition (%)
GABA	40	10.53	89.47
Alanine	40	23.08	76.92
Theanine	40	34	64.00
GHB[a]	40	42.51	57.49

Source: Lin, Z. and Omori, M. 2001. Effects of Gabaron tea components on angiotensin I-converting enzyme activity in rat. In *Proceedings of the 2001 International Conference on O-CHA(tea) Culture and Science*, Shizuoka, Japan, 81–84. With permission.

[a] GHB = γ-Hydroxybutyric acid.

investigated for comparison. Using 28 green teas and 28 GABA teas as material, moisture content, Hunter *L*, *a*, and *b* values, phenolic compounds, and amino acids, including GABA, fatty acids, and ascorbic acid, were determined. The results showed that moisture, total free amino acids, crude fat, Hunter *L* value, total nitrogen, free fatty acids, and reducing sugar did not differ significantly between the two. The major difference was that GABA tea had higher GABA, alanine, ammonia, lysine, leucine, isoleucine, and Hunter *a* and *b* values, while green tea had higher glutamic acid, aspartic acid, phenylalanine, total catechins, and ascorbic acid content ($p < 0.05$).[130]

For the animal test, high-dose GABA (300 mg/100 g) and low-dose GABA (150 mg/100 g) of Taiwan GABA teas together with green tea were made by Chin-Shin Oolong as experimental samples and fed to the spontaneously hypertensive rats (SHR) and compared with the high and low doses of pure GABA solution and water as control. The blood pressure, body weight, serum GABA, and serum lipids (including total cholesterol, triacylglycerol, and high-density lipoprotein (HDL) and low-density lipoprotein (LDL) cholesterols of SHR were measured periodically. The results showed that after 12 weeks of feeding, the blood pressure of SHR fed with either high-dose or low-dose GABA tea solution was reduced significantly, and the effectiveness was greater than those with pure GABA and green tea solutions (table 4.6).

From the results for the effect of feeding GABA tea on the serum triacylglycerol, cholesterol, LDL-C, HDL-C, GABA, and tetrabutylammonium hydrogen sulfate (TBAS) of SHR (table 4.7), the effect of GABA tea solution on reducing serum lipids was the same as that of green tea. As the blood pressure decreased, serum GABA of SHR increased. However, there was no significant difference in serum GABA of SHR fed with a high or low dose of GABA for both pure GABA and GABA tea. In terms of triacylglycerol, cholesterol, LDL-C, and HDL-C, the reading for the control (drinking water) had no significant change, while for all other groups the readings significantly decreased. Thus, these studies indicated that GABA tea extract indeed has good biological functions, especially reduction of blood pressure and serum lipids for SHR.

Furthermore, when extending the feeding period up to 82 weeks (age of 95 weeks), only SHR fed with either high-dose or low-dose GABA tea infusion were all alive (figure 4.5). The number of survivors in the control, green tea, and low-dosage pure GABA solution-treated groups decreased to 50, 57.14, and 66.7%, respectively. Clearly, the survival rate of the GABA tea-treated group at 100% contrasted strongly with those of the other groups. Consequently, the largest survival benefit was conferred by the GABA tea-treated group, with the rest in the following order: pure GABA solution > green tea > control group.

4.7 CONCLUSIONS

GABA is important for health, and its content decreases as we get older. In order to avoid disease, we need to consume GABA. It has been found that fermented soybean, mulberry leaf, turtle, germinated unpolished rice, yogurt, and GABA tea are rich in GABA. Among them, naturally produced GABA tea is the richest source of GABA. In addition, GABA tea is readily available, and drinking GABA tea has no side effects.

TABLE 4.6
Blood pressure and heart rate in SHR with different treatments

	Systolic pressure (mmHg)[2]				Diastolic pressure (mmHg)[2]				Mean pressure (mmHg)[2]				Heart beat (beat/min)[2]			
	Before		After		Before		After		Before		After		Before		After	
Control[1]	238.00^{a*}	±23.78	252.40^{a}	±9.40	170.20^{a*}	±8.35	195.20^{a}	±7.79	198.60^{a*}	±20.80	216.80^{a}	±6.18	604.17^{a}	±56.36	615.20^{a}	±26.20
GT	240.33^{a*}	±16.56	224.50^{b}	±12.14	170.67^{a}	±30.96	167.67^{b}	±6.65	197.50^{a*}	±21.35	187.33^{b}	±21.93	603.17^{a}	±58.50	599.17^{a}	±9.49
LGW	240.17^{a*}	±19.54	219.00^{b}	±8.51	172.00^{a}	±31.64	163.33^{b}	±19.37	202.33^{a*}	±18.16	179.17^{bc}	±8.91	601.33^{a}	±26.85	592.50^{a}	±15.86
HGW	238.50^{a*}	±10.21	219.00^{b}	±6.90	169.00^{a}	±19.32	165.83^{b}	±21.25	198.17^{a*}	±12.45	163.50^{cd}	±18.66	613.83^{a}	±27.89	583.33^{a}	±33.50
LGT	241.33^{a*}	±15.00	213.00^{b}	±25.58	169.50^{a}	±34.03	166.17^{b}	±17.24	202.17^{a*}	±12.40	161.67^{cd}	±13.81	604.00^{a}	±50.78	584.00^{a}	±47.56
HGT	236.71^{a*}	±21.81	207.14^{b}	±21.54	169.71^{a}	±25.99	163.57^{b}	±24.34	196.86^{a*}	±16.97	158.71^{d}	±10.24	606.00^{a}	±35.54	582.29^{a}	±30.19

[1] Control, water; GT, green tea; LGW, low dose of pure GABA solution; HGW, high dose of pure GABA solution; HGT, high dose of GABA tea; LGT, low dose of GABA tea.

[2] The means in each column followed by the same letter are not significantly different at the 5% level. The means in the row between "before" and "after" of the feeding test followed by an asterisk are significantly different at the 5% level.

TABLE 4.7

Concentrations of serum GABA, lipids, and TBAS of six SHR groups before and after feeding test

Variable[1]	Triglyceride (mg/dl)[2]				Cholesterol (mg/dl)[2]				LDL-C (mg/dl)[2]				HDL-C (mg/dl)[2]				GABA (ng/ml serum)[2]				TBAS (ng/ml)[2]			
	Before		After		Before		After		Before		After		Before		After		Before		After		Before		After	
Control	48.54^{a*}	±2.34	41.52^{a}	±1.70	85.98^{a}	±1.25	82.09^{a*}	±0.65	27.99^{a}	±1.88	27.01^{a}	±1.92	48.28^{a*}	±2.52	46.77^{a}	±2.56	17.34^{a*}	±1.87	13.85^{b}	±0.88	5.51^{a}	±1.33	5.90^{a}	±0.54
GT	48.13^{a*}	±5.25	32.77^{b}	±1.19	83.18^{a*}	±0.82	47.41^{b*}	±0.90	27.35^{a}	±1.60	23.05^{ab}	±4.01	46.30^{a*}	±0.96	17.81^{b}	±4.42	17.37^{a*}	±3.46	14.55^{b}	±1.61	5.56^{a}	±1.10	5.56^{a}	±1.12
LGW	48.91^{a*}	±3.96	33.05^{b}	±2.50	85.68^{a*}	±1.70	47.54^{b*}	±1.17	27.47^{a}	±4.62	21.86^{b}	±3.09	48.43^{a*}	±5.37	19.07^{b}	±3.84	17.30^{a*}	±4.13	21.00^{a}	±1.49	6.30^{a}	±2.10	5.56^{a}	±0.78
HGW	48.48^{a*}	±2.52	32.84^{b}	±2.30	83.29^{a*}	±0.92	47.70^{b*}	±0.94	26.18^{a}	±2.04	20.29^{b}	±3.18	47.42^{a*}	±2.16	20.85^{b}	±3.42	17.38^{a*}	±0.74	21.04^{a}	±1.03	4.17^{a}	±1.20	5.66a	±0.94
LGT	48.62^{a*}	±2.59	32.14^{b}	±1.77	84.50^{a*}	±0.92	47.93^{b*}	±1.14	27.45^{a}	±1.48	22.06^{b}	±4.56	47.32^{a*}	±1.86	19.44^{b}	±4.17	17.35^{a*}	±2.36	20.04^{a}	±2.09	6.09^{a}	±0.62	5.77a	±0.40
HGT	48.08^{a*}	±3.30	32.08^{b}	±1.63	85.30^{a*}	±1.36	44.95^{c*}	±1.37	27.47^{a}	±2.96	20.89^{b}	±3.06	48.21^{a*}	±3.46	17.64^{b}	±2.99	17.32^{a*}	±2.90	21.06^{a}	±1.37	6.04^{a}	±0.52	5.86a	±0.94

[1] Same as in table 4.6.

[2] The means in each column followed by the same letter are not significantly different at the 5% level. The means in the row between "before" and "after" of the feeding test followed by an asterisk are significantly different at the 5% level.

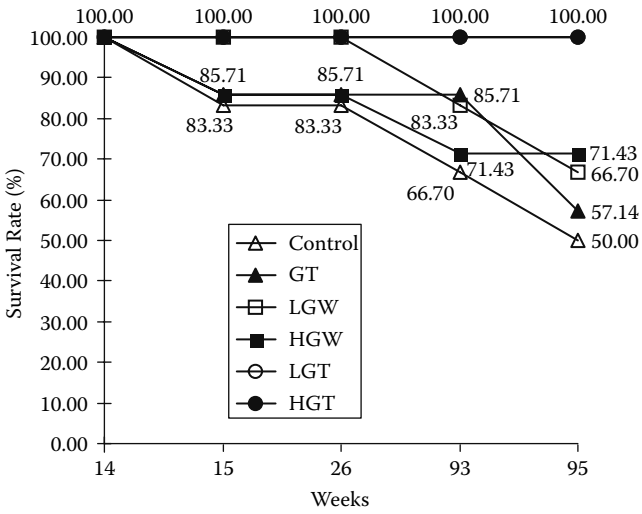


FIGURE 4.5 Survival rate in SHR fed with (Δ) control (distilled water); (▲) green tea (GT; GABA 24.1 μg/day); (□) low-dosage pure GABA solution (LGW; GABA 151 μg/day); (■) high-dosage pure GABA solution (HGW; GABA 301 μg/day); (○) low-dose GABA tea infusion (LGT; GABA 151 μg/day); (●) high-dose GABA tea infusion (HGT; GABA 301 μg/day). The first 12 weeks used normal diets ($n = 4 - 7$).

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5 Production of Theaflavins, Theasinensins, and Related Polyphenols during Tea Fermentation

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CONTENTS

5.1 INTRODUCTION

Tea is made by processing the leaves of the tea tree *Camellia sinensis*, which originated in the southern areas of Yunnan province in China, and is now spread throughout the world. This plant has been used as a medicine for 5,000 years,[1] and was probably first selected because its leaves contain a high concentration of caffeine. Caffeine is a central nervous system stimulant, and other caffeine-containing plant products, such as coffee beans, cacao beans, guarana berries, kola nuts, and mate leaves, have been selected and used to make beverages by people on various continents.

It is well known that tea contains catechins besides caffeine. Although catechins are widely distributed in the plant kingdom, the catechin monomers in many plants coexist with larger amounts of dimeric to polymeric proanthocyanidins, which are comprised of catechin units connected by C–C bonds.[2] However, the polyphenol composition of *C. sinensis* is unique, and mainly comprised of four monomeric catechins—(–)-epicatechin (**1**), (–)-epigallocatechin (**2**) and their galloyl esters,

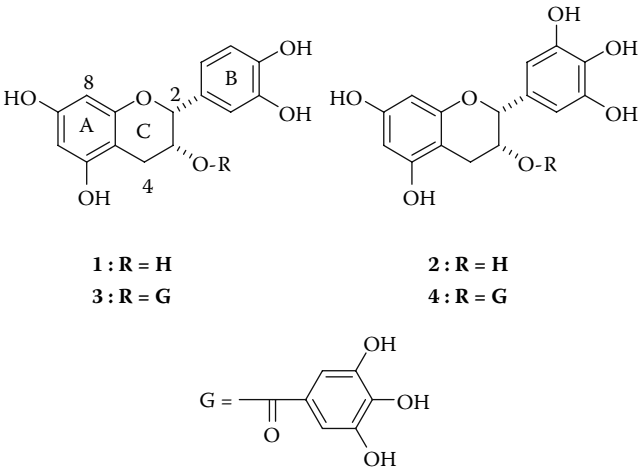


FIGURE 5.1 Structures of major tea catechins.

(–)-epicatechin gallate (**3**) and (–)-epigallocatechin gallate (**4**) (figure 5.1)—and the concentration of proanthocyanidins is much lower than that of the monomers.[3,4] In addition, an abundance of the catechins with pyrogallol-type B-rings and their galloyl esters is also characteristic of tea leaves.[5,6] These catechins are responsible for the characteristic bitterness and astringent taste of green tea.

Tea is usually classified into several types according to different processing methods, typically green tea (nonfermented tea), pu-erh tea (produced by microbial fermentation of green tea), oolong tea (partially fermented tea), and black tea (fully fermented tea).[7] Among these, black tea accounts for almost 80% of the world tea production.[8] During the process of black tea production, moderately withered fresh leaves are crushed to destroy the leaf tissue, and consequently the constituents, including polyphenols and various enzymes, are mixed together. This process is called fermentation, but differs from real fermentation since no microorganisms participate in the process. At this stage of the processing, the tea leaf polyphenols are oxidized to many characteristic black tea polyphenols, and subsequent heating and drying processes inactivate the enzymes and terminate the oxidation reaction. On the other hand, in the process of green tea production, tea leaves are steamed immediately after harvesting and the enzymes are inactivated at the initial stage. Therefore, the composition of green tea polyphenols is simple and similar to that in the fresh tea leaves. Recently, many scientific reports have suggested various health benefits of green tea.[9,10] The presence of a receptor for (–)-epigallocatechin gallate that mediates anticancer activity has also been suggested.[11] Although Japanese and Chinese people prefer green tea, most people in other areas of the world drink black tea. Some epidemiological studies have revealed that black tea is also good for human health,[12–15] although the composition of black tea polyphenols is so complex that further biochemical investigations at the molecular level are difficult to perform. Despite many efforts to understand black tea polyphenols, the majority of the phenolic constituents of black tea remain ambiguous.[16] Hence, black tea is still an attractive target for further research in food chemistry and phytochemistry. During the course

of our research on the chemistry of plant polyphenols, we extended efforts to shed light on black tea polyphenols by elucidating the chemical mechanisms of enzymatic catechin oxidation.

5.2 MODEL FERMENTATION EXPERIMENTS

Since the 1950s, many efforts have been made to isolate polyphenols directly from black tea, and the structures of the major phenolic compounds characteristic of fermented tea have been elucidated.[4,17–20] However, the composition of black tea polyphenols is so complex that the minor phenolic substances, which cumulatively account for a substantial portion of black tea polyphenols, are difficult to separate even by high-performance liquid chromatography (HPLC). This difficulty associated with the purification is mainly due to the presence of uncharacterized substances that are detected as a broad hump on the baseline during HPLC analysis but do not produce any clear spots on thin-layer chromatography. These substances are probably a complex mixture of catechin oxidation products with higher molecular sizes, and usually account for the majority of black tea polyphenols.

Due to the difficulty associated with separating black tea polyphenols, various model fermentation experiments have been devised for understanding the formation of black tea polyphenols.[21–26] We believe that revealing the chemical structures of minor catechin oxidation products one by one is one of the most important and effective approaches, and our experiments are therefore devised in order to obtain sufficient oxidation products to determine their structures by chemical and spectroscopic methods. In our model fermentation experiments, a tea leaf homogenate, from which the polyphenols had been removed in advance by adsorption on polyvinylpolypyrrolidone (Polyclar AT®),[27] or homogenate of various plants, such as Japanese pear and banana fruits, was used as the enzyme source.[28–30] Our preliminary experiments suggested that oxidation with Japanese pear or banana fruit homogenates gave oxidation products essentially similar to those in tea leaf homogenate, as evaluated by comparison of the major products.[27,30] An advantage of the experiments using fruit homogenates is that large-scale experiments can easily be performed by relatively simple procedures. In addition, practically no oxidation products originating from the fruit homogenates themselves were detected by HPLC analysis.[30] A peroxidase–hydrogen peroxide system was also used for this purpose, and some interesting oxidation products were obtained.[31,32] Our results suggested that oxidation enzymes mainly catalyzed the initial oxidation of *o*-diphenols to *o*-quinones, and that the subsequent complex reactions of the resulting quinones proceeded nonenzymatically. Therefore, the products of experiments using polyphenoloxidase and peroxidase from various plants yielded similar oxidation products. However, we sometimes recognized differences between our model experiments and actual tea leaf fermentation, especially in the formation of minor products and polymeric substances, and these differences remain to be clarified.

5.3 PRODUCTION AND OXIDATION OF THEAFLAVINS

Theaflavins (theaflavin and its galloyl esters) are the most important reddish orange pigments of black tea and contain a unique 1',2'-dihydroxy-3,4-benzotropolone moiety.[33] These pigments are formed by oxidative coupling between catechol-type catechins ((–)-epicatechin and (–)-epicatechin gallate) and pyrogallol-type catechins ((–)-epigallocatechin and (–)-epigallocatechin gallate). We examined the production of theaflavin (**5**) from a mixture of purified (–)-epicatechin and (–)-epigallocatechin by model fermentation mainly using a banana fruit homogenate (figure 5.2).[30] When (–)-epicatechin and (–)-epigallocatechin were treated separately with the banana homogenate, the rate of decrease of (–)-epicatechin was faster than that of (–)-epigallocatechin (data not shown). However, when a mixture of (–)-epicatechin and (–)-epigallocatechin was treated in the same manner, (–)-epigallocatechin decreased more rapidly than (–)-epicatechin. Our results were consistent with the coupled oxidation mechanism for theaflavin formation (figure 5.3), in which the plant enzyme preferentially oxidized the B-ring of **1** to its corresponding quinone **1a**, and in turn, the quinone **1a** oxidized the B-ring of (–)-epigallocatechin to quinone **2a**.[25] In this mechanism, (–)-epicatechin appeared to be oxidized very slowly compared to (–)-epigallocatechin, since (–)-epicatechin was regenerated by reduction of **1a**. This rapid oxidation-reduction turnover was chemically proved by a similar experiment in the presence of glutathione, in which (–)-epicatechin was completely converted to glutathione adducts of **1a**, whereas about 70% of (–)-epigallocatechin remained unchanged.[30] This coupled oxidation mechanism can be explained by the higher enzyme specificity for (–)-epicatechin and lower oxidation-reduction potential of (–)-epigallocatechin.[34]

A theaflavin-related benzotropolone pigment having a procyanidin unit was synthesized *in vitro*. Although proanthocyanidin dimers have two B-rings, the condensation occurred regioselectively at the extension (upper) unit of the procyanidin due to steric effect.[35]

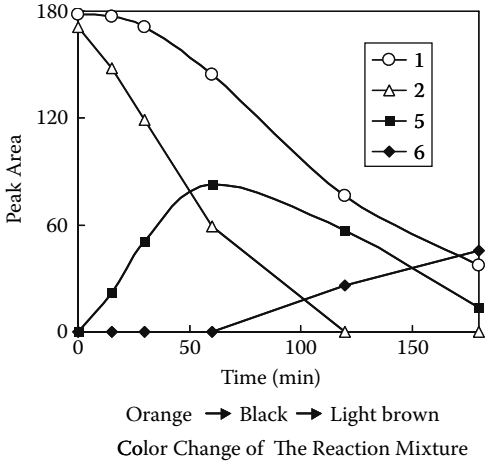


FIGURE 5.2 Time course of catechin oxidation with banana homogenate.

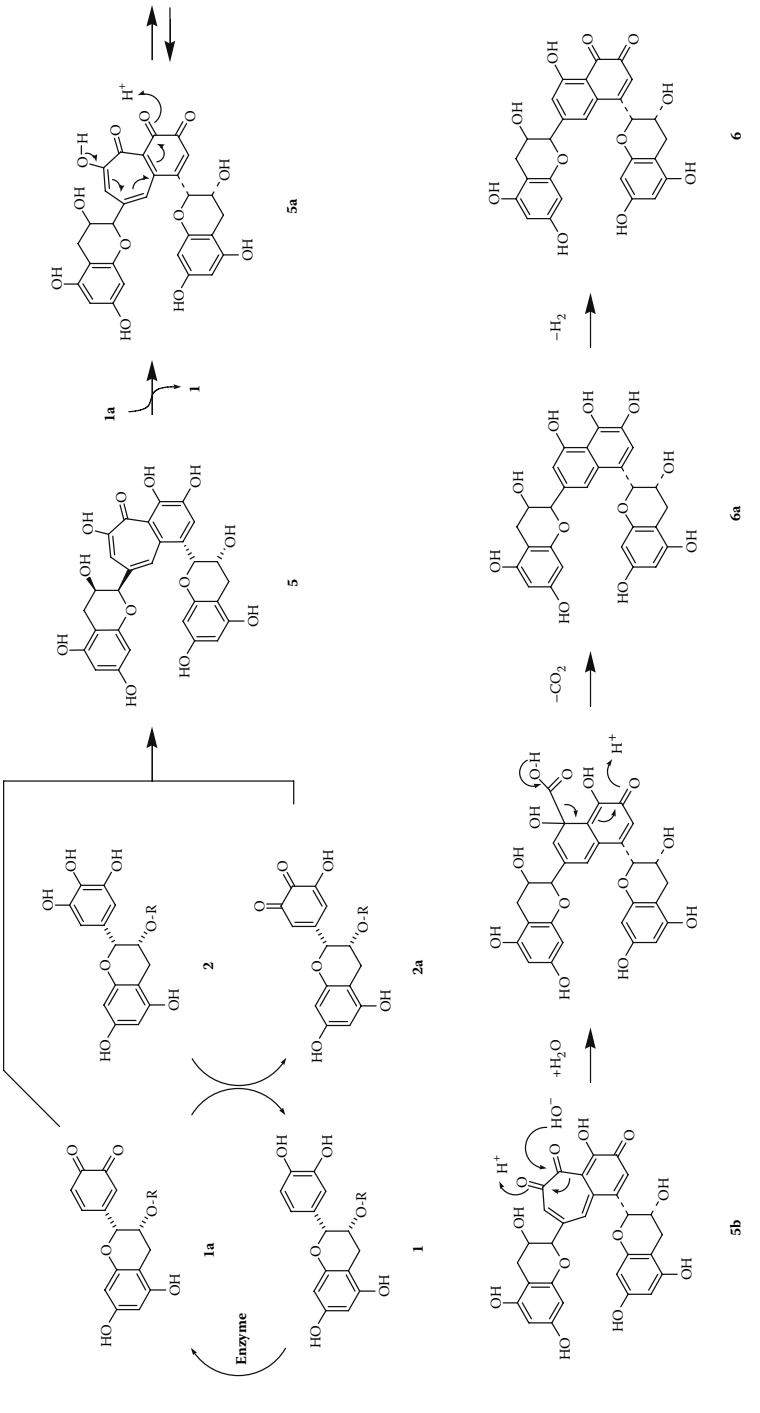


FIGURE 5.3 Production of theaflavin (**5**) and theanaphthoquinone (**6**).

In a series of experiments, an oxidation product of theaflavin (**5**), designated theanaphthoquinone (**6**) (figure 5.3), was obtained.[29] The structure of this yellow pigment was established by two-dimensional nuclear magnetic resonance (NMR) spectroscopic analysis and chemical derivatization, and the oxidation mechanism of the formation of theanaphthoquinone from theaflavin was deduced (figure 5.3). In this mechanism, epicatechin quinone (**1a**) was responsible for the initial oxidation of the benzotropolone ring of theaflavin.[36,37] During oxidation of a mixture of (–)-epicatechin and (–)-epigallocatechin with a banana fruit homogenate, the color of the mixture first becomes reddish orange as the theaflavin concentration increased, and then dramatically changed to greenish black when (–)-epicatechin was exhausted and theaflavin began to be oxidized.[30] A similar color change was observed immediately after the addition of a banana fruit homogenate to a mixture of (–)-epicatechin and theaflavin and was probably caused by the formation of a quinhydrone-type π-π complex between theaflavin and **1a**.[38] The black color gradually changed to light brown as theaflavin was consumed. The oxidation of theaflavins with galloyl esters at the C-3 or C3' hydroxyl groups under similar conditions proceeded differently. Specifically, the color change from reddish orange to greenish black was not observed, and oxidation products corresponding to galloyl esters of **6** were not detected. These findings probably arose because the benzotropolone ring was covered with galloyl groups, thereby interfering with the complexation with **1a**.

In addition to theanaphthoquinone, two new oxidation products of theaflavin, bistheaflavin A (**7**)[39] and dehydrotheaflavin (**8**),[27] were isolated in similar model fermentation experiments using banana fruit and tea leaf homogenates, respectively. On the other hand, auto-oxidation of theaflavin in a phosphate buffer at pH 7.3 yielded a yellow pigment, bistheaflavin B (**9**), along with **6** (figure 5.4).[39] The dimer **9** is deduced to be formed by coupling of the reduced form (**6a**) of theanaphthoquinone with theaflavin-quinone (**5b**). The results demonstrated that theanaphthoquinone was also formed nonenzymatically from theaflavin.

5.4 OXIDATION OF CATECHOL-TYPE CATECHINS

In tea leaves, cross-condensation between catechol- and pyrogallol-type catechins produced theaflavins by the coupled oxidation system as described above. When only catechol-type catechins, (+)-catechin or (–)-epicatechin, are oxidized by banana fruit homogenates or peroxidase–hydrogen peroxide, the initial B-ring *o*-quinone oxidation product acted as an electrophile and reacted with the phloroglucinol A-ring or catechol B-ring of another molecule.[26,30] The resulting dimeric products, typically represented by dehydrodicatechin (**10**),[40] were capable of being substrates for further reactions, yielding oligomeric and polymeric products, such as the trimer **11** (figure 5.5).[30] However, these products were not isolated from fermented tea leaves, since the concentrations of the catechol-type catechins are relatively low. In contrast, the contents of pyrogallol-type catechins, (–)-epigallocatechin and (–)-epigallocatechin gallate, accounted for over 70% of the total tea catechins.[5,6] In addition, pyrogallol-type B-rings were oxidized more easily than catechol-type catechins due to their low oxidation-reduction potential.[41] Thus, oxidation of the pyrogallol-type catechins is important for the production of black tea polyphenols.

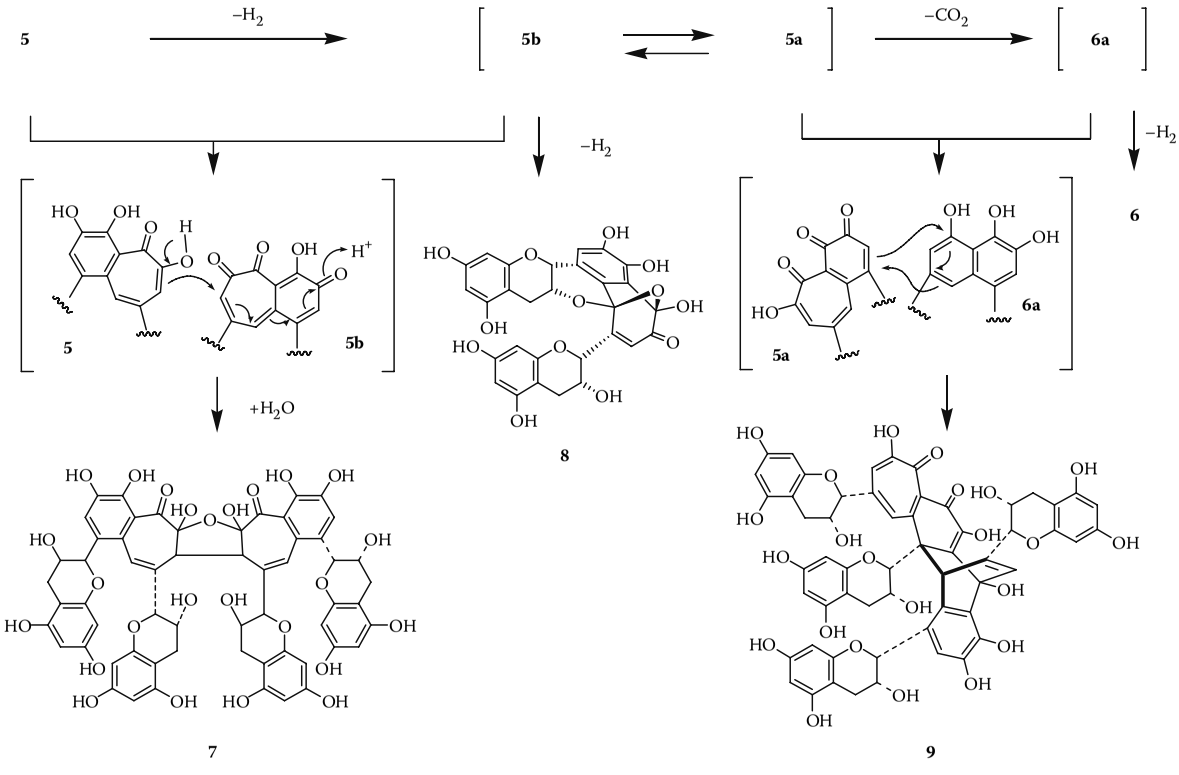
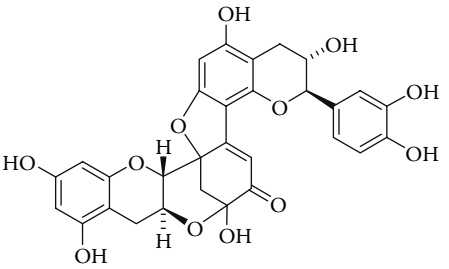


FIGURE 5.4 Oxidation of theaflavin (**5**).



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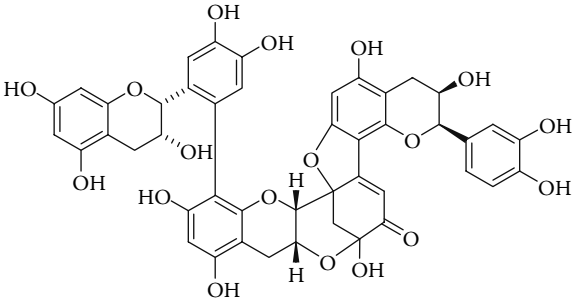

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FIGURE 5.5 Oxidation products of catechol-type catechins.

5.5 OXIDATION OF PYROGALLOL-TYPE CATECHINS AND FORMATION OF THEASINENSINS

In contrast to the oxidation of catechol-type catechins, enzymatic oxidation of (–)-epigallocatechin and (–)-epigallocatechin gallate afforded dimers generated by oxidative coupling between two B-rings (figure 5.6). The hydroxy *o*-quinone (**2a** and **4a**) produced by the initial oxidation was attached to another hydroxy *o*-quinone to produce various quinone dimers. The most important quinone dimers were dehydrotheasinensins C (**12**)[42] and A (**13**),[43] which were found to be accumulated in crushed fresh tea leaves.[44] When the leaves were heated and dried, these products were converted to theasinensins (**14–17**). Theasinensins are the major constituents of black tea and their total concentration is comparable to that of theaflavins.[4,18,45,46] Similar conversion of dehydrotheasinensins to theasinensins was also observed in an aqueous solution at neutral pH.[43] The reaction appeared to be oxidation-reduction dismutation, since theasinensins, the reduction products of dehydrotheasinensins, were accompanied by oolongtheanins (**18**) and compound **19**, which are the oxidation products. Interestingly, direct derivatization of the dehydrotheasinensins accumulated in crushed tea leaves with *o*-phenylenediamine yielded phenazine derivatives with only an *R* biphenyl bond (**20**), indicating that the quinone coupling is highly stereoselective.[44] Dehydrotheasinensins are important as precursors of pigments. Recently, we found a novel pigment in commercial black tea, designated dehydrotheasinensin AQ (**21**) (figure 5.6), which was generated by isomerization of **13**.[47] On the other hand, isomerization

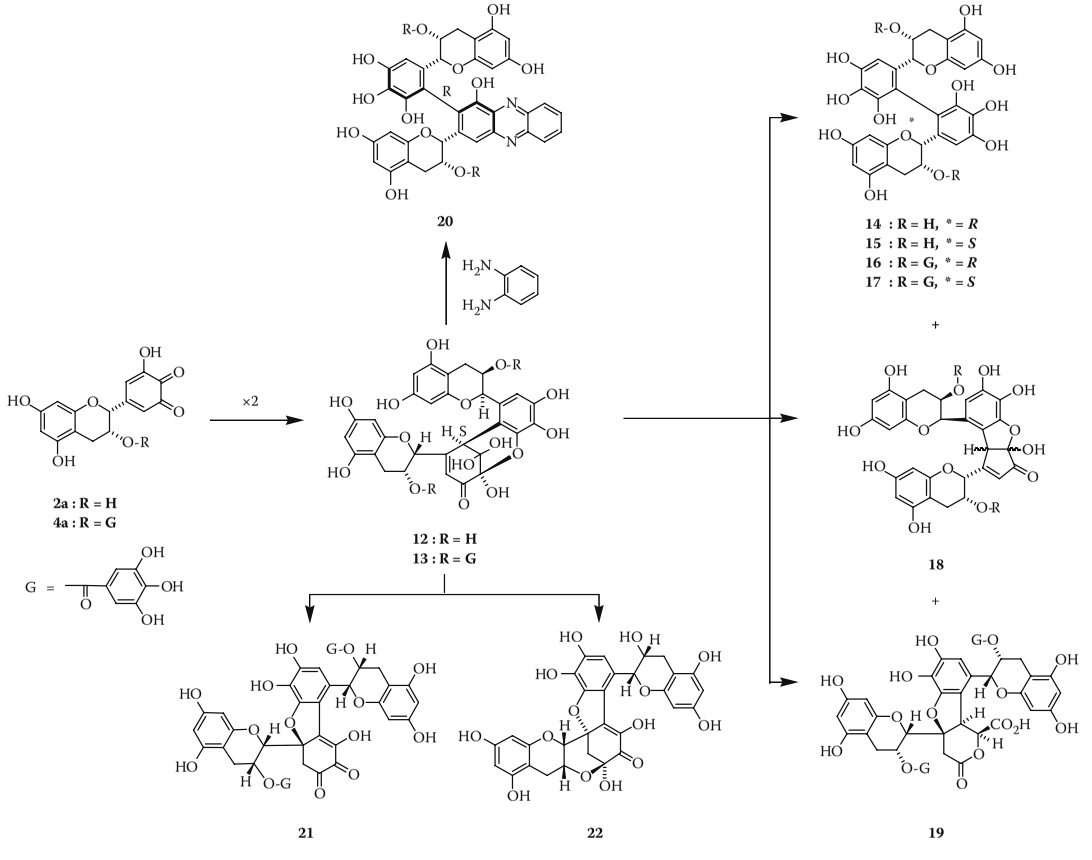


FIGURE 5.6 Oxidation of pyrogallol-type catechins.

of **12** afforded dehydrotheasinensin E (**22**),[30] although its presence in black tea has not yet been confirmed.

We demonstrated that **13** was produced not only by enzymatic oxidation but also by auto-oxidation of (–)-epigallocatechin gallate in a phosphate buffer at pH 7.4. Due to its instability at pH 7, **13** did not accumulate in the reaction mixture and was only detected at an initial stage of the auto-oxidation.[43]

In addition to dehydrotheasinensins, we confirmed the structures of related quinone dimers of (–)-epigallocatechin gallate, which were produced via quinone coupling in model fermentation experiments (figure 5.7). EGCG quinone dimer A (**23**) was originally identified as a product produced by radical oxidation of (–)-epigallocatechin gallate in an organic solvent.[48] This compound was unstable in hot water and gave (–)-epigallocatechin gallate and compound **24**, which corresponds to the A- and C-rings of (–)-epigallocatechin gallate and was isolated from black tea.[43] The production of these compounds may be explained by a retro-Aldol-type reaction of **23** affording the quinone **4a** and subsequent oxidation-reduction dismutation. Another model fermentation experiment yielded EGCg quinone dimer B (**25**) with an interesting caged structure containing two carbonyl groups.[47] Compound **26** was first isolated by a model fermentation experiment using a tea leaf homogenate and later isolated from commercial black tea.[27] Production of this product was not explained by simple dimerization of quinone **4a**, and a relatively complex mechanism including decarboxylation was proposed.

5.6 POLYPHENOL WITH A THEANINE STRECKER ALDEHYDE MOIETY

In addition to caffeine and four tea catechins, *C. sinensis* contains theanine (γ-L-glutamyl ethylamide, **27**), which is a characteristic amino acid only isolated from this plant. Theanine is the major amino acid in tea leaves, and recent studies have suggested that it has various biological activities in the central nervous system.[49,50]

In the heating process of tea production, it has been suggested that some aldehydes contributing to tea aroma are produced by a Strecker reaction between amino acids and the carbonyl groups of sugars or catechin quinones.[51] Recently, we isolated a novel polyphenol **28** with a theanine Strecker aldehyde moiety (figure 5.7).[52] In this structure, an *N*-ethyl-2-pyrrolidinone moiety, equivalent to theanine Strecker aldehyde (**27a**), was attached to the A-ring of theasinensin A (**16**). This structure was confirmed by partial synthesis, and the condensation between the aldehyde with the A-ring was shown to be a reversible reaction. Although reactions of aldehydes with catechins, proanthocyanidins, and anthocyanins have been suggested to occur in persimmon fruits[53] or red wine,[54,55] related adducts of **27a** with monomeric catechins could not be detected in black tea, probably because the products were unstable compared to **28**. These findings may suggest that the aldehyde–A-ring linkage was partially stabilized by another catechin unit in the structure of **28**.

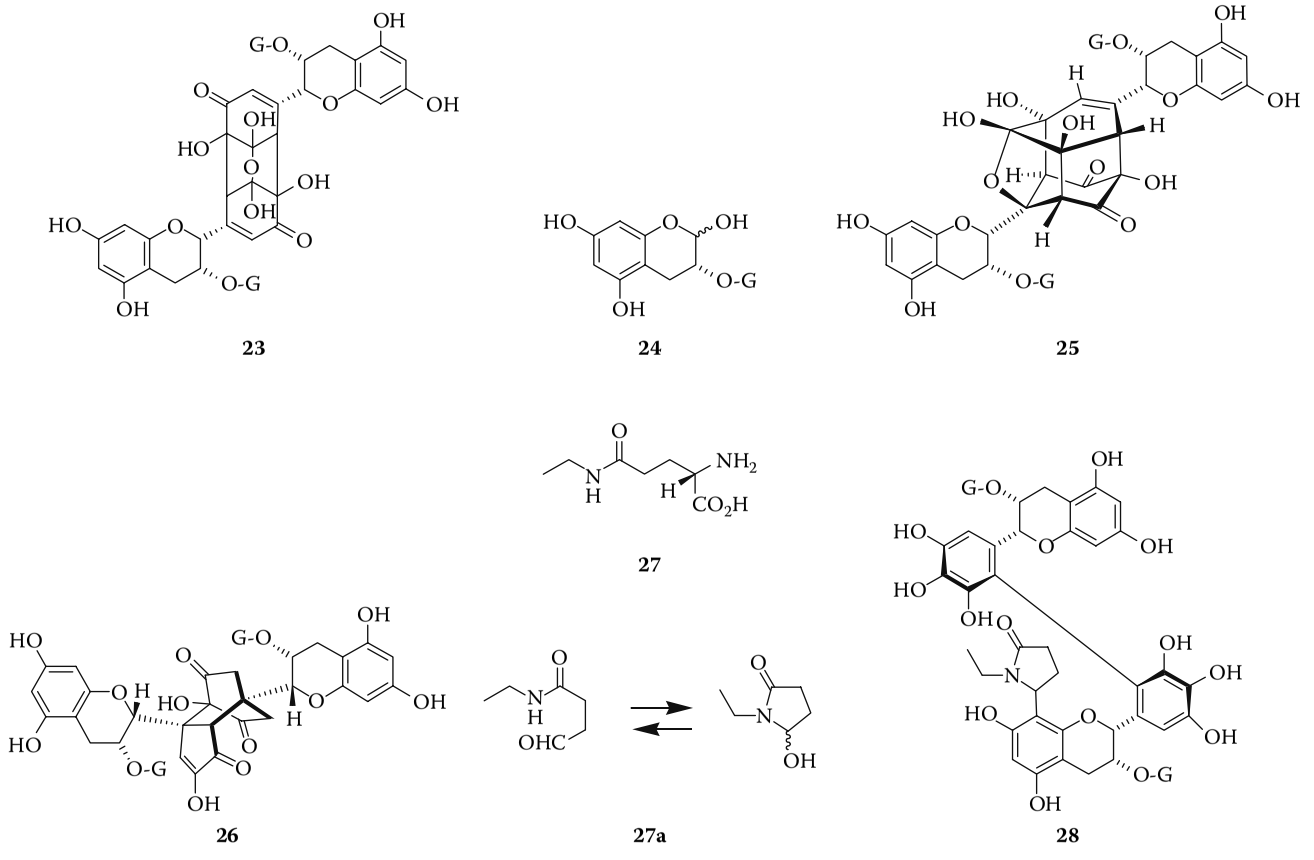


FIGURE 5.7 Oxidation products of epigallocatechin-3-*O*-gallate (**4**).

5.7 ANOTHER PATHWAY FOR BENZOTROPOLONE FORMATION

As described earlier, oxidative coupling between catechol-type catechins and pyrogallol-type catechins produced theaflavins and related pigments with a 1',2'-dihydroxy-3,4-benzotropolone moiety. On the other hand, other types of benzotropolone pigments with different hydroxylation patterns are known to exist in black tea. Epitheaflagallin (**29**) and its 3-*O*-gallate have a 1',2',3'-trihydroxy-3,4-benzotropolone moiety, and were originally synthesized from epigallocatechin and pyrogallol (1,2,3-trihydroxybenzene) or gallic acid by *in vitro* experiments.[20,56] Based on the *in vitro* synthesis results, it was presumed that epitheaflagallins would also be produced in crushed tea leaves by a similar mechanism. However, we found that **29** was directly produced from epigallocatechin. Recently, the mechanism was revealed, in which an unstable quinone dimer of epigallocatechin, designated proepitheaflagallin (**30**), decomposed on heating to yield **29** along with a new benzotropolone pigment designated hydroxytheaflavin (**31**) (figure 5.8).[42] Compared to dehydrotheasinensins, the contribution of **30** to the total catechin oxidation was relatively low. However, this represents the first reported oxidation route for the formation of benzotropolone pigments from epigallocatechin.

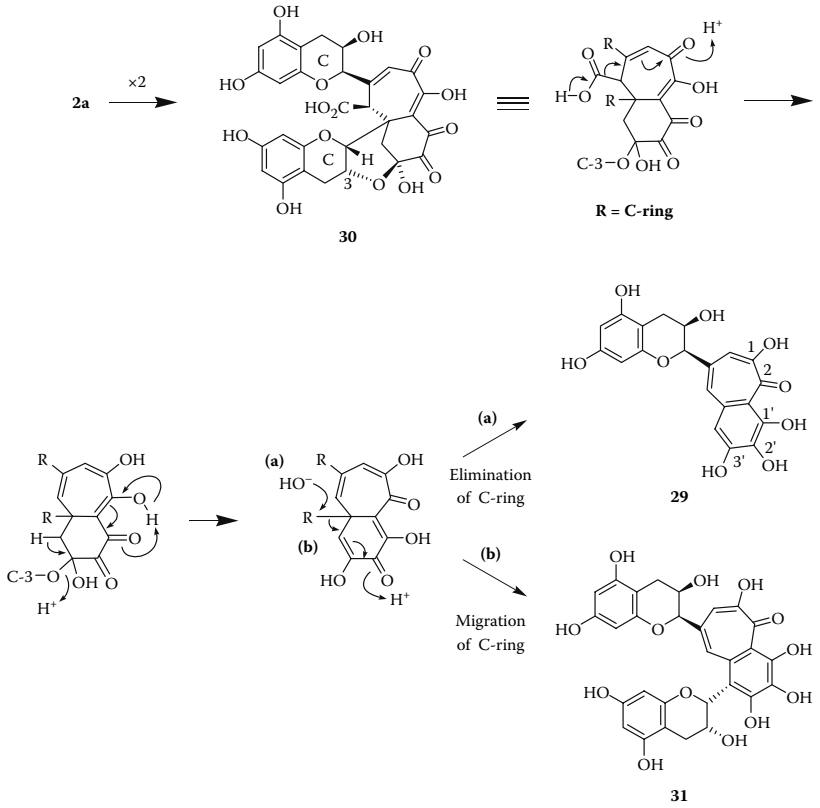


FIGURE 5.8 Structure of proepitheaflagallin (**30**) and formation of epitheaflagallin (**29**).

5.8 WHY ARE BLACK TEA POLYPHENOLS SO COMPLEX?

In the oxidation of tea catechins, the initial reaction gave simple catechin quinones. However, subsequent cross-coupling reactions of the quinones yielded many products. Some of these products were unstable and decomposed to give further complex products. These cascade-type reactions account for the complexity of the black tea polyphenols (figure 5.9). Although many of the oxidation products described here were obtained by model fermentation experiments, the presence of compounds **21**, **24**, **26**, **28**, and **29**, as well as theaflavins (theaflavin and its galloyl esters), theasinensins (**14–17**), and oolongtheanins (**18**), in commercial black tea were confirmed.

As mentioned before, black tea contains uncharacterized substances detected as a broad hump on the baseline during HPLC analysis. Our experimental results for the initial stage of catechin oxidation suggest that oxidative coupling between the pyrogallol-type B-rings of the most abundant tea catechin mainly affords dimeric compounds, and there is no evidence to date that a similar mechanism leads to the formation of trimers and further large molecules. Although the mechanism has not been clarified, the matrix assisted laser desorption ionization time-of-flight mass spectrometry (MALDI TOF MS) analysis of the total mixture of oxidation products of (–)-epigallocatechin suggested the production of a trimer and tetramer of (–)-epigallocatechin.[42] However, the peak intensities for these products were much smaller than those of the dimeric products. Therefore, it is unlikely that the oxidative coupling between B-rings is important in the formation of polymeric substances, at least at the initial stage of tea fermentation. Some recent results have indicated the importance of oxidation of galloyl groups in black tea polyphenols. Participation of galloyl groups in benzotropolone formation, leading to the production of trimeric to oligomeric pigments such as **33**, has been demonstrated (figure 5.10).[32,57,58] In addition, we

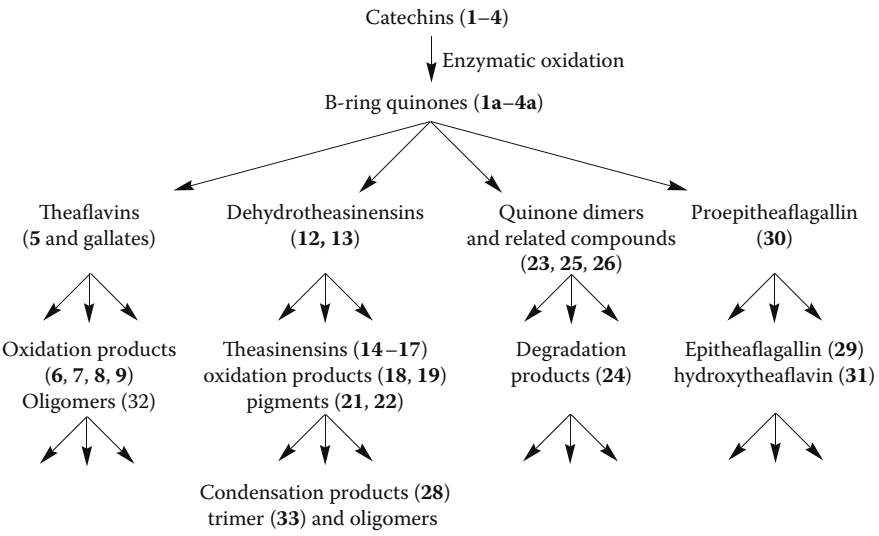
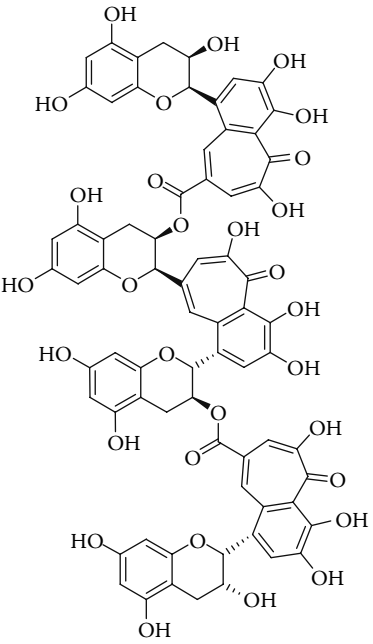


FIGURE 5.9 Catechin oxidation cascade.



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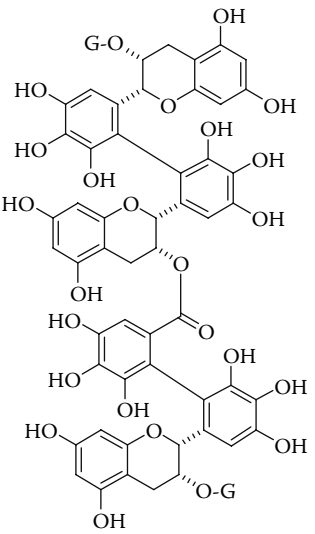

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FIGURE 5.10 Structures of catechin oligomers.

have reported that enzymatic oxidation of (–)-epigallocatechin gallate yielded the trimer **33** as a minor product, in which one of the two galloyl groups of theasinensin A (**16**) was linked to another B-ring of (–)-epigallocatechin gallate through a biphenyl bond.[47] These reactions of galloyl groups extended the molecular sizes from dimers to further large molecules. It should be mentioned here that the oxidation of pyrogallol-type B-rings proceeds much faster than the oxidation of galloyl groups.[34] As an alternative mechanism leading to the polymerization, electrophilic substitution at the catechin A-rings affording dehydrodicatechin-type products possibly occurs in polymer formation.[26,30] In addition, the presence of a proanthocyanidin-type C-4–C-8 interflavan linkage in the polymer fraction of black tea polyphenols has been proposed.[59] However there is, to date, no evidence that structures of the proanthocyanidin type can be generated from phenolic flavan-3-ols by chemical or enzymatic means.[16,60] Consequently, formation of dimers by oxidative coupling of the B-rings and extension of the molecule by oxidative coupling of the galloyl groups are the most reasonable mechanisms for producing the polymeric oxidation products at present.[58] However, chemical evidence obtained to date is still insufficient to construct the general structure of the polymeric oxidation products, and we are still a long way from chemical understanding of all the black tea polyphenols.

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6 Analytical Methods for Bioactive Compounds in Teas

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CONTENTS

6.1 INTRODUCTION

Tea is defined as the hot aqueous infusion of the dried leaves of the plant *Camellia sinensis*. It is one of the most popular beverages worldwide, and its consumption is second only to water.[1] The earliest records of tea date back to 5,000 years ago in China, and since then it has been utilized both as a beverage and as a medicine.[2] Recently, the health-enhancing properties of tea have received much attention. Epidemiological and animal studies suggest that tea is protective against certain cancers, cardiovascular diseases, and neurodegenerative diseases.[3–5] Therefore, tea extracts have gained popularity as ingredients in dietary supplements and functional foods. Depending on the degree of fermentation prior to further heating and processing, teas are categorized into three major varieties: green tea, oolong tea, and black tea. The unfermented green tea and semifermented oolong tea are popular mainly in China, Japan, and India.[6] However, in the rest of the world the fully fermented black tea is the regular choice, such as in the UK, Canada, and the U.S.[1]

Green tea, oolong tea, and black tea differ in their polyphenolic contents.[7] Catechins are the major flavanoids present in green tea, accounting for up to 30% of the dry weight of the leaves.[6] They are flavan-3-ols, which are either dihydroxylated (3,4-OH) or trihydroxylated (3,4,5-OH) on the B-ring. There are eight major catechins in green tea:[8] (+)-catechin (C), (–)-epicatechin (EC), (–)-gallocatechin (GC), (–)-epigallocatechin (EGC), (–)-catechin gallate (CG), (–)-gallocatechin gallate (GCG), (–)-epicatechin gallate (ECG), and (–)-epigallocatechin gallate (EGCG). Catechins are best known for their potent antioxidant activities, being more effective radical scavengers than vitamins C and E.[9] The antioxidant properties of catechins may contribute to the beneficial effects of tea on a number of diseases related to reactive oxygen species (ROS), such as cardiovascular diseases and cancer. Alternative mechanisms to the chemopreventive properties of catechins have also been proposed. *In vitro* studies have demonstrated that EGCG inhibits proliferation of breast and prostate cancer cells via cell cycle arrest.[10] Moreover, EGCG has been found to be antiangiogenic and to block tumor metastasis.[11,12] Thus, catechins are widely regarded as the active components of green tea. On the other hand, black tea has comparatively low catechin content (10–12%), but with substantial quantities of dimeric and polymeric catechins, namely, theaflavins (3–6%) and thearubigins (12–18%), which are absent from green tea.[1] During the fermentation process, polyphenol oxidase (PPO) initiates conversion of catechins to theaflavins and thearubigins.[13] The former are dimers formed by condensation reactions between ungallated catechins and gallated catechins[14]; the four major theaflavins formed are theaflavin, theaflavin-3-gallate, theaflavin-3'-gallate, and theaflavin-3,3'-digallate.[15] Theaflavins are generally regarded as an influential parameter on the quality of black tea[16] and

possess potent antioxidant activities.[17,18] The majority of theaflavins formed, however, undergo further condensation reactions to form polymeric thearubigins with molecular weights in the range of 1,000 to 40,000 Da.[19] The structures and bioactivities of thearubigins are not completely known. Oolong tea is partially fermented and thus contains a mixture of catechins, theaflavins, and thearubigins.

Apart from catechins, tea contains smaller quantities of other phenolic compounds. Flavonols (1–2%) are present mainly in the form of mono- to triglycosides, with quercetin, kaempferol, and myricetin as aglycones.[20] Phenolic acids (2–3%) such as gallic and caffeic acids are also found. In addition, a range of other bioactive components such as purine alkaloids, saponins, lignans, and pigments have been identified from tea leaves. Caffeine is the major purine alkaloid present, together with theobromine and theophylline. Considerable amounts of lignans (~6%), including secoisolariciresinol and matairesinol, have been detected in commercial tea samples.[21] More recently, several novel bioactive triterpenoid saponins have been isolated,[22,23] and they were found to be gastroprotective and possess cytotoxic activity toward certain cancer cells.[24,25] While the black color in black tea is attributed to the presence of theaflavins and thearubigins, in green tea the major pigments are chlorophylls and carotenoids.

Traditionally tea is graded according to its flavor and color by tea tasters; however, analysis of chemical constituents would provide more accurate and reliable parameters for evaluating the quality of tea, and this is related to factors such as the age of tea leaves and the degree of fermentation.[26] Phytochemical analysis of tea is especially important for the production of health-promoting food products that require the necessary bioactive constituents to be delivered in a complete and consistent manner. In addition, the continuously emerging evidence of the beneficial pharmacological effects of tea polyphenolics necessitates investigation of biological samples to derive information about the metabolic fates of these phytochemicals and their pharmacological mechanisms. In other words, chemical analysts are presented with samples that range from simple tea infusion containing target compounds up to 20–30% by weight to those containing target compounds in minute quantities. Therefore, development and validation of analytical methods for qualitative and quantitative determination of tea compounds are crucial. Analysis has been largely facilitated by the availability of various powerful chromatographic techniques (such as high-performance liquid chromatography [HPLC] and capillary electrophoresis [CE]), which may be coupled to different detectors (such as UV, electrochemical, and mass spectrometry [MS]). These coupled analytical tools permit rapid analysis of multiple bioactive constituents in a single run. Near-infrared (NIR) spectroscopy and nuclear magnetic resonance (NMR) spectroscopy have also been proposed as alternative methods. The applications of these methods for the analysis of bioactive components from teas are the focus of this chapter.

6.2 ANALYSIS OF CATECHINS

6.2.1 Sample Preparation

Tea catechins are typically extracted with water or organic solvents, filtered, and then employed directly for analysis.[26] Although water is often a popular solvent employed

for extraction, studies indicate that aqueous solvent mixture results in better extraction efficiencies.[27] An additional advantage with the incorporation of organic solvents into the solvent system is that they efficiently extract the catechins without undesirable epimerization of the major catechins.[28] In cases where organic solvents are not desirable, applying water alone is still suitable through careful adjustment of extraction conditions (extraction time, temperature, etc.). While Perva-Uzunalic et al.[29] showed that the highest extraction efficiency of catechins from green tea was achieved using boiling aqueous acetone (>95%) and 50% acetonitrile (99.8%) for 2 hours, comparable results (97–98% yield) were also obtained using water as a sole solvent via a high-temperature (80°C), short-time (20 minutes) procedure. Extractions for longer periods resulted in lower yields due to degradation of catechins. Moreover, extraction at temperatures higher than 80°C can promote catechin epimerization, such as the formation of GCG from EGCG,[30,31] which significantly alters the catechin composition of the original sample. One method to circumvent this problem is by carrying out extraction at a lower pH.[28] Extraction in alkaline conditions is not recommended, as such conditions greatly enhance catechin degradation. Thus, a judicious choice of the solvents and extraction conditions is important in achieving optimal recovery and minimizing artifacts formation. Following extraction, prepurification of catechins from the crude extract can be performed. In most cases, this step serves to remove caffeine, which may interfere with catechin analysis. Liquid-liquid partition[31,32] is a commonly employed method by which caffeine in a tea infusion is extracted several times using chloroform. Catechins remaining in the water layer can be further purified via an ethyl acetate-water partition step. Other methods, such as solid-phase extraction (SPE) on C_{18} cartridges[33] and precipitation of catechins by addition of calcium carbonate, have also been reported.[34] The same principles are applied to the purification of catechins from different biological fluids such as plasma and tissue homogenates.[35]

6.2.2 HPLC

HPLC is traditionally the method of choice for comprehensive analysis of catechins in teas, as it offers good separation and can be combined with an array of detectors. Reversed-phase C_{18} column is employed in the majority of cases, while the use of C8 columns has only been occasionally reported.[36,37] Aqueous methanol or acetonitrile is the most common component of the mobile phase, and the presence of acids has been found to be crucial for complete resolution of catechins and elimination of peak trailing.[38] Optimization of separation conditions has allowed an increasing number of catechins to be analyzed and quantified in a single analysis. Previously, Dalluge and Nelson[8] noted that the benchmark for comprehensive separation of catechins in tea was complete resolution of the eight major catechins (C, EC, GC, EGC, CG, GCG, ECG, and EGCG) as first reported by Goto et al.[39] The separation utilized a C_{18} column and tea catechins were eluted by a gradient mobile phase consisting of water–acetonitrile–phosphoric acid. Detection was by means of UV absorption at 231 nm, and the limit of detection of this method is about 0.2 ng for all catechins present. Since then, several methods have been developed, and these are capable of simultaneous analysis of minor catechins and methylated catechin derivatives

in addition to the major catechins present in tea infusion. Zeeb and coworkers[40] reported the use of an HPLC-MS method in the separation and identification of twelve catechins, including the eight major catechins, minor catechins, (–)-epiafzelechin (EZ), and (–)-epiafzelechin gallate (EZG), as well as methylated derivatives, (–)-epigallocatechin methylgallate, and (–)-epicatechin methylgallate. LC separations were performed on a Zorbax Eclipse XDB-C_{18} column with a gradient mobile phase of water (0.05% trifluoroacetic acid [TFA]) and acetonitrile (0.05% TFA) for 35 minutes. Coupled to an MS detector, the twelve compounds were determined in a single analysis with a sensitivity down to femtomole to low-picomole levels. However, lack of available standards only allowed tentative assignments of the structures of methylated derivatives via interpretation of their mass spectra. Sano et al.[41] later reported the separation and determination of twelve catechins by HPLC with electrochemical detection. The compounds were resolved on a TSK gel ODS column using isocratic elution with 0.1 M NaH_2PO_4 (pH 2.5) in 0.1 mM Na_2EDTA-acetonitrile (87:13, v/v) as the mobile phase. Besides major catechins, four additional methylated catechins, (–)-epigallocatechin-3-*O*-(4-*O*-methyl)gallate (4"Me-EGCG), (–)-epigallocatechin-3-*O*-(3-*O*-methyl)gallate (3"Me-EGCG), gallocatechin-3-*O*-(3-*O*-methyl)gallate (3"Me-GCG), and (–)-epicatechin-3-*O*-(3-*O*-methyl)gallate (3"Me-ECG), were identified by comparing their retention times with those of authentic standards, although 3"Me-ECG was found to coelute with ECG. With an electrochemical detector, the limits of detection vary between 10 and 40 pmol ml^{-1}. These two studies represent the most comprehensive analysis of tea catechins reported.

As catechins are good UV chromophores with absorption maxima at 210 and 270–280 nm,[26] UV and diode array detectors (DAD) are traditionally the most popular detectors employed for their determination in tea infusions. A typical HPLC chromatogram for green tea extract with low caffeine content is shown in figure 6.1.

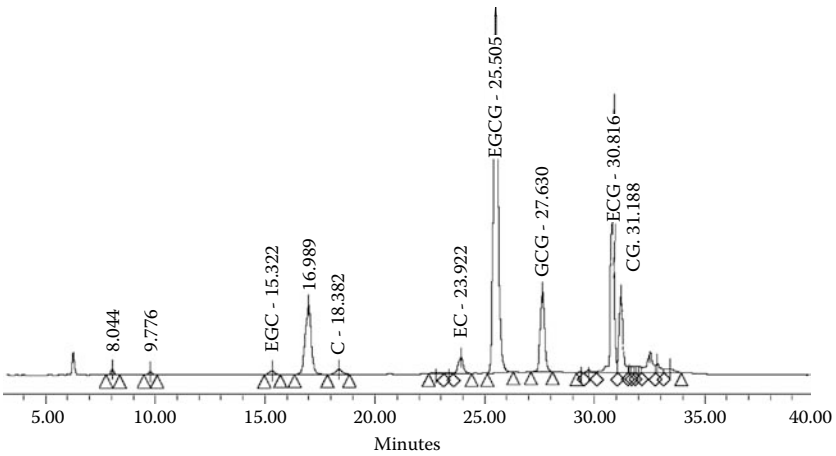

FIGURE 6.1 A representative HPLC chromatogram of a green tea extract run on a Phenomenex C18(2) column (5 µm, 4.6 × 250 mm) with flow rate at 1.0 ml/min, detection wavelength at 280 nm, and water (0.1% phosphoric acid)-isopropanol-acetonitrile gradient as mobile phase.

These detection methods, however, have several pitfalls. First, UV is nonspecific in nature. It cannot distinguish an analyte from a coeluting interference. This renders it less applicable when catechins are present in complex matrices such as human plasma and saliva. Second, quantification necessitates baseline separation of all marker analytes, resulting in long run times (>60 minutes) and low throughput. Highly sensitive and selective methods, for example, MS and electrochemical and fluorescence detection, have therefore been proposed as alternatives to UV detection.

6.2.3 HPLC-MS

Mass spectrometry provides important information regarding the molecular weight and the characteristic structural fragments for the identification of catechins. Thermospray ionization (TSI), electrospray ionization (ESI), and atmospheric pressure chemical ionization (APCI) are commonly applied interfaces between LC and the MS detector. These soft ionization methods generally provide molecular weight data, which in combination with the retention times allow unambiguous peak assignments. Additionally, in cases where standards were unavailable, tandem MS/MS experiments represent a powerful approach for characterization and tentative assignments of the unknowns. LC-MS and LC-MS/MS find uses in three major areas: (1) rapid determination of trace amounts of catechins in extracts, cosmetics, and supplements; (2) structural characterization and tentative assignments of minor unknown catechins; and (3) determination of catechins from biological fluids and characterization of their metabolites. Pelillo and coworkers[42] established an LC-ESI-MS method for the analysis of six catechins. Quantification was carried out in selected ion monitoring (SIM) mode, in which only several $[M+H]^+$ catechin ions were scanned. This enhances signal-to-noise ratio and therefore gives higher sensitivity compared to the full scan mode. Under the HPLC conditions adopted in this study, gallocatechin was found to coelute with gallic acid. In spite of this, the unique selectivity of SIM mode means that only the gallocatechin $[M+H]^+$ ion was observed, thus avoiding the interference that would otherwise affect UV-DAD quantification. The authors also concluded that MS offers superior sensitivity to UV detection. Determination of trace amounts of catechins in cosmetic formulations and in *in vitro* skin extracts using SIM was also reported.[43] All catechins were detected as $[M-H]^-$ ions in the negative ion mode. Analysis was relatively rapid (15 minutes), and limits of detection (LsOD) ranged from 5 to 15 ng/ml. A highly sensitive method was developed by Wu et al.[44] via the coupling of solid-phase microextraction (SPME) to LC-ESI-MS. In this system, 40 µl of samples were repeatedly drawn from the sample vial and injected into a polypyrrole (PPY)-coated capillary tube. Extracted catechins were subsequently eluted using the initial mobile phase directly onto the LC column. By employing SIM in either the positive or negative ion mode, LsOD for all five catechins were <0.5 ng/ml, which represents a tenfold improvement in sensitivity over the direct liquid injection method. In contrast to the above studies, Zeeb et al.[40] observed that fragmentation to the m/z 139 is a common fragmentation pattern of catechins, and SIM analysis was thus performed at m/z 139. This fragment arises from a retro Diels-Alder fragmentation of the unmodified A-ring. It is a simple method for a complete fingerprint of all catechin species present; however, sensitiv-

ity would be compromised in cases where the m/z 139 is not the base peak of the respective catechins.

Apart from providing a highly sensitive and selective method of detection, tandem MS/MS with collision-induced dissociation (CID) would allow identification of substructures and meaningful structural assignments. ESI-MS/MS fragmentation of catechins and its methylated derivatives was investigated in the positive ion mode.[45] Diagnostic ions for the differentiations of different methylated derivatives are shown in figure 6.2.

For catechin, retro Diels-Alder fragmentation of the A-ring gives rise to $^{1,3}A^+$ ion of m/z 139. A mass shift of 14 Da in this ion indicates methylation of ring A. Likewise, B-ring methylation leads to identical mass shifts in B-ring-derived $^{1,2}B^+$ and $^{1,4}B^+$ ions, from m/z 123 and m/z 165, respectively. Through interpretation of these ions, the methylated ring is readily assigned. Moreover, the intensities of major product ions in MS/MS spectra reveal the precise sites of methylation on each ring. Miketova and coworkers[46] also employed ESI-MS/MS for structural elucidation of catechins, albeit in the negative ion mode. Fragmentation of $[M-H]^-$ ions of EGCG resulted in three major ions at m/z 125, 169, and 305. The m/z 125 ion represents an unmodified A-ring, the m/z 169 ion is due to the loss of an intact gallic acid anion, while the ion at m/z 305 results from the neutral loss of gallic acid. Mass shifts in these three ions are diagnostic of the site of methylation. A 14-Da shift of m/z 169 to m/z 183 is characteristic of methylated gallic acid moiety. The position of methylation could also be defined by the presence or absence of this particular ion. If the methyl group is located at the 4'-position of gallic acid, the formation of m/z 183 ion containing the gallic acid group would be blocked. Thus, the absence of m/z 183 indicates 4'-position methylation, while its presence allows assignment of the methyl group to the 3'-position. Such MS/MS methodology has demonstrated irreplaceable efficiency for structural characterization of catechins.

Application of LC-MS to the analysis of catechins in complex biological matrices is an important advance in the sensitivity offered by LC-MS, which simplifies sample preparation procedures such as liquid-liquid extraction or SPE required in traditional methods. Dalluge et al.[47] demonstrated the use of capillary LC-ESI-MS in SIM mode for the determination of EGCG in human plasma, with detection limits of ~20 ng. A rapid and sensitive tandem LC-LC-MS was developed for simultaneous detection of six catechins in human plasma.[48] Turbulent flow chromatography C_{18} column was employed for on-line sample preparation. Using 0.1% formic acid at a flow rate of 5 ml/min, proteins were rapidly removed. Catechins were extracted and then directly eluted in reverse onto a Zorbax Eclipse XDB C_{18} column for separation. As a result of the efficient cleanup process, the limit of detection (LOD) of each catechin ranged from 0.6 to 2 ng/ml. The first LC-MS-SIM method for comprehensive analysis of eight major catechins in biological fluids was applied by Masukawa et al.[49] Samples were pretreated with perchloric acid and acetonitrile to precipitate the proteins, followed by the addition of potassium carbonate solution to neutralize any excess acid. Separation was achieved on a C_{18} column with gradient elution of water (0.1 M acetic acid) and acetonitrile (0.1 M acetic acid). Using this system, the author first demonstrated that all eight major catechins were absorbed into the human plasma following oral ingestion of a commercial tea beverage. Apart

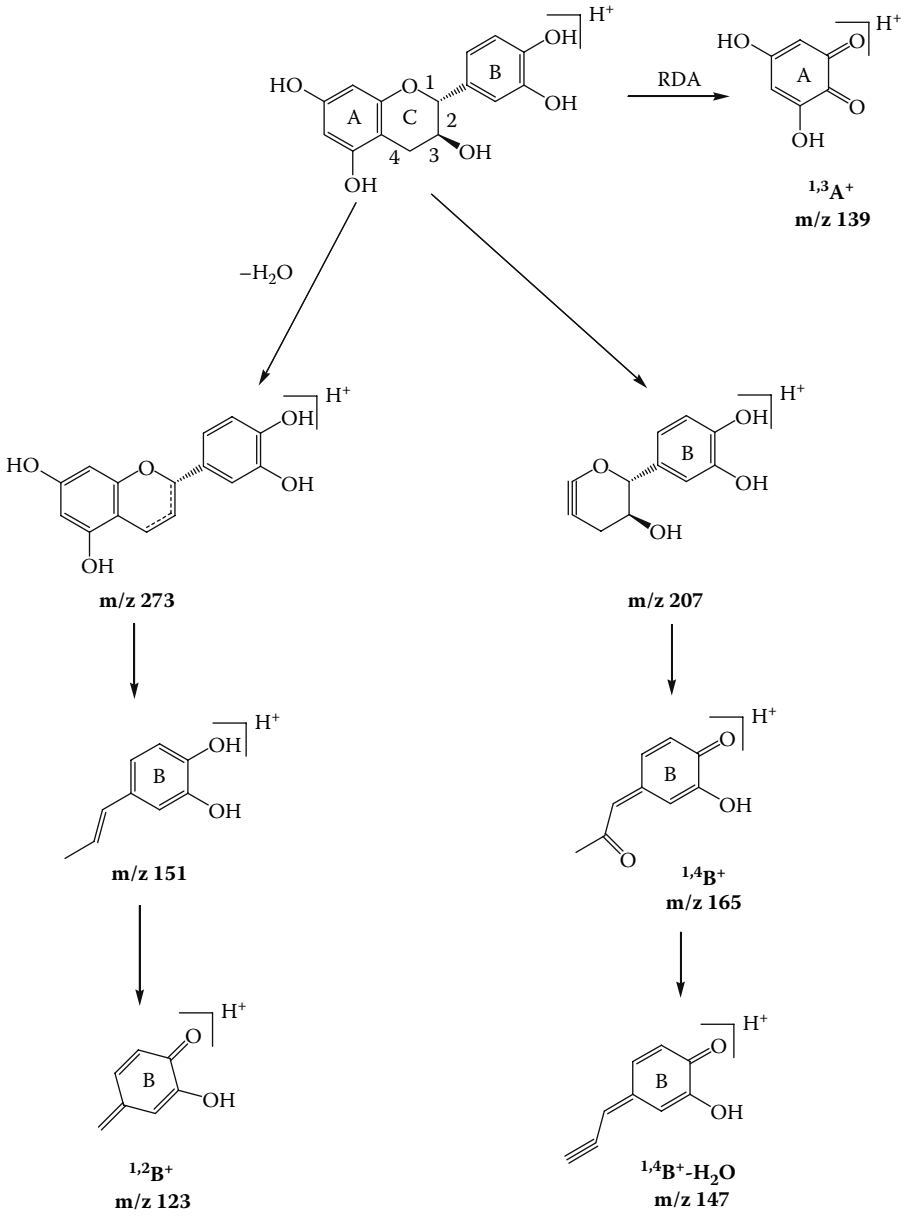

FIGURE 6.2 Major fragmentations of protonated catechin in the generation of diagnostic ions containing the A-ring and B-ring, respectively. (Adapted from Cren-Olive, C., Deprez, S., Lebrun, S., Coddeville, B., and Rolando, C. 2000. Characterization of methylation site of monomethylflavan-3-ols by liquid chromatography/electrospray ionization tandem mass spectrometry. *Rapid Commun. Mass Spectrom.* 14:2312–19.)

from the detection of unmodified catechins, LC-MS finds applications in the rapid elucidation of catechin metabolites. Xenobiotics including catechins are converted to glucuronide and sulfate derivatives, which increase their hydrophilicity and facilitate their excretion. An LC-ESI-MS/MS approach was adopted to study catechin glucuronides and sulfates present in human urine samples after tea consumption.[50] Peaks were tentatively assigned by matching the observed deprotonated ions against the predicted molecular weight of catechin conjugates. Further evidence of identity of the parent catechins was derived from MS/MS, which showed aglycone ions as the predominant peak. Monoglucuronides and monosulfates of EGC and EC were the major conjugates appearing in urine. Other biotransformation products such as the monoconjugates of catechin ring-fission metabolites, (–)-5-(3',4',5'-trihydroxyphenyl)-γ-valerolactone (M4) and (–)-5-(3',4'dihydroxyphenyl)-γ-valerolactone (M6), were also detected. In another study, Meng et al.[51] investigated metabolites in humans, mice, and rats after ingestion of pure EC, EGC, and EGCG using LC-ESI-MS/MS. In a human plasma sample, 4',4"-di-*O*-methyl-EGCG was identified as a major peak after EGCG ingestion by comparing the retention time and MS/MS spectra with those of a synthetic standard. In the urine, M4, M6, and a further unknown peak were detected following ingestion of EGC and EGCG. The unknown has the same molecular mass and m/z 163 $[(M-44)-H]^-$ ion as in M6, suggesting that they have an identical lactone structure. It was postulated that the unknown is (–)-5-(3',5'-dihydroxyphenyl)-γ-valerolactone (M6'), which possesses a meta-substituted phenol ring as opposed to ortho-substitution in M6. As the bioavailability of EGCG is low, these metabolites may make a substantial contribution to the beneficial effects of green tea.

6.2.4 HPLC Electrochemical Detection

Since catechins possess reducing potentials,[14] they are readily detected by an electrochemical detector (ECD). ECD is highly sensitive and more selective than UV detection, but less expensive than MS. Although a recently introduced coulometric array detector allows for gradient elution, many studies continue to use ECD with isocratic elution. Umegaki et al.[52] reported the determination of five catechins using ECD with a sensitivity (40 pmol) of 1,000 times higher than UV detection. Kumamoto et al.[53] compared the use of UV and ECD for analysis of four catechins in green tea. Caffeine, a major component in tea, coelutes with EGC, and EGC cannot be quantified by UV detection, whereas in ECD, interference from caffeine is eliminated as it is electrochemically inactive. Moreover, ECD is ~45 to 320 times as sensitive as UV detection. Determination of eight major catechins by HPLC with ECD detection was reported by Long et al.[54] Catechins were separated on a C_{18} column eluting with 20 mM chloroacetic acid in 11% acetonitrile. EGCG and catechin were detected at 400 mV, while others were detected at 900 mV with LsOD of 0.1–1.5 ng/ml. In general, sensitivity of catechins detection by ECD is affected by the applied voltage, and maximum response occurs at ~750 to 800 mV. However, at such high voltages ECD selectivity is compromised, as many other coeluting substances are also oxidized, and thus a voltage of ~500 to 600 mV is recommended.[52]

Owing to its high sensitivity, ECD is also a popular technique for the determination of catechins in biological fluids. The conjugated catechins cannot be directly analyzed by ECD as authentic standards are lacking; however, the analysis of aglycones can be achieved after an enzymatic hydrolysis step using sulfatase and β-glucuronidase. In order to reduce matrix interferences, sample prepurification strategies have been applied. Lee et al.[55] employed a water–ethyl acetate partition to concentrate catechins present in urine, plasma, or tissue samples. EGCG, EGC, ECG, and EC were detected at levels of 5–10 ng/ml. Kotani et al.[56] reported an improved method based on liquid-liquid extraction and HPLC-ECD. The use of a microbore C_{18} column was found to give superior sensitivity to a conventional column, possibly due to reduced diffusion of samples in the former. An 85% phosphoric acid–water–methanol (0.5:81:19, v/v/v) solution served as the mobile phase with a flow rate of 25 µl/min. At 600 mV, the LsOD for the eight catechins were in the range of 290–460 pg/ml, and this method was more sensitive than other HPLC methods reported previously. On the other hand, Umegaki et al.[57] utilized solid-phase extraction for prepurification. The proteins in plasma samples were precipitated by adding acetonitrile, and catechins in the solution were then extracted by a C_{18} SPE cartridge prior to HPLC analysis. SPE allows for automation, and many samples can be handled in parallel. Using a conventional C_{18} column, the detection limit for each catechin is ~1 ng/ml. In simpler matrices, such as urine, HPLC-ECD without prior sample purification is also feasible. Yang et al.[58] analyzed the eight major catechins in human urine. The urine samples were either hydrolyzed or directly injected onto a C_{18} column and separated with a mobile phase composed of acetonitrile:0.1 M phosphate buffer (pH 2.5) (15:85, v/v). The sensitivity was slightly compromised and the LOD for all the catechins present was 0.5 pmol, which equates to 29–46 ng/ml.

The determination of methylated catechins by HPLC-ECD has also been investigated. As methylated catechins have a hydroxyl group substituted, their oxidation potentials increases considerably. Donovan et al.[59] reported that at 400 mV, LsOD of the methylated catechins were several times higher than those of EC. Although the lower sensitivity could be compensated by increasing the electrode potentials, the selectivity of detection in complex biomatrices would be compromised. Nevertheless, ECD compares favorably to UV detection in terms of both sensitivity and selectivity. Lee et al.[60] analyzed 4'-*O*-methyl-EGC, M4 and M6 in human plasma, and urine using HPLC-ECD. High concentrations of 4'-*O*-MeEGC were found in plasma and urine, and it was suggested that this component may be used as a biomarker for tea consumption. In Sano and coworkers,[41] the study mentioned above, ECD was also employed for the determination of 4"-MeEGCG, 3"-MeEGCG, 3"-MeGCG, and 3"-MeECG. However, the lack of commercially available standards has limited the widespread use of ECD for detection of catechin derivatives.

6.2.5 HPLC–Fluorescence Detection

Several catechins and their derivatives possess fluorescence properties that allow the use of fluorescence detection, at 280 and 310 nm for excitation and emission, respectively. It is preferable to UV when analyzing catechins in biological matrices due to its inherent selectivity of detection. Ho et al.[61] first reported the determination

of (+)-catechin in plasma using HPLC coupled to a fluorescence detector. Sample preparation involved the addition of acetonitrile to remove proteins, followed by sample loading on to alumina. HPLC was performed on an ODS column with acetonitrile:35 mM phosphoric acid (pH 2.5) (12:88, v/v) as the mobile phase. The LOD and limit of quantification (LOQ) were 8 and 20 ng/ml, respectively. The sensitivity of catechin detection by UV fluorescence and ECD was compared by Donovan et al.,[59] who simultaneously determined C, EC, 3'-*O*-MeC, 3'-*O*-MeEC, 4'-*O*-MeC, and 4-*O*-MeEC with a C_{18} column eluted using a gradient of acetonitrile and 50 mM ammonium dihydrogen phosphate (pH 2.5). It was found that fluorescence is a much more sensitive method, with detection limits of ~2 to 3 ng for catechins and their methylated derivatives. This suggests that fluorescence detection is suitable for monitoring catechins and their metabolites in biological fluids. HPLC-fluorescence has also been utilized for the analysis of catechins in fruits and legumes.[62] Fluorescence detection, compared to UV detection at 280 nm, improved the detection limits of EC and C by tenfold, and higher specificity was apparent from the lower noise levels. However, the fact that, except C, EC, and their methylated derivatives, all the other major catechins show little fluorescence has limited this method's usefulness in this application. Efforts to enhance the fluorescence activity of these catechins were unsuccessful.[62] Thus, fluorescence would be the method of choice when only fluorescence-active catechins are to be analyzed.

6.2.6 HPLC–Chemiluminescence Detection

At present there are two known mechanisms for producing chemiluminescence from catechins. The first mechanism is common to polyphenolic compounds that exhibit chemiluminescence (CL) when reacting with hydrogen peroxide and acetaldehyde.[63] Based on this phenomenon, HPLC-CL methods have been developed for the measurement of ECGC and EGC in rat and human plasma.[64–66] In the first series of investigations, EGCG was determined at picomole levels in plasma. The samples were separated on a C_{18} column, followed by post-column derivatization by a combination of 8.2 M acetaldehyde in 50 mM phosphate buffer (pH 2.5) and 8.8 M hydrogen peroxide. A LOD of ~2 pmol indicates comparable sensitivity to ECD. Moreover, the inherent high selectivity enables sample preparation to be accomplished within a single protein precipitation step. In subsequent studies Nakagawa and coworkers[65,66] employed this method for studying the pharmacokinetic behavior of EGCG in a rat model, and measured the absorption of EGCG and EGC into human plasma. However, as this derivatization reaction only occurs with flavanoids with vicinal hydroxyl groups,[63] it would not be useful for methylated catechin derivatives. As a result, a second chemiluminescence method was proposed by Kodamatani et al.[67] They found that aromatic compounds, upon UV irradiation, produce CL when mixed with tris(2,2'-bipyridine)ruthenium(III) ($Ru(bpy)_3^{3+}$). The degradation products from UV photolysis of the aromatic ring may be responsible for the reaction with $Ru(bpy)_3^{3+}$ and the CL reaction. This HPLC-CL method was employed successfully for determination of EC and EGCG in tea samples, with LsOD of 0.8 and 1.2 pmol, respectively. Such a level of sensitivity is promising, taking into consideration that the use of a long coil for UV irradiation actually results in broad peaks, and that further

improvement in sensitivity is possible. Also, an aromatic ring is a property shared by all catechins, and thus this method has the potential for simultaneous determination of all major catechins and their derivatives.

6.2.7 Capillary Electrophoresis

Capillary electrophoresis (CE) is an emerging analytical technique for determination of catechins. The majority of CE studies involve the analysis of catechins in tea infusion, extracts as well as supplements. The three variants of CE suitable for the analysis of catechins include capillary zone electrophoresis (CZE), micellar electrokinetic chromatography (MEKC), and microemulsion electrokinetic chromatography (MEEKC) with UV detection. In general, the resolution of MEKC was found to be superior to CZE for separation of catechins.[8] MEEKC is a relatively new technique, and the few reports available suggest that it offers a performance similar to MEKC.[68,69] CE conditions are often quite complex, and many factors, such as buffer composition, pH, presence of surfactants, and column temperature, can all affect the quality of separation and should be optimized individually. On the other hand, CE offers several advantages over HPLC. The short analysis time (<20 minutes), low running costs, and reduced use of solvents make it an attractive alternative for routine analysis of catechins.

6.2.7.1 CZE

CZE is the simplest and one of the most widely used CE methods. Horie et al.[70] first reported the separation and determination of C, EC, EGC, ECG, and EGCG together with caffeine, theanine, and ascorbic acid using CZE with UV detection. Separation conditions were 20 mM borax buffer at pH 8 and analysis time was <11 minutes. Borate forms complexes with vicinal hydroxyl groups on catechins, which confer one negative charge per complex.[71] The complex formation results in a longer elution time and improved separations of catechins. Arce et al.[72] described another CZE method for the separation of five catechins, caffeine, adenine, and theophylline. It employed a buffer of 0.15 M boric acid at a pH of 8.5; however, separation between catechins was not improved. Later, Lee and Ong[73] applied an improved CZE method that was capable of analyzing six catechins and several theaflavins using a more complex buffer containing 200 mM boric acid, 10 mM potassium dihydrogenphosphate, 4.5 mM β-cyclodextrin, and 27.5% (v/v) acetonitrile. β-CD, a chiral selector, was added to alter the selectivity of the buffer system, while addition of acetonitrile reduced solute-wall interactions. These alterations greatly enhanced electrophoretic efficiency, and the six catechins were well resolved in less than 10 minutes. Despite the improvements, popularity of CZE for catechin analysis has been declining due to the superior separation capabilities of MEKC. Nevertheless, CZE gives more efficient separation of theaflavins[73] and would be preferred over MEKC for simultaneous analysis of catechins and theaflavins in fermented tea samples.

6.2.7.2 MEKC

MEKC incorporates a surfactant, most commonly sodium dodecyl sulfate (SDS), at a concentration higher than the critical micelle concentration (CMC). The micelles form a pseudo-stationary phase which provides an additional means of separation. MEKC analysis of catechins was first reported by Larger et al.,[71] who used a buffer system composed of 20 mM SDS, 50 mM phosphate, 50 mM sodium tetraborate, and 10% acetonitrile. Although separation of the five catechins was accomplished in 16 minutes, the catechins, EGC, C, and EC could not be baseline resolved and the background noise of tea samples was high, particularly in fermented teas. In the same year, Watanabe et al.[74] reported the separation of seven catechins by MEKC under a running buffer consisting of 25 mM SDS and 25 mM phosphate–50 mM borate (pH 7), and all catechins, caffeine, and ascorbic acid were resolved in 8 minutes. This method was compared to the CZE procedure reported previously by Horie et al.[70] Using CZE, the resolution of five catechins (C, EC, EGC, ECG, and EGCG) was not sufficient to provide quantitative analysis. MEKC, on the other hand, provided the baseline separation for all catechins except EGCG and GCG. Recently, Bonoli et al.[75] reported a MEKC method for simultaneous separation of seven catechins, three methyl xanthines, and gallic acid. The highest resolution was obtained using 10 mM potassium dihydrogenphosphate, 66.7 mM SDS, and 8.3 mM sodium tetraborate, adjusted to pH 7, as the running buffer. Satisfactory separation of the eleven compounds was achieved within 4.5 minutes; however, caffeine and (+)-catechin were not completely resolved. With UV detection at 210 nm, LsOD ranged from 0.0011 to 0.0051 μg/ml. To the best of our knowledge, the latter two MEKC methods represent the most comprehensive analysis of catechins reported. Analysis of tea catechins by MEKC and HPLC was recently compared by Bonoli et al.[76] MEKC was performed according to the method described above,[75] while HPLC separations were carried out on an ODS column with a gradient mobile phase consisting of solvent A (water, methanol, formic acid; 74.7:25:0.3, v/v/v) and solvent B (acetonitrile–formic acid; 99.7:0.3, v/v). In spite of the slightly better repeatability (RSD < 2% vs. RSD < 6%) using HPLC, MEKC was found to offer advantages in terms of the speed of analysis (4.5 minutes), sensitivity, and reduced solvent consumption. Thus, MEKC represents a highly desirable analytical tool for routine analysis of catechins.

6.2.7.3 MEEKC

Recently, MEEKC has been applied for the determination of catechins. In a similar fashion to MEKC, MEEKC uses a pseudo-stationary phase for separation of analytes. Instead of having micelles, surfactant-stabilized oil droplets in microemulsion solution serve as the pseudo-stationary phase for the partitioning of analytes. The formation of a microemulsion involves an organic solvent, SDS as a surfactant, and an alcohol as a cosurfactant.[77] Pomponio et al.[78] first developed a MEEKC method for the analysis of six catechins (C, EC, GC, ECG, EGC, and EGCG). This involved the use of heptane (1.36%, w/v), SDS (2.31%, w/v), and 1-butanol (9.72%, w/v), which serve, respectively, for the three functions mentioned above. The medium was a 50-mM phosphate buffer (86.61%, w/v) at pH 2.5. Electro-osmotic force (EOF) was almost absent, and analytes, due to their interactions with SDS-coated oil droplets,

migrated toward the anode. Six catechins were completely resolved in 10 minutes using this method. Detection by UV was anodic, and LsOD of C and GC were 0.391 and 0.781 μg/ml, respectively. In a subsequent study, Pomponio et al.[69] investigated the effects of different types of cosurfactant on the analysis of catechins. At high concentrations (7.38–9.72%, w/v), the choice of cosurfactant is an important factor for optimizing the selectivity of an MEEKC system,[79] in particular, the migration order of catechins. Among the nine alcohols evaluated, all of them allowed satisfactory separation of catechins (peak resolution > 1.5), but good repeatability was obtained only with 1-butanol, 2-hexanol, and cyclohexanol as cosurfactants. The latter two also provided better peak symmetry. Huang and coworkers[68,77] achieved separation of thirteen phenolic compounds, including six catechins by MEEKC and MEKC. The buffer system of MEEKC consisted of 2.89% (w/v) SDS, 1.36% (w/v) heptane, 7.66% (w/v) cyclohexanol, and 2% acetonitrile. Addition of acetonitrile, an organic modifier, was found to effectively enhance separation capabilities of MEEKC.

The separation efficiency of MEEKC was further improved by increasing the voltage and column temperature without affecting its resolution. Comparing MEEKC and MEKC, it was found that MEEKC has higher efficiency (246,000–1,302,000 plates/m), but with LsOD ~3 times higher than MEKC. However, MEEKC has a notable advantage over CZE and MEKC in that it uses acidic separation conditions, while in the latter two techniques alkaline conditions are commonly reported. Such alkaline separation condition promotes decomposition of catechins. The lower sensitivity would not be a limitation in the analysis of tea infusions and extracts; however, in more sensitive applications, such as in analyzing cosmetics and supplements, MEKC would be the preferred choice.

6.2.8 Near-Infrared Reflectance Spectroscopy (NIRS)

Near-infrared reflectance spectroscopy (NIRS) is a fast and nondestructive technique suitable for the routine analysis of a large number of samples. Previously, several studies have reported the use of NIRS in the estimation of total polyphenols, total free amino acids, and tannins in tea.[80,81] More recently, this technique has been applied to predict the content of individual catechins in tea. Schulz et al.[82] reported an NIRS technique for estimating the contents of EG, EGC, ECG, and EGCG along with gallic acid and purine alkaloids. A partial least squares (PLS) algorithm was employed for the NIR calibration, using data from 95 tea leaf samples in one common set. A high correlation was observed between NIRS predicted and HPLC-UV determined values for EC, ECG, EGC, and EGCG ($R^2 > 0.85$). Moreover, this NIRS method was capable of differentiating tea leaves based on their age by applying principal component analysis (PCA). Younger leaves might be reflected by a higher level of EGCG and ECG but lower level of EC than older ones. An important advantage of NIRS over chromatographic techniques such as HPLC is the speed of data acquisition. Individual catechins can be measured simultaneously and analysis time may be as low as 1 minute. Another NIRS method was developed by Luypaert et al.[83] for the analysis of EGCG, EC, and caffeine in tea leaves. The calibration models for EGCG and ECG were developed for whole tea leaves, and the predicted content was compared to that quantified with an HPLC-UV method. A better correlation coefficient

was obtained for EGCG ($R^2 > 0.83$) using standard normal variate transformation (SNV)-corrected spectra. For ECG, SNV-processed samples gave $R^2 > 0.44$, which was considered to be insufficient for accurate quantification. The poor prediction results could be due to the low EC concentration in the tea leaves. On the other hand, NIRS was found to give an acceptable prediction for the total antioxidant capacity ($R^{24} > 0.85$) in ground tea leaves.

6.2.9 NMR

The ^{1}H- and ^{13}C-NMR spectra and the absolute assignments of eight major green tea catechins, EGCMG, and EZ were reported by Davis et al.[84] Thus, tea catechins could be identified on the basis of specific ^{1}H- and ^{13}C-shift patterns. Le Gall et al.[85] reported a high-resolution ^{1}H-NMR method for simultaneous analysis of catechins, amino, organic, phenolic, and fatty acids as well as sugars from a green tea extract. Employing both one- and two-dimensional ^{1}H-NMR techniques, five predominant (C, EGC, EC, EGCG, and ECG) and five unknown catechins were identified. The characteristic signals arising from catechins included H-3 (3.80–5.20 ppm) and H-4 (2.50–3.10 ppm) of the C-ring, which were confirmed by a cross-signal of the two protons in H-H COSY spectra. Using PCA and cluster analysis, the high-quality Longjing-type tea was differentiated from most other Chinese teas, and it appeared that EGCG, ECG, theanine, gallic acid, and caffeine were present in higher amounts in Longjing tea. Van Dorsten et al.[86] investigated the applicability of ^{1}H-NMR for the analysis of catechins/catechin metabolites in biological fluids. After tea consumption, catechin metabolites were observed in the urine samples in regions spanning 6 to 7.3 ppm; however, further characterization was limited by their low concentrations. Gong et al.[87] reported the use of ^{13}C-NMR for identification of four catechins (EC, EGC, ECG, and EGCG) together with purine alkaloids and sugars in green tea extracts. The profiles generated revealed the decreased levels of catechins in fermented tea compared to green tea. Solid-state CP-MAS NMR was first applied in Martinez-Richa and Joseph-Nathan's study for chemical profiling of teas.[88] Analysis of a black tea sample revealed a signal at 64.5 ppm for C-3, and further peaks belonging to catechins were identified in the aromatic region (98.5–155.7 ppm). A carbonyl peak appearing at 168.4 ppm was attributed to the gallate ester moiety in gallated catechins.

6.2.10 High-Speed Countercurrent Chromatography (HSCCC)

HSCCC is a liquid-liquid chromatographic technique that employs a coil planet centrifuge to retain the stationary phase while an immiscible mobile phase is continuously delivered through the column. Solutes are resolved via repetitive partitions between the liquid phases. HSCCC has been applied for the preparative separation of catechins from tea extracts. Amarowicz and Shahidi[89] employed CCC for the preliminary purification of catechins using water-chloroform (1:1, v/v) and water–ethyl acetate (1:1, v/v) as the solvent system. The crude sample obtained was then subjected to Sephadex LH-20, followed by preparative HPLC to obtain pure EC, EGC, ECG, and EGCG. A limitation of this method is that it requires time-consuming multiple chromatographic steps to achieve separation. Degenhardt et al.[90] reported

a one-step HSCCC method for the separation of catechins. Using a solvent system of hexane–ethyl acetate–methanol–water (1.5:5:1.5:5, v/v/v/v) at a flow rate of 2.8 ml/min, multimilligram quantities of EGCG, ECG, and epiafzelechin gallate (EAG) were resolved in 300 minutes. Although EGCG was found to coelute with caffeine, the latter can be conveniently removed by extraction with chloroform. Cao et al.[91] first described the analytical separation of EGCG, GCG, and ECG by CCC. Two solvent systems were evaluated. The first one consisted of ethyl acetate–ethanol–water in different ratios and catechins were separated using a stepwise elution. Initially, the column was eluted with ethyl acetate–ethanol–water (25:1:25, v/v/v) until all polar impurities were washed out. Then, a 10:1:10 (v/v/v) ratio was used to elute EGCG. Finally, a 5:1:5 ratio was employed for the elution of GCG and ECG. The second solvent system consisted of hexane–ethyl acetate–water and the three catechins were well resolved by the following solvent ratios: 1:10:10 (v/v/v), 1:20:20 (v/v/v), and 1:30:30 (v/v/v). The entire analysis takes less than 5 hours, and solvent consumption was reduced compared to preparative CCC. The most comprehensive chemical profiling of tea extracts by analytical HSCCC was demonstrated in Yanagida and coworkers' study.[92] EC, ECG, EGC, and EGCG were analyzed along with theaflavins, gallic acid, and purine alkaloids using a solvent system of tert-butyl methyl ether–acetonitrile–0.1% aqueous trifluoroacetic acid (TFA) (2:2:3, v/v/v), and the analysis was completed in 80 minutes. Theaflavins eluted quickly as a mixture, and were followed by catechins and finally purine alkaloids. Among the five catechins analyzed, only ECG and EGCG were baseline separated, while the peaks of EGCG, EC, and GA partially overlapped with each other. The method has been successfully applied for the identification of catechins and caffeine in green tea and black tea extracts. Two peaks (6 and 60 minutes) were present in black tea but not in green tea, and they were assigned as theaflavins and thearubigins, respectively. In addition, several food-related polyphenols such as procyanidins and phenolic acids have also been successfully separated under identical HSCCC conditions.

6.2.11 Planar Chromatography

Thin-layer chromatography (TLC) is a cost-effective tool for the determination of catechins from tea extracts. Using unmodified silica gel, Zhu and Xiao[93] successfully separated five catechins with a solvent system of chloroform–ethyl formate-n-butanol–formic acid. Quantification was carried out by UV detection at 254 nm. The resolution of silica gel can be further improved by chemically modifying the stationary phase.[94] Comprehensive separation of all eight major catechins by high-performance TLC (HPTLC) was first reported by Vovk et al.[95] Chromatography was performed on Merck precoated HPTLC cellulose plates and catechins were detected using a spray solution of vanillin-H_3PO_4 reagent. Using a solvent mixture consisting of 1-propanol–water–acetic acid (4:2:1, v/v/v), all catechins except EGCG were separated from each other, although the resolution was only good enough for qualitative purposes. On the other hand, a solvent system of 1-propanol–water–acetic acid (20:80:1, v/v/v) gave the best resolution among the five major catechins (EC, GC, EGC, ECG, and EGCG) present in green tea extracts, and densitometric evaluation could be performed. Thus, a combination of the above solvent systems may be

sufficient for profiling of the eight major catechins in tea as well as quantification of the five major ones, though the sensitivity and precision of the method have not been reported. Separation of catechins by two-dimensional paper chromatography has also been described.[96] Six catechins (C, EC, GC, EGC, ECG, and EGCG) were resolved on a Whatman no. 1 chromatographic paper using 10% acetone as the first solvent and the upper layer of n-butanol–acetic acid–water (4:1:5, v/v/v) as the second solvent. Catechins were visualized by (1) exposure to ammonia vapor, followed by short-wave UV analysis, (2) dipping in acidic ferric chloride–ferricyanide, or (3) spraying with diazotized sulfanilanmide (1%, w/v) in acetone (A), followed by HCl (35.4%) diluted with water (30:100, v/v) (B). The latter method is highly sensitive and selective for catechins, with LOD of less than 1 μg of C on the chromatograph. For quantification purposes the catechins were eluted from the paper using 60% acetone, reacted with reagents A and B for 1 hour, and then measured at 425 nm. For (+)-C, the absorbance at 425 nm was linear over the concentration range of 0.4–8 μg/ml.

6.2.12 Gas Chromatography (GC)

GC methods were developed in the 1960s for determination of catechins. In all cases, conversion of catechins to volatile trimethylsilyl (TMS) derivatives was required prior to GC analysis. Although the very early studies carried out GC analysis of catechins on glass columns with packing materials like 3% SE-30 and 3% OV-1, the derivatized catechins were well separated[97,98] and quantification of catechins could be accomplished when two separate runs were done.[97] These methods were time consuming and did not exhibit significant advantages over other techniques, such as HPLC and CE. Recently, however, GC-MS was proposed as a highly sensitive method for the detection of C, EC, and four of their methylated derivatives (3'-*O*-MeC, 3'-*O*-MeEC, 4'-*O*-MeC, and 4'-*O*-MeEC).[59] Separation was carried out on a DB-23 capillary column, and the base peaks of catechins (m/z 368) and methyl catechins (m/z 310) were monitored by electron impact mass spectrometry (EI-MS) in SIM mode. LOD (2–3 pg) of GC-MS is over a thousand times more sensitive than HPLC coupled to fluorescence or electrochemical detector. Besides, the selectivity of MS detection means that GC-MS is readily amenable for analysis of these catechins in biological fluids. However, as the authors acknowledged, one of its limitations is that it cannot directly analyze the polar conjugated catechin metabolites present in biological fluids. In addition, separation of GC, CG, GCG, and other minor catechins have not been reported thus far. Further developments in GC separation are needed in order to expand its applications in the analysis of tea bioactive components.

6.2.13 Spectrophotometry

Diethyl amino cinnamaldehyde (DMACA) can form a green complex with catechins rapidly, with a peak absorbance at 637 nm.[99] Due to its specificity, DMACA was utilized for spectrophotometric determination of total catechins from biological fluids.[100] Catechins were adsorbed onto alumina, reacted with DMACA under strong acidic conditions, and subsequently desorbed. LOD of this method was 0.05 μmol/l. However, since this reagent also reacts with theaflavins and thearubigins, the accuracy of this method may be compromised when black tea is analyzed for its catechin

content. Another specific dye for catechins is diazotized sulfanilanmide.[96] It forms a colored complex (maximum at 425 nm) by reacting with the A-ring of catechins and excludes other types of polyphenols such as flavonols and phenolic acids. The reactivity of this reagent toward theaflavins and thearubigins has not been reported**,** but it is likely due to the structural similarity of catechins, theaflavins, and thearubigins.

6.3 ANALYSIS OF THEAFLAVINS

The extraction conditions of theaflavins are largely identical to those of catechins, with the use of either boiling water[101,102] or organic solvents.[103] Lee et al.[104] reported that under slightly acidic conditions (pH 6.5), theaflavins are stable, but they degrade slowly at pH 7.0 and 7.5, and rapidly at pH 9.0. Nevertheless, Temple and Clifford[101] showed that degradation in water extracts was insignificant even after extended periods (815 minutes). In most studies, only four major theaflavins, TF, TF-3-G, TF-3'-G, and TF-3,3'-diG were analyzed. Other theaflavins, such as theaflavates A and B, theaflavic acids, are rarely characterized. A comprehensive profiling of theaflavins is thus lacking. Recent developments have mainly been focusing on the detection and quantification of the four major theaflavins in different biological fluids and tissues in an effort to understand their bioavailability and assess their relevance to the health-promoting effects of black tea. On the other hand, methods have also been developed to correlate theaflavin levels to the sensory quality of black tea.[105] HPLC is the predominant technique for the analysis of theaflavins. Capillary electrophoresis, particularly CZE, has also been employed. Other alternative techniques include NIR, HSCCC, and spectrophotometry, among others.

6.3.1 HPLC–UV Detection

The first HPLC methods for the analysis of black tea theaflavins were reported by Hoefler and Coggon[106] and Robertson and Bendall.[107] These methods employed C14 or ODS reverse-phase packing materials eluted with a mobile phase of 29% (v/v) aqueous acetone containing 1% acetic acid. However, such systems have poor resolution and all theaflavins were eluted with a certain degree of overlapping, which only allowed quantitative estimation of total theaflavins present. Bailey et al.[108] demonstrated good separation of the four major theaflavins along with four minor theaflavins. The system consisted of a Hypersil ODS column with a citrate buffer. This represents the most comprehensive HPLC method for theaflavins to date. The four major theaflavins possess three absorption maxima at around 280, 373, and 450 nm. Thus, UV detection has been commonly employed. Temple and Clifford[101] studied the levels of theaflavins in decaffeinated black tea with UV detection. The authors observed that quantification of theaflavins at 450 nm was most specific and gave superior repeatability. However, in many studies wavelengths of 205 and 280 nm were selected,[73,109] as these allow the simultaneous analysis of catechins and caffeine. At 205 nm, LsOD of theaflavins were around 0.5 μg/ml.

6.3.2 LC-MS

Mulder et al.[110] developed an LC-ESI-MS/MS method for the analysis of theaflavins in biological fluids. ESI-MS analysis gave the parent ion as the base peak. Positive-mode tandem MS/MS analysis of the parent $[M+H]^+$ ion revealed that the m/z 139 fragment generated from retro Diels-Alder fragmentation of the unmodified A-ring gave the base peaks for TF, TF-3-G, TF-3'-G, and TF-3,3'-diG. Subsequently, the authors evaluated the performance of MS for determination of TF in plasma using two detection modes: SIM and multiple-reaction monitoring (MRM). LC-SIM-MS in negative-ion-mode monitoring of m/z 562.9 ($[M-H]^-$) gave a LOD of 6 ng/ml; however, background noise was high when biological samples were analyzed. In positive ion MRM-MS/MS, m/z 565 and m/z 139 were selected as precursor and daughter ions, respectively. Due to multiple selection events, MRM was found to be highly sensitive (0.05 ng/ml). In addition, its unique selectivity allowed the detection and quantification of TF in urine and plasma samples. TF analysis using an APCI interface has also been reported.[111] At low cone voltage (10 V), $[M+H]^+$ molecular ions were observed in the total ion chromatogram (TIC). More structural information was obtained by raising the cone voltage to 40 V to induce fragmentation. In all theaflavins, a characteristic fragment $[M+H-138]^+$ was found, corresponding to the loss of an unmodified A-ring as a neutral species. Menet et al.[103] utilized matrix assisted laser desorption ionization (MALDI)-MS for the analysis of seven black tea theaflavins, including four major TFs, theadibenzotropolone A, and theaflavates A and B. The adduct ions $[M+H]^+$, $[M+Na]^+$, and $[M+K]^+$ were found in the MS spectra of the theaflavin standards. Noticeable fragmentations included the loss of gallate ester moiety (–169 Da) from TF-3-G, TF-3'-G, TF-3,3'-diG, and theaflavate; retro-Diels-Alder (RDA) rearrangement with neutral loss of 138 Da in TF-3,3'-diG; and cleavage of ester function in theadibenzotropolone A, leading to an ion at m/z 548. Theaflavins in black tea were separated on a Sephadex LH-20 column with acetone–water and identified on the basis of their MS spectra, although TF-3-G and TF-3'-G could not be differentiated since they had identical fragment ions. Direct analysis of crude tea extract is possible, as MALDI is tolerant to impurities. In addition, the MALDI-MS technique is highly sensitive and, when coupled to HPLC, may be useful for the analysis of theaflavins in biological matrices.

6.3.3 LC–Electrochemical Detection

The sensitivity and specificity of HPLC-ECD make it another valuable tool for determination of theaflavins in biomatrices. Analysis of black tea theaflavins in biological tissues and fluids was first reported by Lee et al.[55] The biological samples containing theaflavins were first digested with β-D-glucuronidase and sulfatase, and the theaflavins extracted into ethyl acetate. Separation was performed on a C_{18} column using gradient elution of solvent A (100 mM sodium phosphate, 1.75% acetonitrile, and 0.12% tetrahydrofuran) and solvent B (15 mM sodium phosphate, 58.5% acetonitrile, and 12.5% tetrahydrofuran). Catechins and theaflavins were detected by a coulometric array detector with potentials set at –90, –10, 70, and 150 mV. The four major theaflavins were baseline separated and identified by reference to the retention time and electrochemical profile of reference standards. Quantification was carried

out at 70 mV, with LsOD between 5 and 10 ng/ml. This method also successfully demonstrated the presence of theaflavins in saliva samples.[55,104]

6.3.4 CE

While MEKC is the CE method of choice for catechin analysis, the determination of theaflavins by this technique was met with little success. Larger et al.[71] observed no peaks after injection of theaflavin standards, and proposed that theaflavins were either adsorbed or degraded by the capillary wall. A CZE method was later developed by Lee and Ong[73] for the analysis of catechins and theaflavins. A running buffer of 200 mM boric acid (pH 7.2), 10 mM KH_2PO_4 (pH 4.2), 4.5 mM β-CD, and 27.5% (v/v) acetonitrile separated the four major theaflavins within 6 minutes. However, the reproducibility of analysis was relatively poor with RSD < 32.2%. Wright et al.[112] reported the use of nonaqueous capillary electrophoresis (NACE) for the determination of theaflavins. The authors found that aqueous CZE analysis gave significant peak broadening and high signal variations. Using a nonaqueous running buffer consisting of acetonitrile–methanol–acetic acid (71:25:4, v/v/v) and 90 mM ammonium acetate, the four major theaflavins were baseline separated in 10 minutes. With UV detection at 380 nm, the LsOD varied between 21 and 25 μg/ml and reproducibility was high with intra- and interday variations less than 1.6 and 3.5%, respectively.

6.3.5 HSCCC

Yanagida et al.[92] used HSCCC for analytical separation of theaflavins. As the solvent system was optimized for separation of catechins, theaflavins eluted as a mixture. Meanwhile, several reported preparative methods suggest that the separation of theaflavins by HSCCC is feasible. In an effort to separate TF and TF monogallates from black tea,[113] the tea extracts were first passed through a Sephadex LH-20 column to remove flavanols, and the fraction containing theaflavins was then resolved on an HSCCC machine using a solvent system of hexane–ethyl acetate–methanol–water (1.5:5:1.5:5, v/v/v/v). TF was separated with good purity, while TF monogallates contained a mixture of two isomers. In other studies,[114,115] a similar solvent system of hexane–ethyl acetate–methanol–water with slightly different ratios (1.25:5:1.25:5 or 1:3:1:6, v/v/v/v) was employed for the separation of theaflavins. Pure TF and TF-digallate were obtained using isocratic elution, but acceptable separation of TF monogallate isomers has not been achieved in these studies. Moreover, sample purification was required for the removal of catechins prior to analysis. In Cao et al.'s study,[114] an alternative purification procedure was developed using gradient elution HSCCC. With the same solvent mixture starting with a ratio of 1:5:1:5 (v/v/v/v) and ending with 2:5:2:5 (v/v/v/v), theaflavins were successfully separated from catechins.

6.3.6 Spectrophotometry

The Flavognost method involves a simple spectrophotometric procedure for determination of total theaflavins in black tea extracts.[116] A tea infusion was filtered and mixed with an equal portion of isobutylmethylketone (IBMK). The theaflavins

extracted into IBMK were reacted with Flavognost reagent (2 g of diphenylboric acid-2-aminoethyl ester), and the absorbance read at 625 nm. Recently, Obanda et al.[117] applied this spectrophotometric method for the estimation of total theaflavins, and it was found that the levels of theaflavins are strongly correlated to the liquor brightness of black tea extracts. However, spectrophotometric measurement cannot quantify the amount of individual theaflavins, and has been largely replaced by newer and more specific techniques such as HPLC and CE.

6.4 ANALYSIS OF METHYL XANTHINES

Caffeine (1.5–4%), theophylline (0.2–0.4%), and theobromine (~0.02%) are the three major methyl xanthines present in tea.[118] These methyl xanthines play an important role in determining tea quality, and their compositions are relatively unaffected by the fermentation process. Extractability of the three major alkaloids was found to be low using water or pure organic solvents such as acetone, methanol, and acetonitrile.[119] In water, pure acetone, and acetonitrile extracts, theophylline was not detected. Improved extraction efficiency was obtained using water–organic solvent mixtures, with the best results achieved using 70% methanol. On the other hand, using an elevated extraction temperature, such as boiling water, can also successfully extract all three alkaloids.[118] To facilitate analysis, several strategies have been adopted to remove polyphenols from the sample matrices. The simplest method is liquid-liquid extraction with chloroform–2-propanol (80:20, v/v).[120] Polyphenols in tea extracts can also be eliminated by passing the extracts through a basic Al_2O_3 column[121] or heavy MgO column.[122] However, these methods are tedious and time consuming. Polyvinylpolypyrrolidone (PVPP), an excellent adsorbent of polyphenols,[123] can be added to samples to remove polyphenols prior to the analysis of caffeine. Alternatively, Nakakuki et al.[124] employed a PVPP precolumn for on-line removal of polyphenols, with greater efficiency than the batch treatment method. Cleanup on C_{18} cartridges was reported by Naik and Nagalakshmi.[125] Aqueous extracts were first passed through cartridges and the eluent discarded. Caffeine was subsequently eluted with chloroform. SPE is also a popular technique for preconcentration of methyl xanthines present in biological fluids. These cleanup procedures remove major interferences to facilitate rapid analysis. Major techniques employed for analysis of methyl xanthines are HPLC and to a lesser extent CE.

6.4.1 HPLC–UV Detection

Methyl xanthines in tea extracts have been widely analyzed using reversed-phase C_{18} column with UV detection. They have absorption maxima at 210 and ~274 nm, the latter being more often reported as it is more specific. HPLC conditions employed varied according to the level of interferences in the extracts. Fernandez et al.[126] reported simultaneous analysis of five catechins and three methyl xanthines by RP-18 HPLC. Without pretreatment of samples, complete separation was achieved in 18 minutes using a gradient mobile phase of water–formic acid–acetonitrile. Isocratic elution with methanol–0.1% ortho–phosphoric acid (20:79.9:0.1, v/v) for the analysis of the same components required 60 minutes.[127] Such time-consuming

analysis is not suitable for rapid and routine analysis. Suitable sample pretreatment dramatically shortens analysis time. Nakakuki et al.,[124] using a PVPP precolumn for cleanup, analyzed caffeine and theobromine in 10 minutes under isocratic condition with a mobile phase composed of water–acetonitrile–methanol–phosphoric acid (82.5:11:6:0.5, v/v). Brunetto et al.[128] reported an on-line SPE technique for the prepurification of methyl xanthines. The samples were loaded onto a C_{18} precolumn, washed with 1% methanol, and backflushed with 20% methanol into an analytical C_{18} column for separation. Ten minutes were sufficient for the analysis, including the washing step. Apart from RP columns, these alkaloids can also be separated by ion exchange columns. Chen and Wang[129] proposed two ion chromatographic methods for separation of caffeine, theobromine, and theophylline in tea extracts. In cation exchange mode, the compounds were separated on two serially connected Dionex HPIC-CS3 columns eluting with 100 mM HCl. These alkaloids were also separated on a Dionex OmniPac PAX-100 anion exchange column with 15 mol KOH–1% acetonitrile as the mobile phase. Both analyses were accomplished in 10 minutes and required no sample pretreatment. For routine analysis, however, cation exchange mode was the method of choice due to the better precision and accuracy of quantification. In all these cases, the elution of methyl xanthines was monitored at 272–275 nm with LsOD in the lower μg/ml range. Despite limitations on sensitivity, LC-UV has frequently been employed for analysis of methyl xanthines in biological fluids. To reduce the complexity of sample matrices, liquid-liquid and particularly SPE have been used prior to LC-UV analysis. Zydron et al.[130] reported a SPE-HPLC system for the analysis of three methyl xanthines and eight metabolites present in urine. SPE was done on an RP-18 cartridge eluted with a mixture of chloroform and 2-propanol (80:20, v/v), and HPLC-UV analysis on an ODS column through a gradient mobile phase containing 0.05% TFA and acetonitrile. Methyl xanthines in urine could be detected at levels of ~0.05 μg/ml.

6.4.2 LC-MS

Zhu et al.[131] developed an HPLC-ESI-MS method for simultaneous analysis of catechins, purine alkaloids, theanine, and chlorogenic acid in tea samples. Separation was performed on an ODS column with a gradient mobile phase of 0.5% formic acid and acetonitrile. For the three purine alkaloids, positive ion mode was favorable, producing abundant protonated $[M+H]^+$ ions at a voltage of 20 V. Quantification was carried out in SIM mode at m/z 195 for caffeine, and m/z 181 for theophylline and theobromine. LsOD ranged from 0.02 to 0.03 ng per injection for the three methyl xanthines. Determination of catechins and purine alkaloids by LC-ESI-MS/MS was also reported by Del Rio et al.[20] Tea extracts were separated on a reversed-phase column, and the mobile phase was a gradient of 1% formic acid and acetonitrile. In positive ion mode, caffeine and theobromine were identified by comparison of the molecular ion $[M+H]^+$ and retention time with those acquired from standard compounds. LC-MS methods also find applications in the analysis of methyl xanthines in biological fluids.[132,133] However, these studies required SPE for cleanup of samples prior to analysis. Taking advantage of the unique selectivity of MS/MS detection, Schneider et al.[134] developed a simplified (without sample cleanup) LC-ESI-MS/MS

method for the determination of urinary methyl xanthines. The three major methyl xanthines and eight metabolites were identified and quantified in MRM mode. Due to the two selection events, metabolites were detected without interference even if their elution time did not differ significantly. Sensitivity was high, with LsOD ranging from 0.05 to 1 μM. LC-MS/MS is thus a promising method for the detection of methyl xanthines in complex matrices.

6.4.3 CE

CE methods for determination of methyl xanthines afford rapid separation and short analysis time compared to conventional HPLC. In most cases, a running buffer of alkaline pH is chosen. Since methyl xanthines are weak bases, they remain uncharged and migrate with electro-osmotic force under acidic or neutral pH. On the other hand, incorporation of SDS micelles in MEKC also allows satisfactory separations under acidic pH. Among the two CE methods, MEKC is the more frequently reported separation mode. Zhao and Lunte[135] described a rapid MEKC-UV method for the determination of caffeine, theobromine, theophylline, paraxanthine, and 1,3,7-trimethyluric acid. An optimized buffer of 20 mM phosphate (pH 11.0) and 40 mM SDS allowed baseline separation of all five methyl xanthines. It represents the benchmark for the shortest analysis time for tea methyl xanthines by CE. Beverages, pharmaceuticals, and rat serum could be analyzed directly with no sample preparation other than a simple filtration step, and the detection limit was <1.5 μg/ml. The selectivity and sensitivity of detection of methyl xanthines could be further improved by coupling MEKC with amperometric detection.[136] Caffeine, theobromine, and theophylline were separated by 20 mM phosphate buffer (pH 8.5) containing 35 mM SDS, and detection was performed with a carbon disc electrode set at +1.175 V. The LsOD were in the range of $1.0–2.0 \times 10^{-6}$ mol/l. An advantage of MEKC over CZE for the analysis of tea extracts is that it allows the use of an acidic mobile phase, and thus simultaneous analysis of methyl xanthines and catechins, the latter of which otherwise would be degraded by the alkaline pH employed in CZE. Gotti et al.[137] applied a cyclodextrin (CD)-modified MEKC for separation of six catechins and three methyl xanthines. Under acidic conditions (pH 2.5), the analytes were resolved in a buffer of 90 mM SDS with the addition of 10 mM hydroxyl-β-CD within 15 minutes, and the elution profile was monitored by UV at 220 nm. Although the analysis of methyl xanthines by CZE is less common, emerging studies have indicated that it also constitutes a useful alternative to MEKC. A rapid CZE method for the analysis of five methyl xanthines, including caffeine, theobromine, theophylline, dyphylline, and enprophylline, was developed by Regan and Shakalisava.[138] Separation was performed using 20 mM borate buffer at pH 9.4, and the five components were resolved within 2 minutes. This method was successfully applied to the analysis of chocolate and pharmaceutical samples without significant interference. However, a limitation of the CZE technique is that caffeine, a major methyl xanthine in tea, remains uncharged and migrates with electro-osmotic force. If other neutral components are present in the sample, determination of caffeine has to be performed by other methods, such as MEKC. An alternative CZE buffer system consisting of carbonate–hydrogen carbonate buffer was developed by Geldart and Brown[139] for

the analysis of methyl xanthines and pyrimidines.[139] The authors noted that this CZE buffer is compatible with MS detection with the use of a volatile carbonate buffer (NH_4CO_3). This idea was realized by Peri-Okonny et al.,[140] who proposed a CZE-MS method for the analysis of caffeine and its metabolites in urine. Urine specimens were first acidified and cleaned by SPE. A buffer system of 50 mM ammonium carbonate (pH 11.0) achieved the separation of caffeine and its eleven metabolites in 32 minutes. The total ion current electropherogram (TICE) profile was registered using ESI-MS. With detection limits of 0.02 μg/ml, it is more sensitive than UV detection. Taking into consideration the fact that more selective and sensitive MS modes such as SIM and MRM in MS/MS^n were not applied in this study, CE-MS holds great potential in the identification of minute amounts of methyl xanthines in complex biological matrices.

6.5 ANALYSIS OF THEARUBIGINS

Thearubigins are polymeric catechins present in fermented black and partially fermented oolong teas. Although they constitute a major portion of polyphenols in black tea (47%),[14] the structures of these compounds remain poorly characterized. Isolation and analysis of thearubigins have proven to be difficult, and HPLC analysis is often characterized by an unresolved convex Gaussian hump in the chromatogram.[113] One way to classify thearubigins is based on their solubility in different solvents, as described by Roberts and Smith[141] and further modified by Brown et al.[142] Aqueous extract of black tea was extracted successively with chloroform, ethyl acetate, and n-butanol. The ethyl acetate and butanol extracts were then purified by precipitation with acetone-chloroform, acetone-ether, and methanol-ether to offer five fractions, TB1–TB5. The other way for thearubigin classification is by their order of elution in reverse-phase HPLC, as reported by Bailey et al.[143] Thearubigins excluded from, resolved by, and remaining unresolved by HPLC were classified into group I, II, and III pigments, respectively. Due to difficulties in separation, frequently the total thearubigins of the tea extracts is estimated, with or without liquid-liquid extraction. Thus, simple spectrophotometric assays are the predominant method employed. Recently, Menet et al.[103] also reported the analysis of partially purified thearubigins from black tea extracts using MALDI-MS.

6.5.1 Spectrophotometry

A commonly employed spectrophotometric method for the estimation of thearubigins and theaflavins was first described by Roberts and Smith,[141] and recently modified by Harbowy and Balentine[14] and Yao et al.[144] Based on this method, tea leaves are simmered in boiling water followed by solvent extraction to obtain several fractions. Spectrophotometric measurement of thearubigins was then monitored at 380 nm. Theaflavin and thearubigin contents were calculated using established formulae.[144] However, flavonol glycosides were also extracted and measured in the process, and these resulted in overestimation of thearubigin content.[145] Subsequently, several pretreatment methods have been developed to reduce the levels of interference in this assay. These include the use of C_{18} cartridge,[146] cellulose chro-

matographic column,[143] or solid-liquid extraction.[147] However, chromatographic approaches invariably suffer from substantial interaction of thearubigins with the solid support. In this regard, CCC, being a support-free chromatographic technique, is advantageous and has also been successfully applied for the purification of thearubigin fractions. Following cleanup on an Amberlite XAD-7 column, the tea extract was separated by a solvent system of ethyl acetate–butanol–water (2:3:5, v/v/v), and group I thearubigins were separated from other components in 2 hours.[113] Degenhardt et al.[148] also reported a centrifugal precipitation chromatography for fractionation of thearubigins. Under a centrifugal force field, tea extracts were precipitated in a hexane- or methyl-tert-butyl-ether rich solvent and exposed to an increasing ethanol concentration. This caused repetitive precipitation and dissolution in which the monomers were separated from the more polar polymeric thearubigins.

Thearubigins can also be determined indirectly by Folin-Ciocalteu assay.[149] This assay provides an estimation of the total polyphenol content in the tea extracts, including monomeric polyphenols and polymeric thearubigins. The amounts of monomeric polyphenols, such as catechins, theaflavins, and flavanoids, can be determined by standard HPLC methods. Then, the total thearubigin content can be estimated by subtracting the amounts of monomeric polyphenols from the total; however, this is tedious and time consuming. In addition, the response factor of thearubigins to Folin-Ciocalteu reagent has not yet been made available, leading to errors in the estimation.[113]

6.5.2 Mass Spectrometry

Menet et al.[103] studied a black tea extract using MALDI-MS. First, fragmentation behavior of catechin polymers was evaluated using three model compounds, theaflavate A, theaflavate B, and theadibenzotropolone. Characteristic mass loss of 153 or 169 Da was observed due to loss of gallate ester, while mass loss of 427 Da occurred when it is a theaflavate-type trimer with a benzotropolone nucleus. The structures of polymers in black tea extracts were then proposed based on observed mass loss. The black tea extract was first partitioned into chloroform, ethyl acetate, and n-butanol, and the n-butanol layer was directly analyzed with MS. MS spectra of the n-butanol fraction revealed many spots with m/z higher than 1,000. A peak at m/z 1,173 corresponds to $[M+H^+]$ of one unknown. Mass loss of 427, 153 Da indicates that it contained a galloyl group and a benzotropolone nucleus. This unknown can be derived from condensation of a gallate ester of a catechin dimer (914 Da) and the B-ring of EC/C (290 Da) to form a benzotropolone skeleton with mass loss of 32 Da (914 + 290 – 32 Da). Condensation of two gallate esters of a catechin dimer with EC or C to form a tetramer explained the presence of a peak at m/z 1431 (914 + 290 + 290 – 32 – 32 Da). Other tetramers could also be formed via condensation with EGC or GC, whose free –OH groups can be further esterified with gallic acid to give peaks with higher masses. Given the lack of progress in the separation of thearubigins, direct MS analysis could well be an important tool for the analysis and structural assignments of thearubigins.

6.6 CONCLUSIONS

In recent years, many analytical methods have been developed for the analysis of major bioactive components in tea, including catechins, theaflavins, thearubigins, and methyl xanthines. HPLC methods are by far the most common procedures employed. Versatility of HPLC is due to its high separation power and its capabilities of coupling to different highly sensitive and selective detectors such as ECD and MS. LC-MS, in particular, is a powerful tool for studying these bioactive components in complex biological matrices. CE is also an effective alternative technique for analysis of different bioactive components in tea. It offers rapid, reliable separation and good resolving power in many cases. Currently, the sensitivity of CE methods is limited by the use of UV detectors employed in the majority of the studies. Further developments in CE-ECD and CE-MS methodologies could greatly enhance the usefulness of this technique. Other techniques, such as GC, TLC, HSCCC, NIR, and NMR methods, have also been developed, but at present, HPLC and CE are the most promising tools for routine quality control of tea extracts, as well as for investigating the pharmacokinetic behavior of bioactive tea components and their metabolites.

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7 Bioavailability and Metabolism of Tea Catechins in Human Subjects

Ting Sun, Chi-Tang Ho, and Fereidoon Shahidi

CONTENTS

7.1 INTRODUCTION

Tea is an ancient beverage that has been consumed for thousands of years. Health benefits of tea have been well documented.[1,2] The long safety record of tea consumption makes tea components attractive targets for ingredient development for nutraceuticals and functional foods, as well as for drug discovery. Information related to the chemistry, synthesis, pharmacokinetics, and pharmacodynamics of tea compounds has recently been reviewed.[3]

Green tea and black tea are produced using different processing methods for tea leaves of *Camellia sinensis*. Green tea is produced by drying the leaves of *Camellia sinensis* at elevated temperatures to prevent polyphenol oxidation. The major

components of green tea are catechins, including (–)-epicatechin (EC), (–)-epigallocatechin (EGC), (–)-epicatechin gallate (ECG), and (–)-epigallocatechin gallate (EGCG) (figure 7.1), which are similar to those in the fresh leaves. The total amount of the four catechins in green tea is approximately 25% on dry weight basis. EGCG is the most abundant and well-studied tea catechin and accounts for 65% of the total catechin content in green tea.[4,5] The other compounds in green tea, such as catechin and its other gallates, are present in small amounts.[5] Black tea is produced by withering, rolling, and crushing fresh leaves, which initiate fermentation of polyphenols and conversion of some catechins to theaflavins and thearubigins. The total flavanol level is reduced from between 35 and 50% in green tea to 10% in black tea. Theaflavin and thearubigin contents in black tea are 3–6% and 12–18%, respectively.[6]

In the past 20 years, laboratory research and epidemiological findings suggest that tea catechins may reduce the risk of several diseases, such as cancer and coronary heart disease, which could be due, at least in part, to the presence of catechins that have antioxidant properties and can inhibit cell proliferation, regulate carcinogen-metabolizing enzymes, and induce apoptosis.[7]

Green tea may be available in the market in several forms: green tea (GT), decaffeinated green tea supplement (DGT), Polyphenon E (a decaffeinated and defined green tea catechin mixture), and green tea extract supplement (GTS), among others.[8] Little research has been done on the bioavailability and metabolism of theaflavin, the major component of black tea,[9] because this compound is highly polymerized and less well characterized. This review provides a cursory account of the metabolism and bioavailability of EC, ECG, EGC, and EGCG in humans, although more research on bioavailability of tea catechins has been performed in animals (mouse and rat) than in humans.

7.2 BIOAVAILABILITY AND METABOLISM OF TEA CATECHINS IN HUMANS

It is very important to know the bioavailability and biotransformation of tea catechins in humans because the pharmacokinetics, absorption, distribution, metabolism, and excretion of green tea determine its potential bioactivities in disease prevention *in*

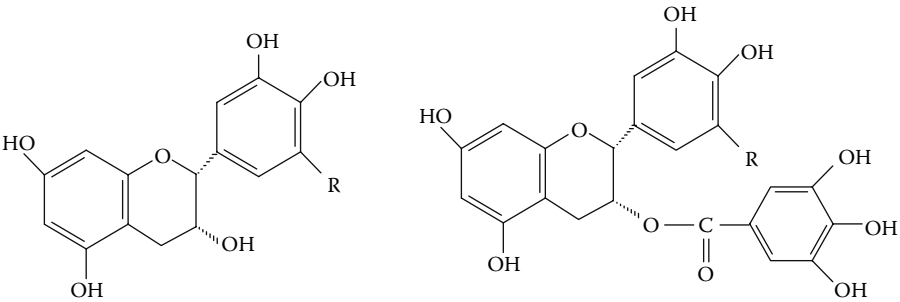

FIGURE 7.1 Structures of tea catechins (EC, EGC, ECG, and EGCG).

vivo. Bioavailability is to describe the concentration of a given compound or its metabolite at the target organ. Due to difficulties in accessing organ sites *in vivo* in humans, it may not be possible to calculate the exact values. Thus, absolute bioavailability is often used to describe the exact amount of a compound that reaches the systemic circulation, which is calculated as the fraction of the area under the curve (AUC) after oral ingestion compared with the AUC after intravenous administration. Bioavailability includes several interlinked processes: liberation, absorption, distribution, metabolism, and excretion (LADME).[10] Although there are no data on the exact amount of catechins that are absorbed, the plasma kinetic profile and urine excretion amount suggest that only small proportions are absorbed. As metabolism and possible elimination in the bile can change amounts excreted in the urine, urinary excretion does not provide an accurate measure of the absorption. The bioavailability of catechins is affected by several factors, such as their chemical property, conjugation form in the intestine, intestinal absorption, and available enzymes catalyzing the metabolism.[11] Approximately 1.68% of the ingested catechins were found to be present in the plasma, urine, and feces in humans drinking black tea, and the apparent bioavailability of the gallated catechins was lower than that of the nongallated forms.[12]

7.2.1 Absorption

The major absorption of tea catechins is the small intestine. EC, ECG, EGC, and EGCG were disposed at the intestinal epithelium by similar efflux and metabolism mechanisms. Intestinal epithelial membrane transport of catechins was studied via perfusion of EC and ECG in the human Caco-2 cell line.[13,14] The absorption and secretion transports of the four catechins, in the form of individual pure compounds, pure compound mixtures, and green tea extract, were studied in the human Caco-2 cell model.[15] The results showed that the absorption transport of the four catechins in different doses was similar, but the green tea polyphenol mixtures could affect the secretion transport of individual catechins. It was suggested that the transporter competition may be responsible for the reduced efflux of EC, and metabolic competition may bring about a reduction in the formation of EGC sulfate and methylated EGC sulfate.[15]

7.2.2 Bioavailability and Biotransformation

7.2.2.1 Pharmacokinetic Study and Metabolism *In Vivo*

EC, ECG, EGC, and EGCG have relatively high molecular weights (290–458) and more than five hydroxyl groups. Due to their large apparent size, these catechins have a relatively low bioavailability.[16] Plasma levels of intact tea catechins and other flavonoids did not exceed 1 μM after consumption of catechins with amounts similar to those in the diet.[17] The total peak plasma concentrations of EGCG, EGC, and EC (free plus conjugated forms) were around 2–3 μM or lower.[18] These concentrations of catechins in plasma are much lower than those used in many *in vitro* cell line studies. The biological activities of glucuronide, sulfate, and methylated tea catechins

and their ring-fission metabolites need to be determined in order to understand the relationship between tea consumption and human cancer risk.[11]

After absorption, tea catechins (EC, ECG, EGC, and EGCG) are metabolized *in vivo* mainly through glucuronidation and sulfation pathways, which are important phase II metabolic reactions that can change catechins to more hydrophilic compounds.[19,20] In addition, methylation and microbial metabolism of tea catechins also occur (figure 7.2). The metabolic reactions are catalyzed by several enzymes. Microsomal UDP-glucuronosyltransferases (UGT) catalyze the glucuronidation of catechins with UDP-glucuronic acid. Cytosolic sulfotransferases (SULT) catalyze the sulfation of catechins with 3'-phosphoadenosine 5'-phosphosulfate (PAPS). Catechol-*O*-methyltransferases (COMT) catalyze the methylation of catechins with the methyl group from S-adenosylmethionine (SAM). The three reactions can occur at different sites of the same molecule of catechins, and catechins can be methylated followed by glucuronidation or sulfation at other sites of the same molecule.[11]

The metabolites of tea catechins that have been identified in humans are listed in table 7.1. Only the structures of some metabolites of EC, EGC, and EGCG have been determined. Other metabolites of these catechins in humans, especially glucuronide and sulfate conjugates, need to be identified. Little work has been done for identification of ECG metabolites in humans, and only free ECG and conjugated ECG after deconjugation have been determined.

The pharmacokinetics parameters of catechin metabolites are listed in table 7.2. Intact tea catechins, EC, ECG, EGC, and EGCG in human plasma and urine, are detected but with low concentrations. To determine the content of conjugated metabolites of catechins, blood and urine samples are treated with β-glucuronidase and sulfatase to remove the glucuonic or sulfate group prior to high-performance liquid chromatography (HPLC) analysis.

One major metabolite observed in human blood and urine samples after green tea administration was identified as 4'-*O*-methyl-EGC (4'-*O*-MeEGC). The human plasma level of 4'-*O*-MeEGC reached its peak value within the first 2 h of tea ingestion, which was four to six times higher than that of EGC.[19] The half-lives of EGC and 4'-*O*-MeEGC in the blood were 1.02 ± 0.07 and 4.39 ± 1.14 h, respectively. The amount of 4'-*O*-MeEGC excreted in urine was about three times higher than that of EGC, and 88% of 4'-*O*-MeEGC was excreted in the urine within 8 h.[19]

An EGCG metabolite, 4',4"-di-*O*-methyl-EGCG (4',4"-DiMeEGCG), was detected in human plasma and urine by LC/MS/MS following green tea ingestion.[21] The compounds 4',4"-DiMeEGCG and EGCG reached peak plasma values of 20.5 ± 7.7 and 145.4 ± 31.6 nM, respectively, after 2 h of tea consumption and had half-lives of 4.1 ± 0.8 and 2.7 ± 0.9 h, respectively.[21] Concentration of 4',4"-di-*O*-methyl-EGCG was 15% of EGCG in human plasma.[21] The cumulative urinary excretion of 4',4"-

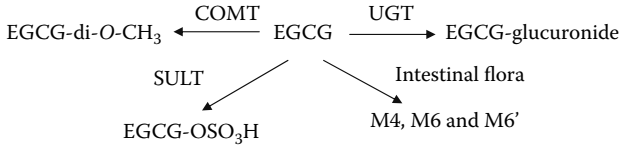

FIGURE 7.2 Biotransformation of EGCG *in vivo* by different pathways.

TABLE 7.1
Metabolites of tea catechins identified in humans after oral intake

Dietary catechin	Metabolites	Reference
EC	Free EC	43, 51
	(–)-EC-3'-*O*-glucuronide	43
	(–)4'-*O*-methyl-(–)-EC-5 (or 7)-*O*-glucuronide	43
	4'-*O*-methyl-(–)-EC-3-*O*-glucuronide	43
	Free and conjugate M6	21, 25, 28
ECG	Free ECG	51
EGC	Free EGC	51
	4'-O-methyl-EGC	19, 26, 28, 51
	Free and conjugate M4	21, 25
	Free and conjugate M6	21
	Free and conjugate M6'	21
EGCG	Free EGCG	29, 30, 50, 51
	4',4"-di-*O*-methyl-EGCG	8, 21
	Free and conjugate M4	21
	Free and conjugate M6	21
	Free and conjugate M6'	21

DiMeEGCG during a 24-hour period was about five times higher than that of EGCG, but the excreted 4',4"-DiMeEGCG and EGCG in urine only accounted for about 0.1% of the ingested EGCG.[21] Some 77.6% of EGCG and 60.6% of 4',4"-DiMeEGCG were in the free form in the plasma. In human urine, both EGCG and 4',4"-DiMeEGCG were mostly in the glucuronide-conjugated form. The compound 4',4"-DiMeEGCG only had about one-tenth of the activity of EGCG in scavenging 2,2'-diphenyl-1-picrylhydrazyl (DPPH) radical, but had a stronger activity than EGCG in inhibiting the spontaneous release of arachidonic acid in human colon cancer HT-29 cells.[21]

The major EGC and EC conjugated in human urine were monoglucuronides and monosulfates. For EC, methylation tended to occur at the 3'-OH or 4'-OH position,[22] and the sulfate conjugate could be the major metabolite in humans.[23] EGC-3'-*O*-β-glucuronide was the major glucuronide metabolite of EGC in human urine; the other EGC-*O*-monoglucuronide was less than 2% of the major one, and only a trace amount of EGC-*O*-sulfate was detected.[24] Ring-fission products of EGC, such as (–)-5-(3',4',5'-trihydroxyphenyl)-(-valerolactone (M4), were found in human plasma and urine mostly in glucuronide and sulfate forms.[25] Further degradation of the valerolactone to propionic acid and carboxylic acid is also expected.[11]

In human plasma, the percentage of methylation for EGC was higher than for ECG and EGCG.[26] The methylation of EGC could be catalyzed by catechol-*O*-methyltransferase in the liver or kidney.[27] In contrast, ECG and EGCG were methylated at much lower rates by this enzyme. After absorption, EGC is partly *O*-methylated and excreted in the urine at a large percentage (13.6%).[26] 4'-*O*-methyl-EGC accounted for 30–40% of the total metabolites of EGC.[26] ECG and EGC increased the antioxidant activity of human plasma, but EGCG did not increase it significantly.[26]

TABLE 7.2
Pharmacokinetic parameters of tea catechin metabolites in human plasma after oral consumption

Dietary catechin (mg)		Source	Metabolites in plasma	C_{max} (μM)	T_{max}(h)	AUC(μM·h)	$T_{1/2}$(h)	Absorption in plasma (%)	Excretion in urine (%)	Ref.
EC	32	DGT	Free + conjugated EC	0.2 at 1 h					6	31
EC	38	DGT	Free + conjugated EC	0.19	1.4	0.96	5.7		4	18
EC	75	DGT	Free + conjugated EC	0.65	1.8	3.46	3.4		5	18
EC	113	DGT	Free + conjugated EC	0.65	1.8	4.13	3.2		2	18
EC	76.5	GT	Free + conjugated EC	0.33	1.2	1.01		0.56	0.5	8
EC	39.8	BT	Free + conjugated EC	0.08	1.4	0.27		0.23	0.52	8
EC	38.3	GTS	Free + conjugated EC	0.24	2.5	0.89		0.75	1.26	
EC	32.0	GT	Free + conjugated EC						2.0	25
EC	45	GT	Free + conjugated EC	0.43	1.3	1.82	2.0	1.1	4.6	28
EC	17	DGT	Free + conjugated EC	0.11	1.0	0.53	2.5	0.71	8.9	28
EC	66.8	BT	Free + conjugated EC	0.17	7			0.06	0.44	12
EC	1050	Pure EC	Free EC	1.53 at 1 h						43
EC	1050	Pure EC	(–)-EC-3'-*O*-glucuronide	11.2 at 1 h						43
EC	1050	Pure EC	(–)4'-*O*-methyl-(–)-EC-5 (or 7)-*O*-glucuronide	0.26 at 1 h						43
EC	1050	Pure EC	4'-*O*-methyl-(–)-EC-3'-*O*-glucuronide	0.61 at 1 h						43
ECG	40	Polyphenon E	Free ECG	0.04 with food						51
ECG	40	Polyphenon E	Free ECG	0.20 no food						51
ECG	80	Polyphenon E	Free ECG	0.08 with food						51
ECG	80	Polyphenon E	Free ECG	0.39 no food						51
ECG	120	Polyphenon E	Free ECG	0.26 with food						51
ECG	120	Polyphenon E	Free ECG	0.87 no food						51

ECG	664	Pure ECG	Free + conjugated ECG	3.1	4	39.9	6.9		ND	26
ECG	119.3	GT	Free +conjugated ECG	0.082	1.4	0.32		0.12	0.02	8
ECG	122.5	BT	Free +conjugated ECG	0.07	1.5	0.29		0.11	0.01	8
ECG	130.0	GTS	Free+Conjugated ECG	0.16	2.8	0.7		0.2	0.02	8
ECG	124.6	BT	Free + conjugated ECG	0.05	24			0.03	0.04	12
EGC	97	Polyphenon E	Free EGC	0.13 no food						51
EGC	97	Polyphenon E	Free EGC	0.05 with food						51
EGC	97	Polyphenon E	Free + conjugated EGC	0.27 no food					2.67	51
EGC	97	Polyphenon E	Free + conjugated EGC	0.42 with food					4.85	51
EGC	459	Pure EGC	Free + conjugated EGC	5.0	1.4	20.1	1.7		9.8	26
EGC	118	GT	Free + conjugated EGC	1.0	0.5		1.0		14–52 EGC + 4'-*O*-methyl-EGC	19
EGC	82.3	GT	Free + conjugated EGC						1.5	25
EGC	269.6	GT	Free + conjugated EGC	0.74	1.3	2.59		0.36	0.19	8
EGC	103.4	BT	Free + conjugated EGC	0.22	1.5	0.97		0.26	0.27	8
EGC	24.9	GTS	Free + conjugated EGC	0.11	2.6	0.58		0.53	1.17	8
EGC	154	GT	Free + conjugated EGC	0.73	1.3	3.09	1.7	0.58	2.3	28
EGC	56	DGT	Free + conjugated EGC	0.26	1.1	0.96	1.5	0.53	3.3	28
EGC	61.9	BT	Free + conjugated EGC	0.17	5			0.06	0.57	12,26
EGC	459	Pure EGC	4'-*O*-methyl-EGC	1.9	2.0	12.6	2.5		3.8	25
EGC	118	GT	4'-*O*-methyl-EGC	5.4	2		4.4		14–52 EGC + 4'-*O*-methyl-EGC	19
EGC	154	GT	4'-*O*-methyl-EGC	5.3	1.7		4.4		8–31	28
EGCG		GTE	Free EGCG	0.22–0.49	1–3				ND	50
EGCG	200	Pure EGCG	Free EGCG	0.16	2.1	0.82	1.27			29
EGCG	400	Pure EGCG	Free EGCG	0.24	1.8	3.67	6.00			29

—continued

TABLE 7.2 (continued)
Pharmacokinetic parameters of tea catechin metabolites in human plasma after oral consumption

Dietary catechin	(mg)	Source	Metabolites in plasma	C_{max} (μM)	T_{max}(h)	AUC(μM·h)	$T_{1/2}$(h)	Absorption in plasma (%)	Excretion in urine (%)	Ref.
EGCG	600	Pure EGCG	Free EGCG	0.37	3.0	2.0	2.7			29
EGCG	800	Pure EGCG	Free EGCG	0.96	4.0	3.1	1.9			29
EGCG	50	Pure EGCG	Free EGCG	0.26	1.3	0.9	1.8			30
EGCG	100	Pure EGCG	Free EGCG	0.41	1.9	2.9	3.3			30
EGCG	200	Pure EGCG	Free EGCG	0.68	1.3	2.5	2.3			30
EGCG	400	Pure EGCG	Free EGCG	1.24	1.4	5.1	2.9			30
EGCG	800	Pure EGCG	Free EGCG	2.10	1.8	7.6	3.6			30
EGCG	1600	Pure EGCG	Free EGCG	6.35	1.4	21.2	4.9			30
EGCG	144	Pure EGCG	Free + conjugated EGCG	0.10	2	0.52	2.5			21
EGCG		GT	Free + conjugated EGCG	0.15	2	1.46	2.7		0.1(EGCG+4',4''-di-*O*-methyl-EGCG)	21
EGCG	688	Pure EGCG	Free + conjugated EGCG	1.3	2.9	12.1	3.9		ND	26
EGCG	146.2	BT	Free + conjugated EGCG	0.02	5			0.01	0.05	12
EGCG	50	Pure EGCG	Free + conjugated EGCG	0.28	1.4	1.0	1.9			30
EGCG	100	Pure EGCG	Free + conjugated EGCG	0.39	2.2	3.0	2.7			30
EGCG	200	Pure EGCG	Free + conjugated EGCG	0.72	1.4	2.7	1.9			30
EGCG	400	Pure EGCG	Free + conjugated EGCG	1.36	1.4	5.6	3.1			30
EGCG	800	Pure EGCG	Free + conjugated EGCG	2.33	1.8	8.4	4.4			30
EGCG	1600	Pure EGCG	Free + conjugated EGCG	7.40	1.3	22.6	4.6			30
EGCG	213.6	GT	Free + conjugated EGCG	0.08	1.3	0.27		0.07	0.06	8
EGCG	230.8	BT	Free + conjugated EGCG	0.1	1.4	0.37		0.08	0.05	8
EGCG	193.3	GTS	Free + conjugated EGCG	0.15	2.7	0.62		0.14	0.06	8

EGCG	195	Pure EGCG	Free + conjugated EGCG	0.08	1.6	0.47	3.7	0.1	ND	28
EGCG	195	GT	Free + conjugated EGCG	0.17	1.6	1.11	3.4	0.16	ND	28
EGCG	72.9	DGT	Free + conjugated EGCG	0.05	1.2	0.20	2.3	0.13	ND	28
EGCG	144	EGCG	4',4"-di-*O*-methyl-EGCG	0.02	2	0.10	2.8			21
EGCG		GT	4',4"-di-*O*-methyl-EGCG	0.02	2	0.16	4.1		0.1(EGCG+4',4"-di-*O*-methyl-EGCG)	21
EGCG, EGC, or EC	200	Pure EGCG, EGC, or EC	Free + conjugated M6	4.7	3–6					21
EGCG, EGC, or EC	200	GT	Free + conjugated M6	31.2	8–24				1.5–16 (M4 + M6 + M6')	21
EC	45	GT	Free + conjugated M6	0.36	10				11.2	21
EC	32.0	GT	Free + conjugated M6						17.5	25
EGC	82.3	GT	Free + conjugated M4						14.9	25
EGCG	200	Pure EGCG	Free + conjugated M4	8.3	3–6					21
EGC	200	Pure EGC	Free + conjugated M4	14	6–9					21
EGC		GT	Free + conjugated M4	2.8	3–8				1.5–16 (M4 + M6 + M6')	21
EGCG	200	Pure EGCG	Free + conjugated M6'	8.3						21
EGC	200	Pure EGC	Free + conjugated M6'	6.6						21
EGC		GT	Free + conjugated M6'	36.7	8–24				1.5–16 (M4 + M6 + M6')	21

Note: C_{max} is the maximum plasma concentration; T_{max} is the time to reach the maximum plasma concentration; AUC is the area under the plasma concentration–time curve; $T_{1/2}$ is the elimination half-life; absorption in plasma (%) is the percentage of the total dose of ingested tea catechins in plasma; and excretion in urine (%) is the percentage of the total dose of ingested tea catechins excreted in urine.

In human plasma, more EGCG was in the free form than EGC and EC, and EGC and EC were mostly in the conjugated form.[21,28–30] In the human plasma at 1 h after ingestion, 77% of EGCG was in the free form, while 31% of EGC and 21% of EC were in the free form.[21] In human plasma after consumption of DGT or pure catechins, more than 80% of green catechins in the plasma were in conjugate form, with two-thirds in sulfate form and one-third in glucuronide form. ECG was not detected in human plasma. The glucuronide, sulfate, and free form of EGC in plasma were 57–71%, 23–36%, and 3–13%, respectively. The glucuronide, sulfate, and free form of EGCG in plasma were 8–19%, 58–72%, and 12–28%, respectively.[31] More than 90% of EGC and EC in urine were in the conjugate form, mainly as sulfated.

Higher doses of catechins may saturate specific metabolic pathways, such as intestinal glucuronidation and *O*-methylation, and change the profile of conjugated metabolites. After oral intake of 200, 400, 600, and 800 mg of EGCG, the AUC for unconjugated EGCG increased disproportionately at 800 mg, which is probably due to saturation of the conjugation pathways.[29]

7.2.2.2 Microbial Metabolism

Tea catechins that are not absorbed in the small intestine reach the colon. Animal and human colons contain around 10^{12} microorganisms/cm^3. Bacterial enzymes catalyze many reactions that can break down tea polyphenols to simple compounds of phenolic acids and their glycone conjugates. The low absorption of green tea catechins in the intestine indicates that most catechins are metabolized by colon microbial metabolites in the large intestine and then absorbed. Some microbial metabolites may have a physiologic effect. The identification and quantification of microbial metabolites constitutes an important field of research. Data on metabolites of tea catechins in humans are still limited, and new microbial metabolites need to be identified. The microbial metabolites, such as M4, (–)-5-(3',4'-dihydroxyphenyl)-(-valerolactone (M6), (–)-5-(3',5'-dihydroxyphenyl)-(-valerolactone (M6'), and hippuric acid, seem to have a lag time and longer half-lives than catechins and increase the action time of catechins.[28]

After ingestion of a single dose of green tea, two major tea catechin metabolites, M4 and M6, were identified in human urine and blood.[25] The cumulative amounts of M4 and M6 excreted in urine were 8 to 25 times higher than those of EGC and EC in some of the studied subjects, and accounted for 6–39% of the amounts of ingested EGC and EC. In addition, the cumulative amount of renal excretion of M4 and M6 was more than nine times higher than that of EGC or EC during and after repeated consumption of green tea. The peak levels of M4 and M6 in the plasma were lower than those of EGC and EC, and exhibited kinetics similar to those of their urinary metabolites. Both M4 and M6 were also detected in human urine following ingestion

of black tea, but the levels were less than one-tenth of those produced by green tea due to the lower EGC and EC levels in the black tea.[25]

M4, M6, and M6' were detected in human urine after green tea ingestion.[21] The combined excretion of these three metabolites accounted for 1.5–16% of ingested catechins. M4, M6', and M6 were all observed after the ingestion of pure EGCG or EGC by human subjects, but only M6 was detected after EC ingestion.[21] The excreted amount of M4 and M6 accounted for 1.4% of ingested EGC and 11.2% of ingested EC.[28] The excreted amount of M4 and M6 was 10 and 8.7 times that of excreted EGC and EC in urine, respectively.[28] EGCG and ECG were present in urine in trace amounts.[28] The fact that renal excretion of M4 or M6 had a 3-h lag time and peaked at 7.5–13.5 h after a single dose of green tea consumption indicated that they are formed in the colon, absorbed, and excreted in urine mostly in the conjugate form.[28]

Hippuric acid was identified as a major metabolite associated with black tea consumption.[33,34] The compound 1,3-dihydroxyphenyl-2-*O*-sulfate (sulfate conjugate of pyrogallol) was also identified as a black tea metabolite in humans.[34] Very low levels of theaflavins in plasma and urine were reported in humans after consumption of black tea.[35] It was reported that the ingestion of either green tea or black tea results in a major increase in the excretion of hippuric acid into urine.[33] The authors concluded that green tea and black tea consumption result in similar amounts of microbial degradation products that are absorbed by the body. They speculated that these microbial metabolites, not the native tea flavonoids, may be responsible for at least some of the health effects attributed to tea consumption.[33]

Studies reported massive increases in urinary hippuric acid excretion using a black tea intervention, that is, a mixture of monomeric and polymeric flavonoids.[32,36] About 43% of the tea phenols were metabolized to hippuric acid.[32] The authors calculated the total amounts of catechins, theaflavins, gallic acid, and flavonols consumed by their volunteers and showed that these simple polyphenols could not fully account for the increased urinary excretion of hippuric acid. Consequently, they suggested that the complex thearubigins were also converted to hippuric acid. This assumption was later confirmed.[33] It was suggested that the lactones and phenolic acids produced from black tea and green tea polyphenols in the human colon are probably quantitatively similar, irrespective of the flavonoid source from which they are derived.[33] On the other hand, hippuric acid can be derived from other sources besides catechins, such as quinic acid and aromatic amino acids. Not only tea drinking but also wine,[37] cider,[38] and coffee[32] consumption can cause increased urinary excretion of hippuric acid. Thus, hippuric acid is not a suitable biomarker of tea catechins intake.[37] It was reported that 3-hydroxyhippuric acid may be a more valid biomarker of polyphenol intake.[39]

7.2.3 Tissue Distribution

Bioavailability of tea catechins in tissues may be much more important than that in plasma, but little published data are available on tissue distribution of catechins in humans after tea consumption, even in animals.[7] The nature of tissue metabolites

may be different from that of blood metabolites, and more studies need to be carried out for identification of metabolites in tissues.

Henning et al.[40] studied the tissue bioavailability of green tea or black tea in humans. Twenty men consumed green tea, black tea, or a caffeine-matched soda control daily for 5 days before radical prostatectomy. Tea catechins (free and conjugated EGC, EGCG, and the total of all four catechins) in prostate samples of men consuming black tea and green tea were significantly higher than in men consuming caffeine-matched soda.

7.2.4 Excretion

Metabolites of tea catechins are excreted in bile or urine. In general, small conjugates, such as monosulfates, tend to be excreted in urine, and extensively conjugated metabolites are more likely to be excreted in bile.[41] The total amount of metabolites excreted in urine correlated roughly with maximum plasma concentrations.[41] The exact half-lives of tea catechins in plasma were calculated to be in the order of 2–3 h, except for EGCG, which is eliminated more slowly.[41] Relative urinary excretion data were used to estimate the minimal absorption rate and were consistent with the plasma kinetic data for most catechins, but for EGCG that mostly excreted in bile, the urinary excretion rate was very small (0–0.1%), and its absorption was underestimated. The urinary excretion rates of EC and EGC were 18.5 and 11.1%, respectively. The low cumulative excretion of tea catechins in human urine, which was 0–9.8%, suggested that they were extensively metabolized in the human body.

7.2.5 Comparison of Pharmacokinetic Parameters of Tea Catechins

The pharmacokinetics data of C_{max}, AUC, absorption percentage in plasma, and excretion percentage in urine listed in table 7.2 were converted to the same supply of catechins, a single 50-mg dose of aglycone equivalent, assuming that the bioavailability parameters increased linearly with the dose.[42] Only data on the sum of free and conjugated intact catechins were used for calculation. The average value and range of the pharmacokinetics data of catechins are summarized in table 7.3. As only unchanged catechins were measured in most studies and methylated metabolites were not analyzed, the mean values of the data are probably underestimated.[42]

Based on the results from Warden et al.[12] and Henning et al.,[8] the amounts of EC, ECG, EGC, and EGCG in the diet, plasma, and urine of humans after tea consumption were analyzed and compared with principal component analysis (figure 7.3). Principal component analysis demonstrated that only principal component 1, which is mostly contributed by these four compounds, is significantly more than principal component 2 (98.7% vs. 1.1%). The eigenvectors (coefficients in the linear combinations of variables making up principal components) of these four components are almost equally the same. Therefore, the pathway of these four compounds is very similar: high concentration in the diet but very low concentration in the plasma and urine. This shows that most of the dietary EC, ECG, EGC, and EGCG have been metabolized into other new compounds during digestion and absorption in the human body.

TABLE 7.3
Mean and range of pharmacokinetics data of total tea catechins (free and conjugate) in humans

	EC		ECG		EGC		EGCG	
	Mean	**Range**	**Mean**	**Range**	**Mean**	**Range**	**Mean**	**Range**
C_{max} (μM)	0.28 ± 0.13	0.10–0.48	0.08 ± 0.09	0.02–0.23	0.24 ± 0.14	0.11–0.54	0.10 ± 0.09	0.01–0.28
T_{max} (h)	2.15 ± 1.87	1.0–7.0	6.74 ± 9.71	1.4–24	1.84 ± 1.40	0.5–2.6	1.95 ± 0.96	1.2–5
AUC (μM·h)	1.39 ± 0.67	0.34–2.31	0.88 ± 1.42	0.13–3.00	1.03 ± 0.63	0.47–2.19	0.50 ± 0.42	0.06–1.50
$T_{1/2}$ (h)	3.36 ± 1.42	2.0–5.7	6.9[a]		1.47 ± 0.33	1–1.7	3.09 ± 0.92	1.9–4.6
Absorption in plasma (%)	0.83 ± 0.76	0.04–2.09	0.05 ± 0.03	0.01–0.08	0.33 ± 0.39	0.05–0.47	0.03 ± 0.05	0–0.09
Excretion in urine (%)	5.11 ± 7.51	0.33–26.18	0.01 ± 0.01	0–0.02	1.25 ± 1.02	0.04–2.95	0.01 ± 0.01	0–0.02

[a] Only one datum was available for $T_{1/2}$ of ECG.

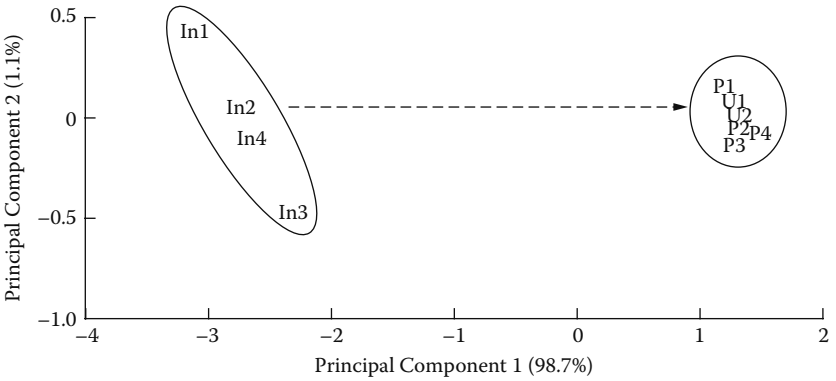

FIGURE 7.3 Principal component analysis of the intake, absorption in plasma, and urinary excretion amount of EC, ECG, EGC, and EGCG. In, intake; P, plasma; U, urine. The data of In1, P1, U1, In2, P2, U2, In3, and P3 were from Henning et al. (2004),[8] and In4 and P4 were from Warden et al. (2001).[12] Raw data were log transformed to generate a normally distributed data set for principal component analysis.

7.3 DIFFERENCE BETWEEN HUMAN AND RAT IN THE METABOLITES OF TEA CATECHINS

Chemical structures of EC metabolites in human and rat urine are different.[24,43] In humans, EC is conjugated with glucuronide mainly at the 3' position of the B ring, forming (–)-EC-3'-*O*-glucuronide, 4'-*O*-methyl-(–)-EC-3'-*O*-glucuronide, and 4'-*O*-methyl-(–)-EC-5 or 7-*O*-glucuronide, but in rat, glucuronidation of EC seems to occur at the 7 position of the A ring, forming 3'-*O*-methyl-(–)-EC, (–)-EC-7-*O*-glucuronide, and 3'-*O*-methyl-(–)-EC-7-*O*-glucuronide.

Although hippuric acid was detected in urine of humans consuming both green and black tea,[33] studies in rats indicated that these animals do not seem to produce hippuric acid from pure catechin, but a species difference between humans and rats cannot be excluded.[37]

Chow et al.[29] did not find significant differences in the pharmacokinetics of EGCG using pure EGCG or Polyphenon E in the human body after oral consumption, but a significant difference was found in rats.[44] When administered i.g., EGCG in DGT showed a 3.6 times higher absorption rate constant (Ka) than pure EGCG in rat. According to the AUC and C_{max} value per unit of EGCG, DGT seems to deliver EGCG into the blood more effectively than pure EGCG, which is possibly due to the complex formation between EGCG and other components in DGT enhancing the absorption of EGCG.[44]

7.4 BIOAVAILABILITY OF CATECHINS AFFECTED BY PURE OR MIXED FORM AND FOOD MATRIX

There may be a difference in the pharmacokinetics parameters when catechins are given in pure or mixed form orally to humans. For 1,050 mg EC in the pure form, C_{max} was 1.53 μM, but it was 0.11 μM based on 75 mg intake,[43] while for 75 mg EC

in the DGT form, C_{max} was 0.65 μM.[18] For 459 mg EGC in the pure form, AUC was 20.1 μM·h, or 13.4 μM, based on 306 mg[26], but for 306 mg EGC in DGT form, its AUC was 10.7 μM·h.[18] For 400 mg pure EGCG, its AUC was 1.71 μM·h on day 1. For EGCG in Polyphenon E form (200 mg EGCG, 37 mg EGC, and 31 mg EC), AUC was 2.59 μM·h. At a higher dose of 800 mg, its AUCs for pure form (3.48 μM·h) and Polyphenon E form (3.57 μM·h) on day 1 were similar.[45] Significant differences in the pharmacokinetic parameters (C_{max}, AUC, T_{max}, and $T_{1/2}$) were not observed when EGCG was given in decaffeinated green tea or in the pure form.[28] Similarly, T_{max} and $T_{1/2}$ were observed when catechins were consumed in the pure or mixed form.

Henning et al.[8] concluded that polyphenols administered in the form of GTS showed enhanced bioavailability compared with that of green tea (GT) or black tea (BT), which led to a small but significant increase in antioxidant capacity. This confirmed previous findings of a lower plasma bioavailability of EGCG than EGC and EC. When the same dose of flavanols was administered through GT, BT, or GTS, the GTS resulted in significantly higher increases in C_{max} and AUC values. T_{max} was always extended by >1 h for all four flavanols after GTS consumption, compared with those after GT and BT consumption. In addition, the percentage of EGC and EC intakes excreted in the urine was significantly higher after GTS consumption than after GT and BT consumption. These results strongly suggest that the absorption of polyphenols from a GTS, delivered in a standard gelatin capsule, was delayed but was higher than after GT or BT administration.[8] It can be postulated that GTS increases the bioavailability of tea flavanols at the absorption phase. More studies are needed to understand this phenomenon.

The effects of the food matrix on the bioavailability of catechins have not been studied in much detail. Possible interactions between catechins and food components, such as binding to proteins and carbohydrates, may affect the absorption of catechins *in vivo*.[41] The effect of black tea consumption with or without milk on antioxidant activity of human plasma determined by ferric reducing antioxidant power (FRAP) assay was studied.[46] Subjects consumed no tea on day 1 and drank either black tea with milk or black tea alone at hourly intervals between 9:00 A.M. and 2:00 P.M. on the other two days. Subjects consuming no tea exhibited no significant change in FRAP across the study day. Similarly, consumption of tea with milk produced no change in FRAP between 9:00 A.M. and 12:00 P.M., and the 50% increase in FRAP between 12:00 and 3:00 P.M. was not statistically significant. When the subjects consumed black tea without milk, the FRAP value was increased by 65% between 9:00 A.M. and 12:00 P.M. ($p = 0.02$), and the FRAP value at 3:00 P.M. was 76% higher than at 9:00 A.M. ($p = 0.002$). Heavy consumption of black tea thus appears to increase the circulating antioxidant potentials *in vivo*.[46]

7.5 *IN VIVO* BIOLOGICAL ACTIVITIES OF TEA CATECHINS

Rietveld and Wiseman[47] summarized the available evidence of antioxidant effects of tea from human clinical trials. The authors concluded that tea flavonoids are potent antioxidants that are adsorbed from the gut after consumption. They showed that human intervention studies with green tea and black tea demonstrated a significant increase in plasma antioxidant capacity in humans about 1 h after consumption of a

moderate amount of tea (1–6 cups/day). Meng and colleagues[21] found that tea catechins are metabolized to ring-fission products and methylated catechins. These ring-fission products can also be further metabolized in the colon to simple phenolic acids such as 4-hydroxybenzoic acid, 3,4-dihydroxybenzoic acid, 3-methoxy-4-hydroxyhippuric acid, and vanillic acid, which have been detected in human urine after tea consumption.[48] Some phenolic acids showed considerable *in vitro* antioxidant activity. In addition, dimerization of EGCG may increase antioxidant capacity, because it has been shown that dimerization of EGCG at an alkaline pH will lead to new dimerization products (theasinensins A and D) with an antioxidant activity two- to threefold higher than that of EGCG.[49]

Inhibition of tumorigenesis by green tea in different organs of animals has been observed,[11] but clear evidence of tea drinking and cancer prevention in humans was little reported.

7.6 TOXICITY OF TEA CATECHINS

A recent clinical study of green tea extract in cancer patients determined the maximum tolerated dose (MTD) of green tea extract at 4.2 g/m^2 once daily or 1.0 g/m^2 three times per day.[50] These studies demonstrated that green tea extract is well tolerated and is relatively nontoxic at a dose up to 4 g/m^2 per day. There is a great need to establish MTD for black tea extract and theaflavins.

7.7 CONCLUSION

After oral ingestion of pure catechins or green tea and black tea, plasma levels of unchanged tea catechins in humans are in the low-micromolar or submicromolar concentration range, and metabolites of tea catechins become major components *in vivo* and may play an important role in inhibiting cancer or other cells.

Some tea catechin metabolites have been identified in humans after oral ingestion, including the glucuronide, sulfate, and methylation forms of catechins and their microbial metabolites. Some of these metabolites have higher plasma concentrations than their parent compounds. However, the structures of other metabolites of tea catechins still need to be identified, and bioavailability of catechins in humans still needs to be determined in order to understand the biological activities of tea catechins *in vivo.*

Pharmacokinetics of tea catechins in humans need to be studied further. Large inter- and intraindividual variations in the pharmacokinetic parameters of tea catechins and their metabolites have been reported in humans. To better understand benefits of tea against cancer and other diseases, future research may focus on the target tissue levels of specific catechins and their metabolites.

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8 Antioxidant Properties and Mechanisms of Tea Polyphenols

Xiaochun Wan, Daxiang Li, and Zhengzhu Zhang

CONTENTS

8.1 INTRODUCTION

The tea bush and in particular its young leaves contain a high concentration of polyphenols and oxidative enzymes, thus the young leaves are better for tea manufacture. Tea polyphenols, previously called tea tannins, are also known as tea flavonoids. Among the polyphenols in fresh tea leaves, catechins are the predominant form of polyphenols, which account for 12–24% of the dry weight. Besides catechins, flavonol, and their glycosides, anthocyanidin and leucoanthocyanidin, phenolic acids and depsides are also present. Their typical concentrations are shown in table 8.1.[1] These phenolic compounds are directly or indirectly associated with the characteristics of tea, including its color, taste, and aroma.

TABLE 8.1
Polyphenols in young tea shoots and their typical contents

Polyphenols	Content (dry weight basis)
Total	18–36%
Flavan-3-ols (catechins)	12–24%
Flavonol and glycosides	3–4%
Anthocyanins and leucoanthocyanidin	2–3%
Phenolic acids and depsides	~5%

Source: Wan, X. C., Huang, J. Z., and Shen, S. R., Eds. 2003. *Tea biochemistry*, 3rd ed., 9–20, 180–94. Beijing: China Agriculture Press. With permission.

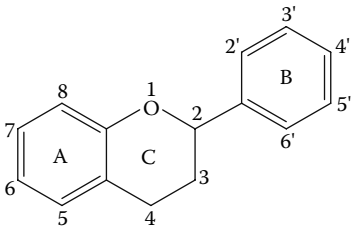


FIGURE 8.1 Basic flavonoid structure.

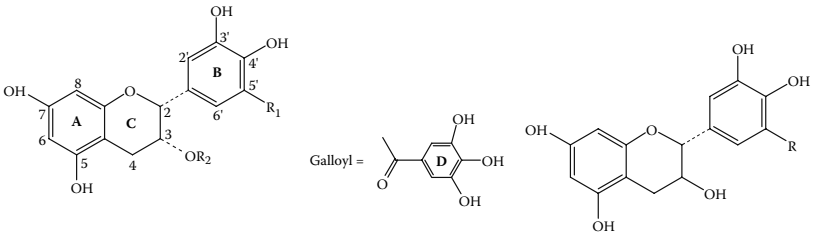
8.1.1 Catechins

Catechins are a group of flavonoids (figure 8.1) belonging to flavan-3-ols. In fresh tea leaves, the principal catechins are (–)-epicatechin (EC), (–)-epigallocatechin (EGC), (–)-epicatechin gallate (ECG), (–)-epigallocatechin gallate (EGCG), catechin (C), and gallocatechin (GC) (figure 8.2) (*1*). EGCG is the most abundant catechin, followed by EGC, ECG, and EC (table 8.2).

Catechins are responsible for the astringent taste and strength of green tea infusion. During green tea manufacture, most catechins and other polyphenols are preserved owing to the inactivation of the endogenous enzymes by dry heating or steaming at the initial step. Green tea quality correlates positively with the concentration of polyphenols. However, a high concentration of polyphenols or catechins, which makes the infusion strongly bitter and astringent, is not necessarily required for high-quality green tea. High-quality green tea is characterized by high contents of free amino acids with appropriate concentrations of catechins and caffeine.[2]

8.1.2 Flavonol and Glycosides

In fresh tea leaves, a total of 20 flavonols and glycosides have been reported.[3,4] The flavonols found in fresh tea leaves include kaempferol, quercetin, and myricetin (figure 8.3), which account for 0.14–0.32%, 0.27–0.48%, and 0.07–0.2% of the dry weight of tea leaves, respectively.[1] Flavonol glycosides are flavonols bound to a monosaccharide (e.g., glucose, rhamnose, galactose, arabinose) or disaccharide (e.g., rutinose). The principal flavonol glycosides are rutin, quercetin glycoside. and kaempferol glycoside (figure 8.4). Their contents in tea are, respectively, 0.05–0.15%, 0.2–0.5%, and 0.16–0.35% of dry weight.[1] Flavonol and its glycosides are often bright yellow and thought to contribute to the yellowish green color of green tea liquor.[1] Moreover, some flavon-3-ol glycosides induce a silky, mouth-drying, and mouth-coating sensa-



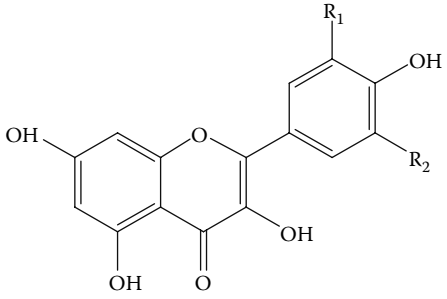
1. $R_1 = R_2 = H$ **Epicatechin**
2. $R_1 = OH, R_2 = H$ **Epigallocatechin**
3. $R_1 = H, R_2 =$ galloyl **Epicatechin gallate**
4. $R_1 = OH, R_2 =$ galloyl **Epigallocatechin gallate**
5. R = H **Catechin**
6. R = OH **Gallocatechin**

FIGURE 8.2 Structures of major catechins.

TABLE 8.2
Catechins in young tea leaves

Catechins	Content[a] (dry weight base)
(–)-Epicatechin (EC)	1–3%
(–)-Epigallocatechin (EGC)	3–6%
(–)-Epicatechin gallate (ECG)	3–6%
(–)-Epigallocatechin gallate (EGCG)	8–12%
(+)-Catechin (C)	1–2%
(+)-Gallocatechin (GC)	3–4%

[a] Data adapted from www.tocklai.net/teachem/index.htm.



1. $R_1 = R_2 = H$ **Kaempferol**
2. $R_1 = OH, R_2 = H$ **Quercetin**
3. $R_1 = R_2 = OH$ **Myricetin**

FIGURE 8.3 Structures of major flavonols.

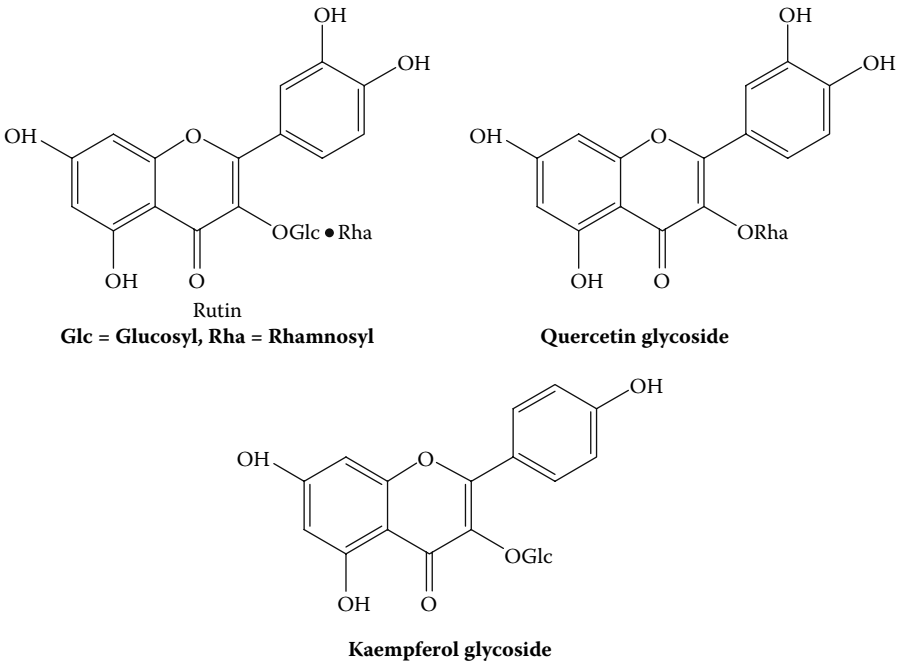
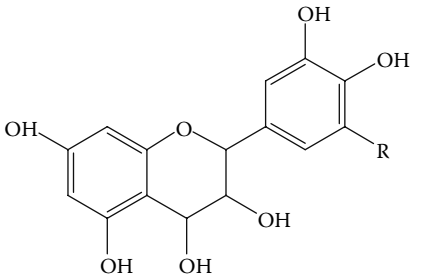


FIGURE 8.4 Structures of major flavonol glycosides.

tion with extremely low taste thresholds, ranging from 0.001 to 19.8 μmol/l.[5] Other studies demonstrate that flavanol-3-glycosides not only impart a velvety astringent taste sensation to the oral cavity, but also contribute to the bitter taste of tea infusions by amplifying the bitterness of caffeine.[6]

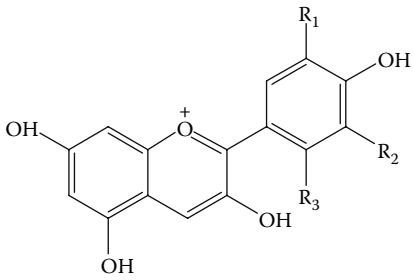
8.1.3 Anthocyanidin and Leucoanthocyanidin[1]

Leucoanthocyanidins (flavan-3,4-diols) are distributed widely in plants. In fresh tea leaves, leucoanthocyanidins, which account for 2–3% of the dry weight of fresh tea leaves, are the principal leucocyanidins and leucodelphinidins present (figure 8.5a). Leucoanthocyanidins are colorless and easy to be oxidized and condensed to pigments during tea fermentation. They are precursors for the important classes of colored anthocyanidins and anthocyanins (anthocyanidin glycosides). Anthocyanidins, including pelargonidin, cyanidin, delphinidin, and tricetinidin (figure 8.5b), account for ~0.01% of the dry weight of tea leaves, whereas they reach up to 1.0% in purple tea shoots, which give undesirable bitterness and color to tea quality. Anthocyanidins are not very stable, and usually glycosylation transfers a glycoside (usually glucose) to position 3 of the anthocyanidin to form a stable anthocyanin. With heat or acidic hydrolysis, anthocyanins release anthocyanidins. Anthocyanins are localized in the cell vacuole. As cellular pH increases, the anthocyanins' structural changes produce red, purple, or blue colors of many blooms, fruits, leaves, and stems of plants.



1. R = H **Leucocyanidin**
2. R = OH **Leucodelphinidin**

(a)



1. $R_1 = R_2 = R_3 = H$ **Pelargonidin**
2. $R_1 = OH, R_2 = R_3 = H$ **Cyanidin**
3. $R_1 = R_2 = OH, R_3 = H$ **Delphinidin**
4. $R_1 = R_3 = OH, R_2 = H$ **Tricetinidin**

(b)

FIGURE 8.5 Structures of major leucoanthocyanidins and anthocyanidins.

8.1.4 Phenolic Acids and Depsides[1]

Phenolic acids are a diverse group that includes hydroxybenzoic and hydroxycinnamic acids and depsides, the latter being intermolecular esters formed from two or more molecules of the same or different phenolic benzoic acids. In fresh tea leaves, phenolic acids and depsides account for about 5% of the dry weight of young tea leaves. Phenolic acids are mainly gallic acid, chlorogenic acid, and theogallin (figure 8.6), which account for 0.5–1.4%, 0.3%, and 1–2% of the dry weight of tea leaves, respectively, whereas depsides are mainly ellagic acid and m-digallic acid as trace compounds. In tea plants, phenolic acids are precursors of catechin gallates. In manufactured tea, phenolic acids associated with other polyphenols contribute to the astringent taste of tea liquor.

8.2 STRUCTURAL CHARACTERISTICS OF TEA POLYPHENOLS

During the tea fermentation process, polyphenols located within the vacuoles of the intact leaf cells are released and oxidized catalytically with polyphenol oxidases located in cytoplasm. Polyphenol oxidase can use any of the catechins as a substrate to form complex polyphenolic constituents. The catechins in fresh tea leaves undergo enzymatic and chemical oxidation leading to oxidized, condensed, and polymerized polyphenols known as theaflavins and thearubigins, which contribute to the color and taste of liquors of black tea. The oxidative fermentation of catechins results in the development of appropriate flavor and color. It will cause a darkening of the leaf and a decrease in astringency. Theaflavins account for 1–3% of the dry weight of black tea. Thearubigins are by far the major components of black tea extract. They constitute as much as 10–20% of the dry weight of black tea.[1]

Catechins are characterized by di- or trihydroxyl group substitution of the B-ring and meta-5,7-dihydroxyl substitution of the A-ring of the flavonoid structure

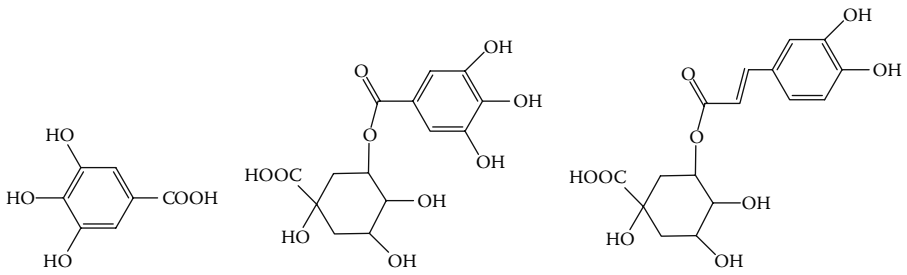


FIGURE 8.6 Structures of major phenolic acids.

(figure 8.1).[1] Catechins possess a 1,3-dihydroxy phenyl group (A-ring) and an *o*-dihydroxy phenyl group (B-ring). Both groups can be oxidized, with the *o*-dihydroxy phenyl group being more susceptible. Oxidation can be mediated with oxidase and metal ions, and occurs autocatalytically at pH values of approximately 6.0 and above. One-electron oxidation generates a free radical that can couple with other radicals to form C–C or C–O bonds from which theasinensins (C–C bond formation) and related derivatives are probably derived. *O*-Dihydroxy phenyl rings and *o*-trihydroxy phenyl rings can, through two one-electron oxidations, lead to the highly reactive *o*-quinones. These species can react further via intermolecular cycloaddition reactions to form products that contain a benzotropolone ring system and have intense yellow/orange colors. These are the basic structural requirements and mechanisms for the formation of dimeric flavonoid-derived pigments.[7] In some conditions, the trihydroxyl group of galloyl moiety (D-ring) can be oxidized as the way of the trihydroxyl group of the B-ring to produce a benzotropolone skeleton by its reaction with the dihydroxyl group of the B-ring of another molecule.[8–10]

A recent study further proves that the trihydroxy group of the B-ring of catechins is more active and could be oxidized to several types of catechin dimers. It has demonstrated that enzymatic oxidation of epigallocatechin affords three quinone dimers, dehydrotheasinensin C, proepitheaflagallin, and an unnamed symmetrical quinone dimer. Dehydrotheasinensin C has a hydrated cyclohexenetrione structure and its oxidation-reduction dismutation reaction yields black tea polyphenols, theasinensins C and E, and desgalloyl oolongtheanin, while proepitheaflagallin is decomposed upon heating to afford epitheaflagallin and hydroxytheaflavin (figure 8.7).[11]

Theaflavins possess a hydroxyl-substituted benzotropolone ring system, which is formed principally from oxidative coupling of one *o*-dihydroxy group and an *o*-trihydroxy group of the B-ring in another. The major theaflavins (figure 8.8) in black tea are theaflavin (TF), theaflavin-3-gallate (TF-3-G), theaflavin-3'-gallate (TF-3'-G), and theaflavin-3,3'-digallate (TF-3,3'-DG)[1]; their precursors and contents in black tea are shown in table 8.3, and their formation mechanism is shown in figure 8.9.[12] Compared with EGCG or other catechins, theaflavins have higher molecular weights, have more phenol groups, and possess a benzotropolone moiety. It is reasonable that these compounds have similar biological activities to their precursor catechins. It has been shown that theaflavins are more active than EGCG in inhibiting 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced mice ear edema.[12]

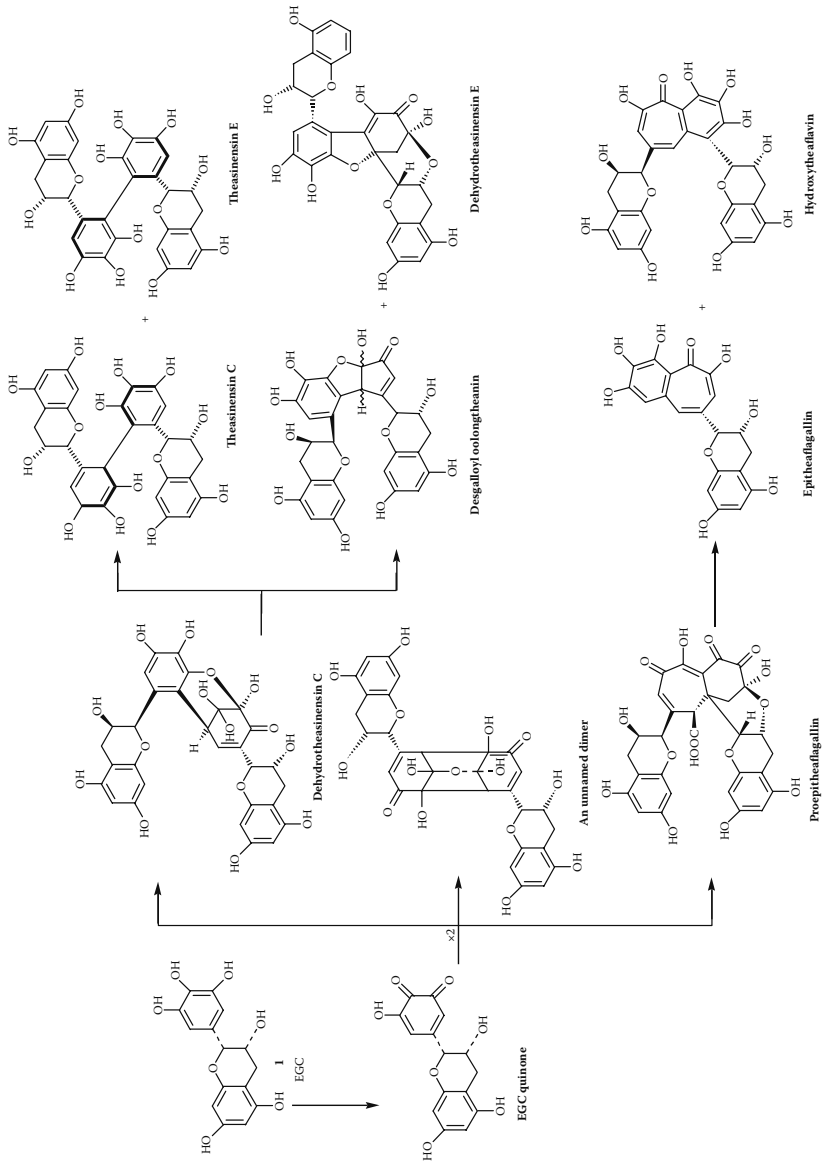
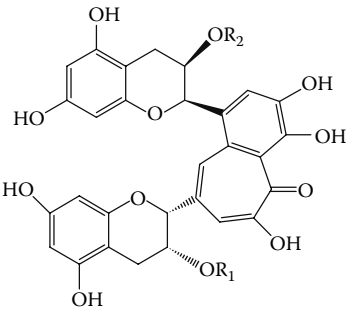


FIGURE 8.7 Enzymatic oxidation products of epigallocatechin. (From Matsuo, Y., Tanaka, T., and Kouno, I. 2006. A new mechanism for oxidation of epigallocatechin and production of benzotropolone pigments. *Tetrahedron* 62:1–10. With permission.)



		R_1	R_2
Theaflavin	**TF**	**H**	**H**
Theaflavin-3-gallate	**TF-3-G**	**Galloyl**	**H**
Theaflavin-3′-gallate	**TF-3′-G**	**H**	**Galloyl**
Theaflavin-3, 3′-digallate	**TF-3, 3′-DG**	**Galloyl**	**Galloyl**

FIGURE 8.8 Major theaflavins in black tea.

TABLE 8.3
Theaflavins in black tea

Precusors	Theaflavins	Content (dry weight base)
EC + EGC	TF	0.2–0.3%
EC + EGCG	TF-3-G	1.0–1.5%
ECG + EGC	TF-3'-G	
ECG + EGCG	TF-3,3'-DG	0.6–1.2%

Theasinensins (biflavanols) could be formed from the paired condensation of two gallocatechins, EGCG and EGC, during tea processing. Their mechanism of formation is shown in figure 8.7. Theasinensins are a group of colorless substances, and are reactive compounds that may rearrange to form other undefined polymers.[13]

Thearubigins are the major oxidation products of catechins during fermentation. They could also be partly produced by further oxidation of theaflavins. The possible formation mechanism of thearubigins (TRs) is shown in figure 8.10.[14] It is noteworthy that up to 75% of catechins in tea leaves may ultimately find their way into thearubigins during black tea processing. Thearubigins are heterogeneous groups of orange-brown phenolic pigments. However, due to the difficulty encountered in their separation, the chemistry of thearubigin is poorly understood.[15] Spectra of TRs by analysis of matrix-assisted laser desorption ionization (MALDI) with a linear time-of-flight (TOF) mass spectrometry have shown that some TRs are polymers of catechins in which the 3-OH group is more or less esterified with gallic acid; others are derivatives of dimers of catechins for which the gallate moiety has been condensed with "B-ring" catechins to form a benzotropolone skeleton.[16]

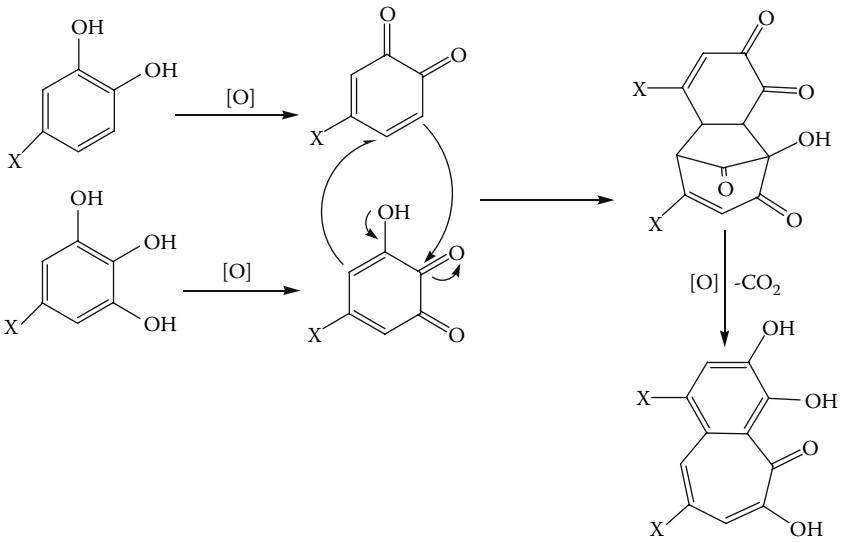


FIGURE 8.9 Scheme of the formation mechanism of theaflavins. (From Sang, S., Lambert, J. D., Tian, S., Hong, J., Hou, Z., Ryu, J., Stark, R. E., Rosen, R. T., Huang, M. T., Yang, C. S., and Ho, C.-T. 2004. Enzymatic synthesis of tea theaflavin derivatives and their anti-inflammatory and cytotoxic activities. *Bioorg. Med. Chem.* 12:459–67. With permission.)

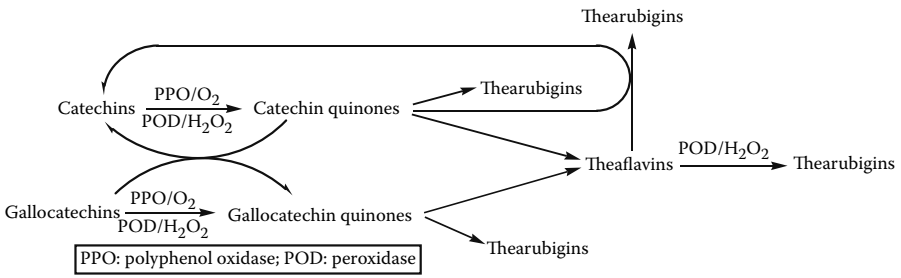


FIGURE 8.10 Scheme of the possible formation mechanism of thearubigins. (From Finger, A. 1994. In-vitro studies on the effect of polyphenol oxidase and peroxidase on the formation of polyphenolic black tea constituents. *J. Sci. Food Agric.* 66:293–305. With permission.)

8.3 ANTIOXIDANT PROPERTIES AND MECHANISMS OF TEA POLYPHENOLS

Free radicals are very important both in food systems and in biological systems. In food, the process of lipid auto-oxidation and development of rancidity involves a free radical chain mechanism proceeding via initiation, propagation, and termination steps. This lipid peroxidation process is responsible for the development of off-flavors and undesirable chemical compounds in food. *In vivo*, free radical–initiated auto-oxidation of cellular membrane lipids can lead to cellular necrosis and is an

accepted important factor of a variety of pathological conditions, such as cancer, cardiovascular disease, and even aging.[17]

The potential health benefits associated with tea consumption have been partially attributed to the antioxidant properties of tea polyphenols.[18]

8.3.1 Antioxidant Properties of Tea Polyphenols

The reduction potentials of catechins have shown that gallocatechins have lower redox potentials than simple catechins; catechins have lower redox potentials than catechin gallates (table 8.4). The 5'-OH group in the B-ring of gallocatechins and catechins without gallate ester into the C-ring is thought to have a better antioxidant activity. Differences in redox potential between catechins and epicatechins were also observed with all *epi* forms having significantly lower values. The reduction potentials of theaflavins are similar to those of simple catechins and higher than those of gallocatechins (table 8.4). Their potentials are similar, although gallate esterification tends to increase the redox value. TF had the lowest first reduction potential; however, the TF gallates have higher antioxidant activity than TF in the lipid phase, demonstrating that redox potential is an indicator, but not a perfect one, of antioxidant activity.[15]

The structural features thought to be responsible for antioxidant activity of tea polyphenols are the *o*-dihydroxy catechol (3',4'-OH) arrangement on the B-ring, either di- or trihydroxy-substituted catechins or flavonols, and the C2–C3 double bond in the C-ring conjugated with a C4 carbonyl group found in flavonols. This structure also allows electron delocalization, conferring high reactivity to quench free radicals.[18] The antioxidant activity of catechins is determined by the B-ring catechol structure and is further enhanced in gallocatechins by the 5'-hydroxyl group on the B-ring. Various structure-antioxidant activity studies have concluded that the

TABLE 8.4
Redox potentials of catechins, gallocatechins, and theaflavins

Component	First redox potential vs. SCE (V)
Epigallocatechin (EGC)	0.09
Gallocatechin (GC)	0.13
Epigallocatechin gallate (EGCG)	0.14
Gallocatechin gallate (GCG)	0.15
Epicatechin (EC)	0.19
Epicatechin gallate (ECG)	0.20
Catechin (C)	0.20
Theaflavin (TF)	0.16
Theaflavin-3-gallate (TF-3-G)	0.20
Theaflavin-3'-gallate (TF-3'-G)	0.19
Theaflavin-3,3'-digallate (TF-3,3'-DG)	0.19

Note: SCE, saturated calomel electrode.

Source: Balentine, D. A., Wiseman, S. A., and Bouwens, L. C. M. 1997. The chemistry of tea flavonoids. *Crit. Rev. Food Sci. Nutr.* 37:693–704. With permission.

presence of a gallate group in the 3-position and a trihydroxy B-ring plays the most important role in the free radical scavenging abilities of catechins.[19–21] Antioxidant function is also thought to be promoted by C5, C7 dihydroxylation on the A-ring.[15] Generally, the A-ring is very insensitive to oxidation, and therefore unlikely to participate directly in the antioxidant reactions. However, Zhu et al.[22] reported that ring A of catechins may also serve as an antioxidant site. The functional group of catechins for antioxidant activity is shown in figure 8.11. It is worthy to note that there is a synergistic effect on antioxidation among catechins as well as catechins with vitamin E[23] or Trolox.[24]

It should be pointed out that catechins may undergo auto-oxidation and behave like prooxidants under certain conditions. A recent review by Yang et al. [25] points out that EGCG is unstable in cell culture medium, with a half-life of less than 30 min due to its auto-oxidation. Dimers as well as superoxide radicals and hydrogen peroxide are formed. Many of the reported biological activities of EGCG could be caused by these reactive oxygen species. For example, EGCG can cause the apoptosis of H661 lung cancer cells through the hydrogen peroxide produced. Reactive oxygen species produced during auto-oxidation of EGCG could lead to the inhibition of epidermal growth factor receptor (EGFR) phosphorylation and EGFR protein degradation in human esophageal squamous cell carcinoma KYSE 510 cells.[25]

8.3.2 Antioxidant Mechanisms of Tea Polyphenols

There are three proposed mechanisms by which tea polyphenols act as antioxidants. These mechanisms are explained in the following sections.

8.3.2.1 Scavenging Reactive Oxygen Species

Tea polyphenols have been shown to scavenge reactive oxygen species, such as superoxide radical, singlet oxygen, hydroxyl radical, nitric oxide, nitrogen dioxide, and peroxynitrite, which may play important roles in carcinogenesis.

Catechins can trap peroxyl radicals and thus suppress radical chain reactions and terminate lipid peroxidation.[26] Catechins also inhibit metmyoglobin-initiated peroxidation of low-density lipoproteins (LDLs) and the consumption of α-tocopherol.[19] Among tea catechins, EGCG is most effective in reacting with most reactive oxygen

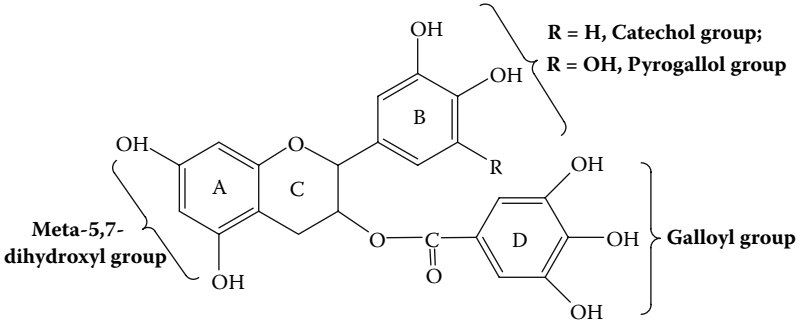


FIGURE 8.11 The functional group of catechins for antioxidant activity.

species.[18] Yang et al.[27] reported that the centers of scavenging reaction of EGCG are B-, D-, and A-rings, and each EGCG traps six superoxide anions ($O_2^{\cdot-}$) or hydroxyl radicals (·OH) *in vitro* as reflected in the electron spin resonance (ESR), whereas EC only traps two free radicals. The scavenging mechanism of EGCG is as follows:

$$O_2^{\cdot-} (\cdot OH) + EGCG \rightarrow H_2O_2 + EGCG\cdot$$
$$5O_2^{\cdot-} (\cdot OH) + EGCG\cdot \rightarrow \text{Nonradical products}$$

A number of studies have shown that the antioxidative properties of theaflavins manifest themselves in their abilities to scavenge reactive oxygen species and to inhibit their generation.[28] Theaflavins also show strong antioxidant activity against lipid oxidation as detected in rabbit erythrocyte ghost system[29] and rat liver homogenates[30] against LDL oxidation in mouse macrophage cells,[31] in preventing DNA oxidative damage in cell-free systems,[29] in the inhibition of xanthine oxidase and suppression of intracellular reactive oxygen species in HL-60 cells, and through H_2O_2 scavenging ability.[32]

8.3.2.1.1 DPPH

The DPPH (2,2-diphenyl-1-picrylhydrazyl) system offers a stable radical-generating procedure. It is sensitive enough to detect active principles at low concentrations. The antioxidant process of catechins is thought to be divided into the following two stages:

$$DPPH\cdot + AH \leftrightarrow DPPH\text{-}H + A\cdot$$
$$H\cdot + X\cdot \rightarrow \text{Nonradical materials}$$

AH is the antioxidant, A· is the antioxidant radical, and X· is another radical species or the same species as A·. Although the first stage is a reversible process, the second stage is irreversible and produces stable radical termination compounds. Structural information about these nonradical products would provide important information about antioxidant mechanism studies.[33]

Sang et al.[33] reported that catechin and epicatechin in the DPPH oxidant system could be oxidized to compounds 3 and 4 and compounds 5 and 6, respectively. From the elucidation of the chemical structures of these compounds, the antioxidant mechanisms of catechin and epicatechin were proposed. As shown in figure 8.12, an initial one-electron oxidation of catechin on the B-ring by a DPPH radical generates a catechin (or epicatechin) phenoxyl radical. This phenoxyl radical can be tautomerized to the corresponding *o*-quinone, which is then subjected to nucleophilic attack by the reactive C-8 (or C-6) carbon of another catechin unit in a Michael-type addition reaction to the quinone B-ring to form compounds 3 and 4 or 5 and 6. This suggests that the configuration of position 3 for catechin and epicatechin does not affect the oxidative reaction. Both the B-ring and A-ring are the principal sites of antioxidant activity of catechin and epicatechin in the DPPH oxidant system.[33] However, the antioxidant mechanism of gallocatechin and epigallocatechin was different from that of catechin (or epicatechin) in the DPPH oxidant system. As shown in figure 8.13, attacked with DPPH radical, epigallocatechin (or its ester) phenoxyl radical was generated and tautomerized to *o*-quinone. This quionone attacks the C-2' carbon

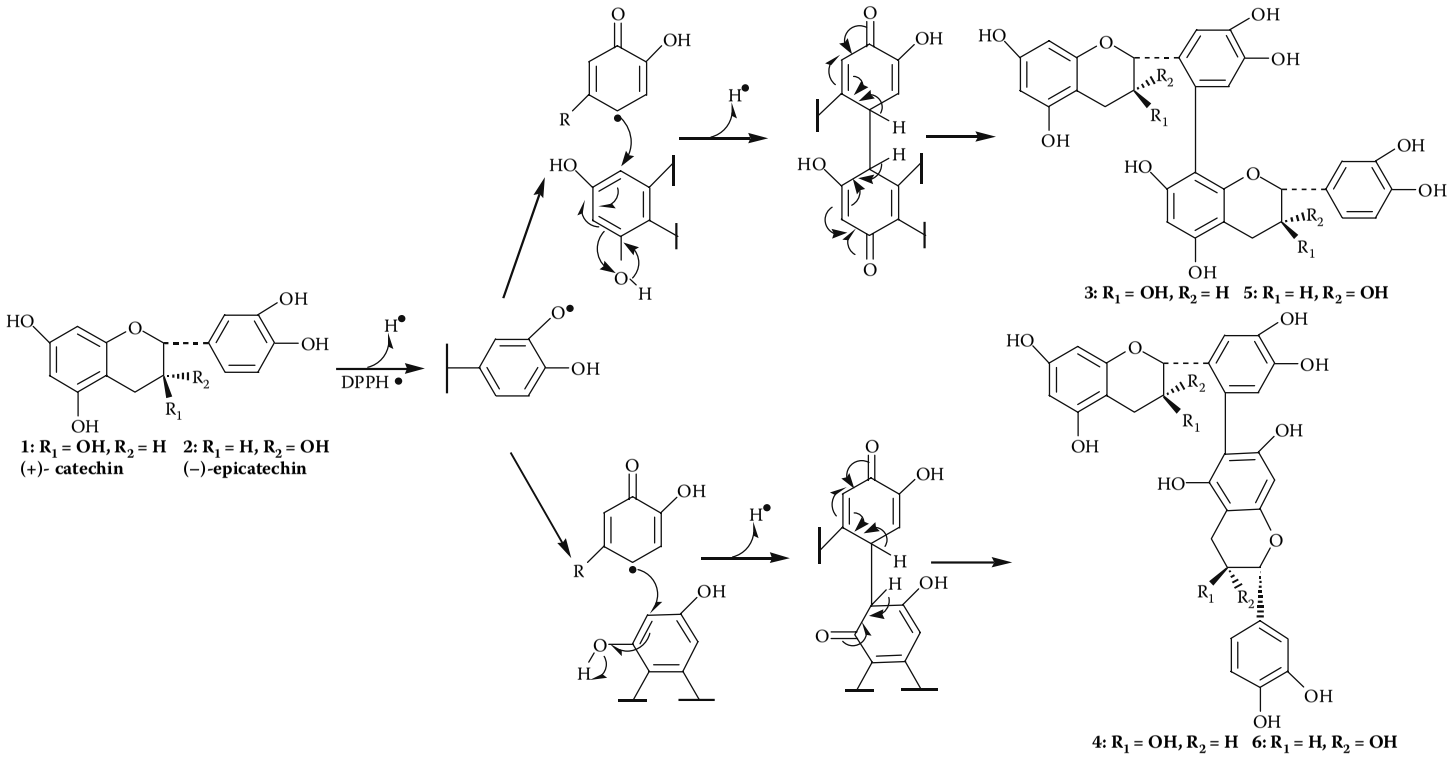


FIGURE 8.12 Proposed scavenging mechanism of epicatechin and catechin to 2,2-diphenyl-1-picrylhydrazyl. (From Sang, S., Cheng, X., Stark, R. E., Rosen, R. T., Yang, C. S., and Ho, C.-T. 2002. Chemical studies on antioxidant mechanism of tea catechins: Analysis of radical reaction products of catechin and epicatechin with 2,2-diphenyl-1-picrylhydrazyl. *Bioorg. Med. Chem.* 10:2233–37. With permission.)

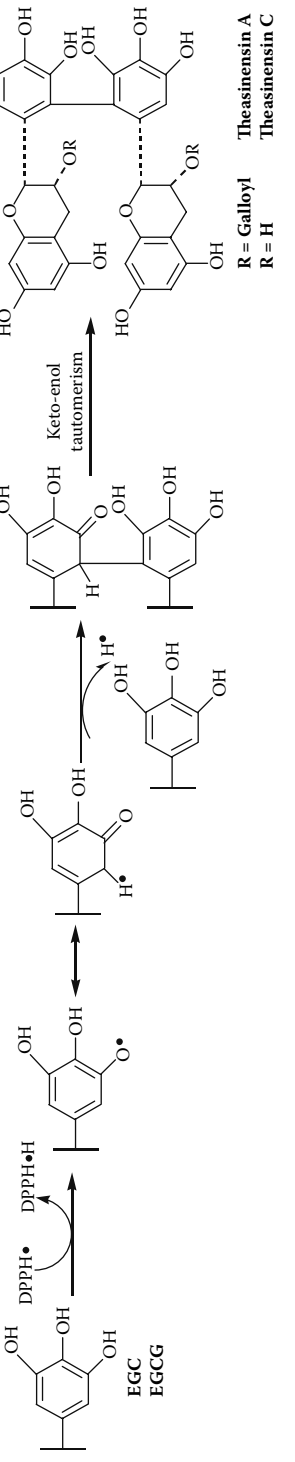


FIGURE 8.13 Proposed scavenging mechanism of epigallocatechin and epigallocatechin gallate to 2,2-diphenyl-1-picrylhydrazyl. (From Zhu, N., Wang, M., Wei, G. J., Lin, J. K., Yang, C. S., and Ho, C.-T. 2001. Identification of reaction products of (–)-epigallocatechin, (–)-epigallocatechin gallate and pyrogallol with 2,2-diphenyl-1-picrylhydrazyl radical. *Food Chem.* 73:345–49. With permission.)

of another epigallocatechin (or its ester) to form theasinensin A or C. This suggests that the trihydroxyphenyl B-ring, rather than the gallate moiety, is the active site of antioxidant reaction in catechins. Moreover, the trihydroxyphenyl B-ring is more active than the dihydroxyphenyl B-ring in the DPPH oxidant system.[17]

Jhoo et al.[34] reported that the oxidation of theaflavin in either DPPH or the peroxidase–hydrogen peroxide model system leads to the generation of theanaphthoquinone as the major oxidation product. The proposed radical scavenging mechanism of theaflavin is shown in figure 8.14. Theanaphthoquinone is formed through one-electron oxidation. This indicates that the benzotropolone moiety of theaflavin is the active site for scavenging of radicals.[34]

8.3.2.1.2 Peroxyl Radical

Sang et al.[35] studied the antioxidant mechanism of epicatechin upon reaction with a peroxyl radical generated by thermolysis of the initiator 2,2'-azo-bisisobutyronitrile (AIBN). The progress of the reaction was thought to proceed via the following stages:

(1) Radical generation

$$RN{=}NR \xrightarrow{\Delta} 2R\cdot + N_2$$

$$2R\cdot + 2O_2 \rightarrow 2ROO\cdot$$

(2) Radical trapping

$$ROO\cdot + AH \leftrightarrow ROOH + A\cdot$$

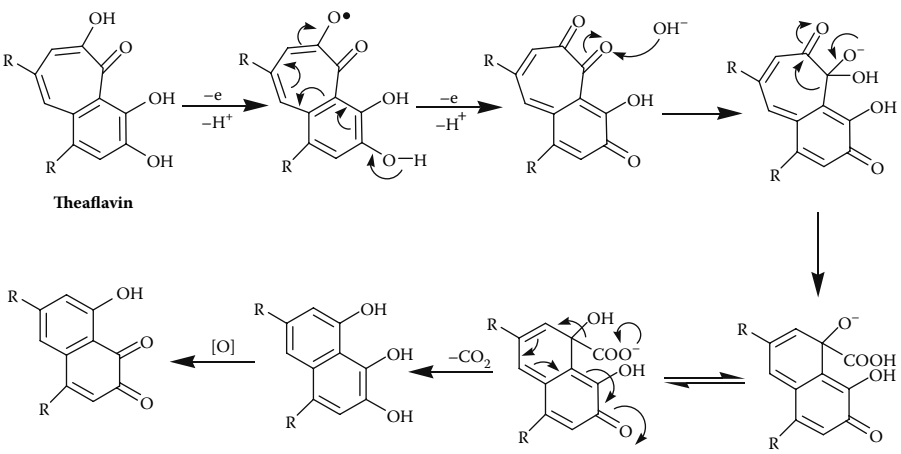


FIGURE 8.14 Proposed scavenging mechanism of theaflavin to 2,2-diphenyl-1-picrylhydrazyl and hydrogen peroxide. (From Jhoo, J., Lo, C., Li, S., Sang, S., Ang, C. Y. W., Heinze, T. M., and Ho, C.-T. 2005. Stability of black tea polyphenol, theaflavin, and identification of theanaphthoquinone as its major radical reaction product. *J. Agric. Food Chem*. 53:6146–50. With permission.)

(3) Radical termination

$$A\cdot + X\cdot \rightarrow \text{Nonradical material}$$

AIBN decomposes thermally to yield alkyl radicals (R·), which then react rapidly with oxygen to generate peroxyl radicals (ROO·). AH is the phenolic antioxidant, A· is the antioxidant radical, and X· is another radical species or the same species as A·. Although the second stage is a reversible process, the third stage is irreversible and produces stable radical termination compounds. Structural information about these nonradical products can lead to the elucidation of antioxidant mechanisms. The chemical structures of the reaction products (compounds 2–5) of epicatechin with alkylperoxyl radicals from AIBN in a homogeneous solution suggest the antioxidant mechanisms for epicatechin illustrated in figure 8.15. Epicatechin is proposed to react with peroxyl radicals by a single-electron transfer followed by deprotonation from the hydroxyl group of the B-ring to form a resonance pair. If the reaction is initiated at the hydroxyl group of C-3', compounds 2 and 4 should be formed; conversely, compound 5 should be formed if the reaction is initiated at the hydroxyl group of C-4'. Compound 3 could be formed by further oxidation of compound 4. These results suggest that the B-ring is the initial site for formation of reaction products. Both the B-ring and A-ring exhibit antioxidant activity observed for epicatechin in the peroxyl radical oxidant system.[35]

Valcic et al.[36,37] studied the reaction of EGCG and EGC with peroxyl radicals generated upon thermolysis of the azo initiator 2,2'-azobis(2,4-dimethylvaleronitrile) (AMVN) to produce several oxidation products. The antioxidant progress of the reaction is similar to that of AIBN described above. The antioxidant pathway is illustrated as figure 8.16. The B-rings of EGC and EGCG are transformed into ring-opened unsaturated dicarboxylic acid moieties. The oxidation products also include a seven-membered B-ring anhydride and a symmetrical dimer and an unsymmetrical dimer. These changes occur solely in the B-ring of EGCG or EGC, and suggest that the principal site of antioxidant reaction in EGCG and EGC is the trihydroxyphenyl B-ring, regardless of the presence of a 3-galloyl moiety. The antioxidant mechanism involves an initial one-electron oxidation of EGCG or EGC by a peroxyl radical that generates a EGCG or EGC phenoxyl radical. This phenoxyl radical either reacts with a second peroxyl radical to form an unstable AMVN adduct and cleaves to compounds 3 and 8, or attacks a second EGCG or EGC molecule to form a dimer radical, then forms an adduct with a second peroxyl radical, and cleaves and rearranges to compounds 4, 6, and 7 with loss of an AMVN-derived alcohol fragment. Compound 3 undergoes hydrolysis and decarboxylation to produce compound 2.[36,37]

8.3.2.1.3 Hydrogen Peroxide

As an important oxidant, hydrogen peroxide (H_2O_2) can normally be produced from many physiological sources in the aerobic environment of mammalian cells and tissues. For example, H_2O_2 is generated during NADH oxidation by cell wall peroxidase, a process that can be stimulated by monophenolic compounds. Catechins exhibit a strong capacity for scavenging hydroxyl radicals and suppressing cytotoxicity induced by H_2O_2.[22]

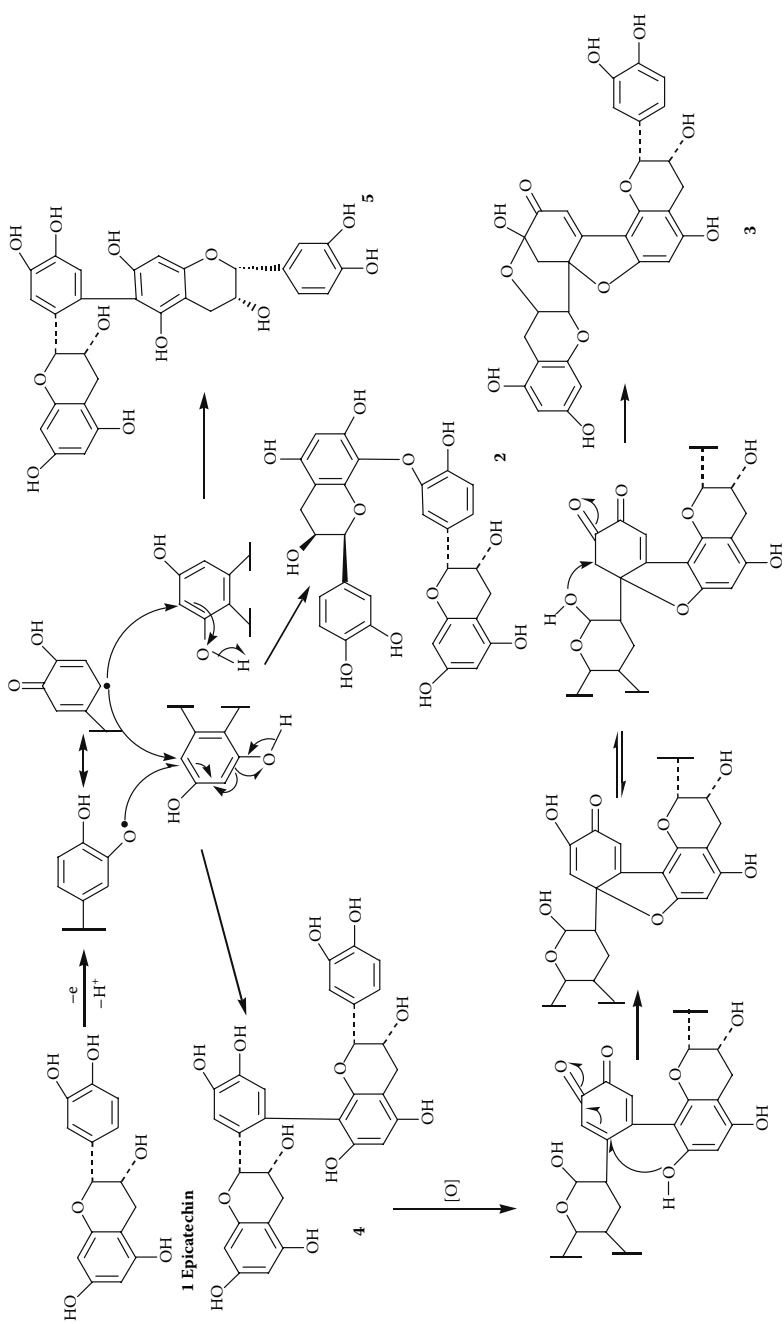


FIGURE 8.15 Scavenging mechanism of EC to peroxyl radicals. (From Sang, S., Tian, S., Wan, H., Stark, R. E., Rosen, R. T., Yang, C. S., and Ho, C.-T. 2003. Chemical studies of the antioxidant mechanism of tea catechins: Radical reaction products of epicatechin with peroxyl radicals. *Bioorg. Med. Chem.* 11:3371–78. With permission.)

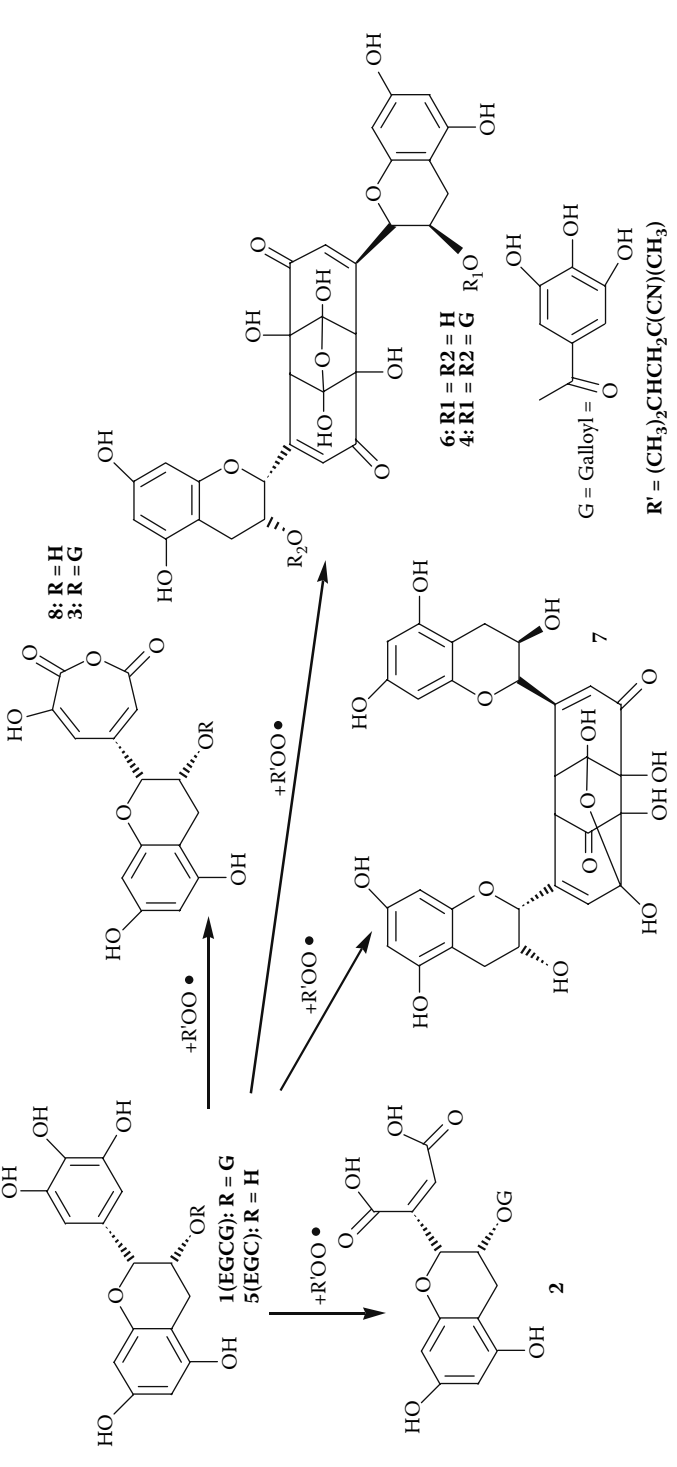


FIGURE 8.16 Oxidation products of epigallocatechin and epigallocatechin gallate with peroxyl radicals. (Adapted from data in Valcic, S., Muders, A., Jacobsen, N. E., Liebler, D. C., and Timmermann, B. N. 1999. Antioxidant chemistry of green tea catechins. Identification of products of the reaction of (–)-epigallocatechin gallate with peroxyl radicals. Chem. Res. Toxicol. 12:382–86; Valcic, S., Burr, J. A., Timmermann, B. N., and Liebler, D. C. 2000. Antioxidant chemistry of green tea catechins. New oxidation products of (–)-epigallocatechin gallate and (–)-epigallocatechin from their reactions with peroxyl radicals. *Chem. Res. Toxicol.* 13:801–10.)

EGC and EGCG oxidized in the H_2O_2 oxidant system were studied by Zhu et al.[22] It was demonstrated that the oxidation products are formed by the oxidation and decarboxylation of the A-ring in the catechin molecule, as shown in figure 8.17. This study provides unequivocal proof that the A-ring of EGCG and EGC may also serve as an antioxidant site. However, the oxidant mechanism remains unclear.[22] Theaflavin-3,3'-digallate may be oxidized with hydroxyl radicals generated by hydrogen peroxide and produce two A-ring fission products as illustrated in figure 8.18.[38] For the oxidant mechanism, the possible initial step is the attack by the hydroxyl radical on the A-ring. The A-ring radical then undergoes a series of further reactions, including cleavage of the A-ring. If the hydroxyl radical attacks the A-ring of the flavan-3-ol connected to the benzene moiety of the benzotropolone group, 2 will be formed; conversely, 3 should be formed if the reaction is initiated at the A-ring of the flavan-3-ol connected to the tropolone part of the benzotropolone group. It is noteworthy that the initial site for the formation of these two major reaction products is the A-

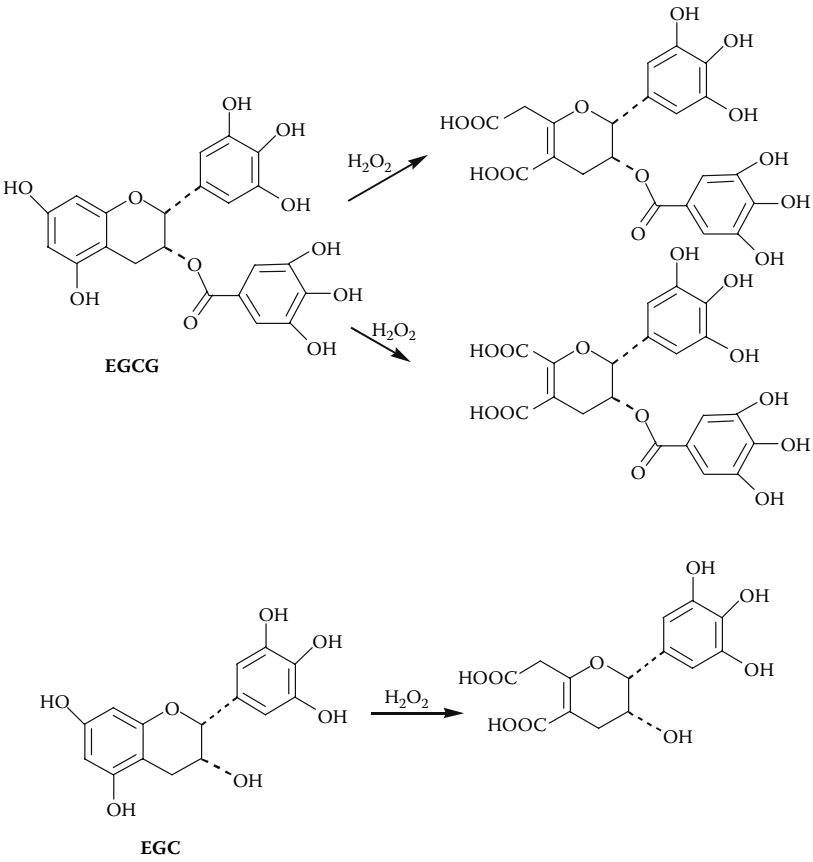


FIGURE 8.17 Oxidation products of epigallocatechin gallate and epigallocatechin with hydrogen peroxide. Zhu, N., Huang, T. C., Yu, Y., LaVoie, E. J., Yang, C. S., and Ho, C.-T. 2000. Identification of oxidation products of (–)-epigallocatechin gallate and (–)-epigallocatechin with H2O2. *J. Agric. Food Chem.* 48:979–81. With permission.)

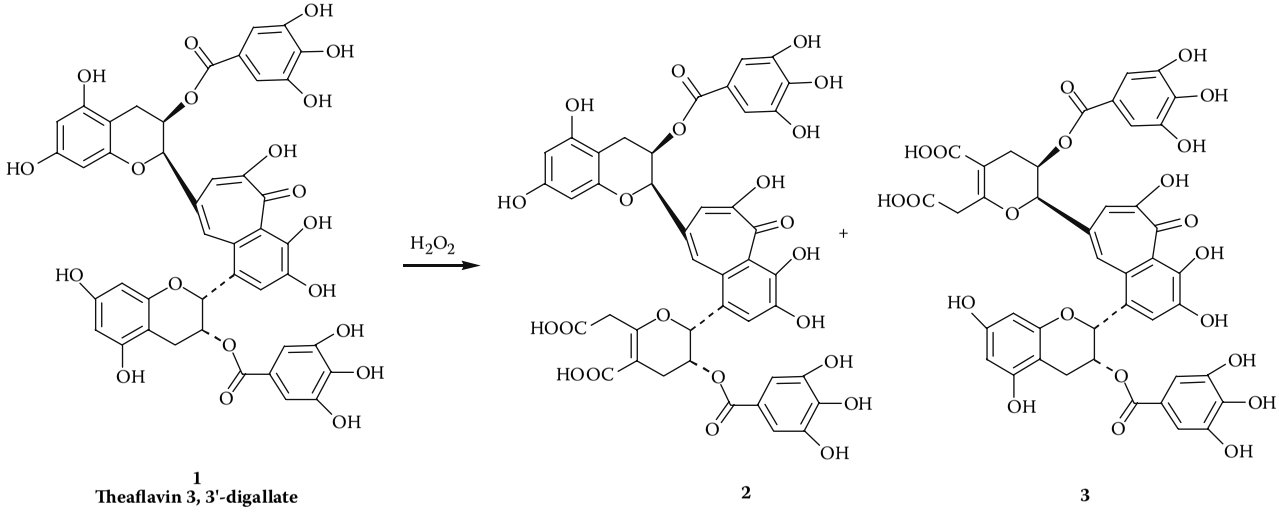


FIGURE 8.18 Oxidation products of theaflavin-3,3'-digallate with hydrogen peroxide. (From Sang, S., Tian, S., Jhoo, J. W., Wang, H., Stark, R. E., Rosen, R. T., Yang, C. S., and Ho, C.-T. 2003. Chemical studies of the antioxidant mechanism of theaflavins: Radical reaction products of theaflavin 3,3'-digallate with hydrogen peroxide. T*etrahedron Lett.* 44:5583–87. With permission.)

ring, not the benzotropolone group or the gallate group, in the hydrogen peroxide oxidant system.[38]

8.3.2.1.4 Superoxide Anion

Superoxide anion radicals are generated in living cells by a single-electron reduction of oxygen under physiological conditions, and play harmful roles as precursors of more reactive oxygen species, contributing to the pathological processes of many diseases. Studies by Nanjo et al.[39] and Unno et al.[40] have shown that the presence of at least an *o*-dihydroxyl phenyl group and a galloyl moiety at the 3-position was important in maintaining the scavenging ability of the superoxide anion.

8.3.2.1.5 Hydroxyl Radical

The hydroxyl radical is a highly reactive free radical (diffusion rate limited) that can react with most living organisms. Hydroxyl radicals damage lipids, polypeptides, proteins, and DNA.[41] Hanasaki et al.[42] reported that (+)-catechin and (–)-epicatechin display a hydroxyl radical scavenging effect 100–300 times superior to that of mannitol, a typical hydroxyl radical scavenger. The hydroxyl radical scavenging activity of these compounds was investigated in a photolysis of the H_2O_2 system. It was found that their ability to scavenge hydroxyl radicals decreased in the order of ECG > EC > EGCG >> EGC.[43]

8.3.2.1.6 Nitrite Ion

Under acidic pH, nitrite ions are converted to nitrous acid (pKa = 3.25), which decomposes to afford a range of species according to the following equations:

$$NO_2^- + H^+ \leftrightarrow HNO_2$$
$$HNO_2 + H^+ \leftrightarrow NO^+ + H_2O$$
$$NO^+ + NO_2^- \leftrightarrow N_2O_3$$

The acid-promoted reaction of EGCG and nitrite ions is initiated by one-electron oxidation of EGCG via the NO_2 generated by decomposition of nitrous acid, as detailed above. Disproportionation or further oxidation of the resulting semiquinone might then lead to the formation of the quinone with concomitant conversion to nitric oxide (NO), as illustrated in figure 8.19. It is worth noting that quinone 1a, when exposed to excess nitrite ions in an acidic environment, is susceptible to nitrosation through competing pathways involving nucleophilic sites on the A- and B-rings. This indicates the potential role of epigallocatechin quinones in the protective effects of tea polyphenols against nitrite-induced gastric cancer.[44]

8.3.2.1.7 Peroxynitrite

Peroxynitrite ($ONOO^-$) is a cytotoxic species generated by the reaction between superoxide and nitric oxide (NO), which is a very strong oxidant and can cause oxidation of cell membrane protein and lead to cell damage and diseases. It can also generate hydroxyl radicals and NO_2 under acidic conditions. Pannala et al.[45] found that the scavenging effect of ECG and EGCG on $ONOO^-$ was more pronounced than that of EC and EGC. Catechins were also found to protect from peroxynitrite-

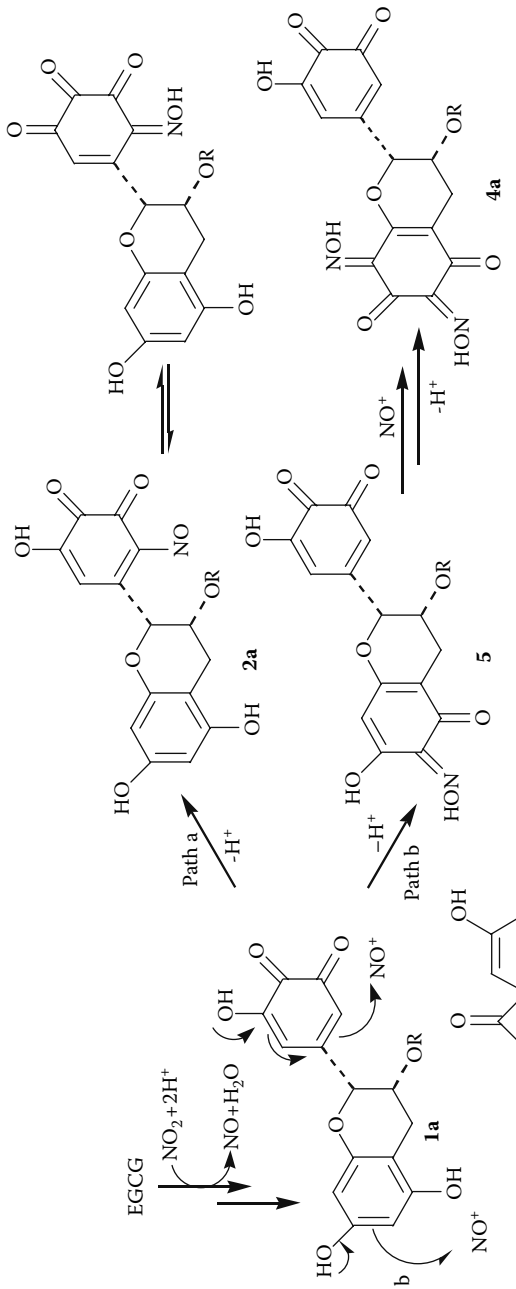


FIGURE 8.19 Proposed scavenging mechanism of epigallocatechin gallate to nitrite ions. (From Panzella, L., Manini, P., Napolitano, A., and d'Ischia, M. 2005. The acid-promoted reaction of the green tea polyphenol epigallocatechin gallate with nitrite ions. *Chem. Res. Toxicol.* 18:722–29. With permission.)

induced modification of critical amino acids of apolipoprotein B-100 of LDL, which contribute toward its surface charge.[45]

8.3.2.2 Chelating Metal Ions to Prevent Oxidation

Catechins can chelate metal ions, mainly Fe and Cu, which are catalysts of free radical reactions, because of their vicinal dihydroxy or trihydroxy structures, and thus prevent the generation of free radicals. Green and black tea polyphenols reduced cell-mediated low-density lipoprotein oxidation induced by Cu^{2+} *in vitro*, which is proposed to contribute to the prevention of atherosclerosis and other cardiovascular diseases.[18]

Guo et al.[43] found that EGC, EGCG, ECG, or EC could protect synaptosomes from the damage of lipid peroxidation in part through their iron-chelating activity. The chelated ratios of EGC, EGCG, ECG, and EC with iron (III) were 3:2, 2:1, 2:1, and 3:1, respectively.

Sugihara et al.[46] reported the effects of EC, EGC, ECG, and EGCG on lipid peroxidation induced by either ferrous, copper, or vanadium ions in normal and α-linolenic acid–loaded (LNA-loaded) cultured rat hepatocytes. Each catechin displayed a marked variation in its antioxidative potency depending on the added metal ion species. However, butylated hydroxytoluene (BHT), a typical lipid radical scavenger, exhibited a similar antioxidative potency with all metal ions. These findings suggest that the metal-chelating property of catechins may play a major role in determining antioxidative activity in cultured hepatocytes.[46]

Both catechins and theaflavins can chelate iron (III). O'Coinceanainn et al.[47] have shown that theaflavins can chelate iron (III) to form a complex, subsequently to be oxidized to *o*-quinone and converted to dehydrotheaflavin, theanaphthoquinone, and polymers, as illustrated in figure 8.20.[47]

8.3.2.3 Regulation Enzymes or Genes Related to Oxidation or Antioxidation

Tea polyphenols perform their antioxidant activity through regulation enzymes or genes related to oxidation/antioxidation. Tea polyphenols also enhance the expression of intracellular endogenous antioxidants such as glutathione, glutathione reductase, glutathione peroxidase, glutathione-S-reductase, catalase, and quinine reductase.[48,49] Lin et al.[50] found that long-term oral feeding of green tea leaves to Wistar rats resulted in the enhancement of the activities of superoxide dismutase (SOD) activity in serum and phase II enzyme, and glutathione S-transferase (GST) and catalase in liver. A recent publication[51] reports that Ang II- and pressure-overload-mediated cardiac hypertrophy was attenuated by EGCG with the suppression of ROS generation and NADPH oxidase expression. Yu et al.[52] reported that tea polyphenols may regulate antioxidant response element (ARE)–mediated phase II enzyme expression through a mitogen-activated protein kinase C pathway. Recently, a well-documented review demonstrated that dietary polyphenols, including tea polyphenols, could stimulate antioxidant transcription and detoxification defense systems through ARE with the coordination of endogenous antioxidants involving glutathione and its related enzymes to execute their antioxidative abilities in biological systems.[53] Kuzuhara et

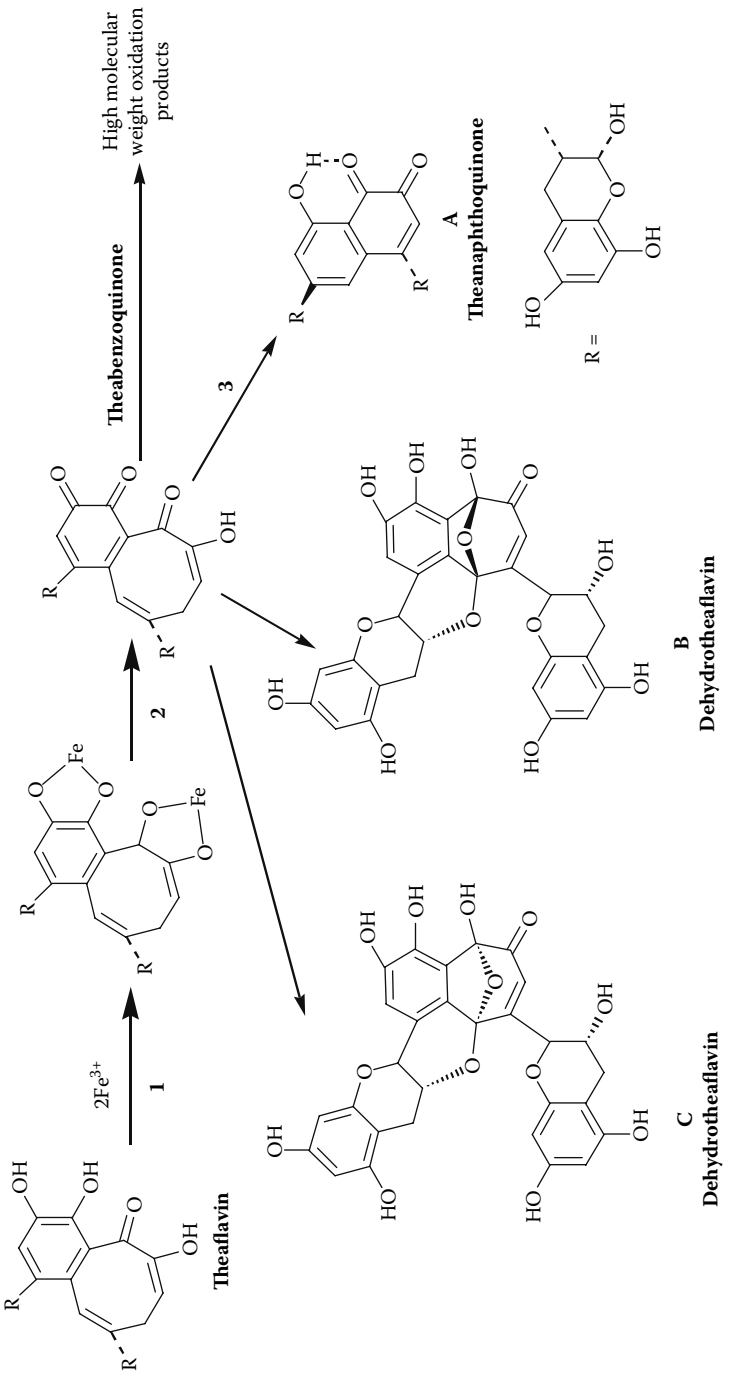


FIGURE 8.20 Proposed oxidation products of theaflavin with iron. (From O'Coinceanainn, M., Bonnely, S., Baderschneider, B., and Hynes, M.J. 2004. Reaction of iron(III) with theaflavin: Complexation and oxidative products. *J. Inorg. Biol.* 98:657–63. With permission.)

al.[54] reported that EGCG could bind directly to single-stranded 18 mers of DNA and RNA, and double-stranded (AG-CT) oligomers of various nucleotide lengths. These indicate that tea polyphenols may regulate the expression of related genes through direct binding.

An experiment on human leukemia cells HL-60 proved that TF-3,3'-DG, TFMG, TF, and EGCG with TF-3,3'-DG most effectively inhibited the prooxidative enzyme–xanthine oxidase activity, which catalyzes oxidation of hypoxanthine and xanthine to uric acid, accompanied by oxygen reduction to superoxide radical and hydrogen superoxide.[32] Moreover, it was shown that EGCG influenced free radical generation through reduction of NADPH–cytochrome P-450 reductase activity.[55] Tea polyphenols also inhibited the activity of cyclooxygenase COX-2 and 5-, 12-, and 15-lipoxygenase, enzymes participating in enzymatic lipid peroxidation in human colon mucosa and colon tumor tissues.[56] Studies on RAW 264.7 mice macrophages revealed that theaflavins, in particular TF-3,3′-DG, effectively inhibit the activation of transcription factor NFκB, preventing expression of an inducible nitric oxide synthase (iNOS) gene in mRNA and, as a consequence, contributing to a decrease in the synthesis of inducible nitric oxide synthase to prevent NO generation.[57]

8.4 CONCLUSIONS

Tea polyphenols, especially catechins and theaflavins, can execute their antioxidant activities principally through scavenging free radicals, chelating transition metal ions, and modulating oxidant/antioxidant enzymes or genes. The main sites of antioxidant action of catechins are the catechol or pyrogallol group of the B-ring, the meta-5,7-dihydroxyl group of the A-ring, and the galloyl group of the D-ring. The main antioxidant sites of theaflavins are similar to those of catechins. However, the benzotropolone skeleton of theaflavins participates in the antioxidant activity. The main sites of antioxidant action of tea polyphenols depend on the oxidant used. Different oxidants can result in distinctly different oxidation products.

It should be pointed out that catechins may display prooxidant activity under certain conditions, in particular, in the presence of copper ion (II) or ferric ion (III). Antioxidant/prooxidant activity of polyphenols is dependent on many factors, such as metal-reducing potential, chelating behavior, pH, solubility characteristics, bioavailability, and stability in tissues and cells.[58]

Future studies will need to clarify further the antioxidant mechanisms of tea polyphenols, especially thearubigins.

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9 Mechanisms of Cancer Chemoprevention by Tea and Tea Polyphenols

Jen-Kun Lin

CONTENTS

9.1 INTRODUCTION

Black tea and green tea are major beverages worldwide and have been shown to possess some beneficial health effects. Polyphenolic compounds and caffeine are major constituents of tea. Following a single topical application, 12-*O*-tetradecanoylphorbol-13-acetate (TPA) was found to induce a time- and dose-dependent increase in edema as well as formation of the proinflammatory cytokines interleukin-1 (IL-1)

and IL-6 in mouse ears. Topical application of epigallocatechin 3-gallate (EGCG), theaflavin (TF-1), TF-2, and TF-3 before TPA treatment inhibited TPA-induced ear inflammation.[1] Furthermore, the effect of a defined extract of black tea (black tea theaflavins [BTTs]) consisting predominantly of theaflavins such as theaflavin (TF-1), theaflavin monogallates (TF-2), and theaflavin 3,3'-digallate (TF-3), and that of green tea extract (green tea catechins [GTCs]) containing mainly catechins such as EGCG on the induction of carcinogenesis in animal models has been investigated. The pharmacological properties of BTTs and GTCs were explored in the C3(1)/SV40T/t-antigen transgenic (Tag) mouse model mammary carcinogenesis.[2] Female Tag mice develop mammary tumors characterized by inactivated tumor suppressor genes p53 and Rb, thus mimicking an independent hormone-insensitive form of human breast cancer. Intervention with either BTTs or GTCs increased Tag mouse survival (control, 143.5 ± 8.25; BTTs, 154 ± 16.83; and GTCs, 151.2 ± 8.02) and decreased Tag mouse mammary carcinogenesis.[2]

Tea (*Camellia sinensis*) originated in southern China and is consumed by over two-thirds of the world population. Tea has an attractive aroma, good taste, and health-promoting effects, and these benefits combined make it one of the most popular drinks in the world. As early as 3000 B.C., tea was used by the Chinese as a medicinal drink and as a beverage by the end of the sixth century. Because tea is an excellent beverage and has an effective pharmaceutical activity, tea plants are now widely cultivated in Southeast Asia, including mainland China, India, Japan, Taiwan, Sri Lanka, and Indonesia, and in a number of countries in Central Africa. Hundreds of teas are now produced and are generally classified into three categories: nonfermented green tea, partially fermented oolong and paochong teas, and fully fermented black and pu-erh teas. The composition of tea varies in a complex manner, according to species, season, age of the leaves, climate, and horticultural practices.[3]

Most commercial teas are derived from the young leaf buds of the tea plant,[4] which are plucked and treated in one of several ways to convert them into the appropriate form for the tea markets. The bulk of the leaf is processed by one of three distinctly different methods, depending upon the characteristics of the end product. Most tea leaves are used for the manufacture of green or black teas, but a smaller, though still significant, quantity is processed to yield oolong tea.[5]

In case of green tea, it is necessary to avoid oxidative enzymatic activity during the operations between plucking and a subsequent heating step; a particular effort is made to avoid exposure to the sun. Black tea is manufactured through a series of fermentation processes that include withering, bruising, rolling, rerolling (several times), and firing (which stops oxidative processes by heat-inactivating relevant enzymes). Oolong teas represent a middle ground between black and green teas; they are considered semifermented, and therefore, their processing closely resembles that of black tea. The processes involved in oolong tea manufacturing are complicated and must be carefully controlled in order to produce the characteristic, and prized, aroma and taste. There are a number of varieties of oolong teas, and their sensory characteristics depend upon the practices of individual factories[6]; these can cause great difficulty when using chemical analysis of tea polyphenols to characterize oolong teas from different sources.

9.2 HEALTH EFFECTS OF TEA AND TEA POLYPHENOLS

The majority of the biological functions of teas may be attributed to their polyphenolic components (see figures 9.1 and 9.2); the chemical structures of the main polyphenols isolated from green, black, oolong, and pu-erh teas are structurally related but not identical. The monomeric catechins from green tea may be considered the precursors of the more complex polyphenols found in other teas as a result of fermentation.

The major polyphenols in green tea are catechins, including catechin itself, gallocatechin, (–)-epicatechin, (–)-epigallocatechin, (–)-epicatechin-3-gallate, and (–)-epigallocatechin-3-gallate (EGCG) (figure 9.1), the latter being the most abundant. In northern China, most green teas are blended with jasmine flower to promote their aroma and flavor. Many intermediate oxidation products of tea polyphenols have been found in oolong teas, and their isolation, characterization, and particularly analysis have been a challenge for many chemists for many years. Theasinesin A (figure 9.2), a new tea polyphenol, has been isolated from oolong tea, and its structure has been confirmed by synthesis involving free radical oxidation of EGCG. In Taiwan, it is estimated that approximately 80% of tea produced is consumed as oolong tea, although paochong tea, which may be considered a lightly fermented oolong tea, is quite popular in the northern regions. Partially fermented oolong or paochong teas contain catechins, theaflavins, and possibly thearubigins; other components, such as proanthocyanidins, are less well characterized but may also have a role in disease prevention.

Pu-erh tea, highly fermented, possesses a rich flavor and was long recognized as a "tribute" tea in the imperial court of China. Its major polyphenols are not well characterized, because of their insolubility in most extracting solvents, but are considered to be polymerized catechins of high molecular weight. The aqueous extract of pu-erh tea is active in inhibiting lipopolysaccharide (LPS)-induced inducible nitric oxide synthase activity.[7]

9.3 BIOCHEMICAL AND MOLECULAR MECHANISMS OF CANCER CHEMOPREVENTION BY TEA AND TEA POLYPHENOLS

Tea polyphenols exhibit a variety of biological properties, including antioxidative effects,[8] inhibition of extracellular mitotic signals,[9] inhibition of cell cycle at the G1 phase,[10] suppression of inducible nitric oxide synthase (iNOS),[9,11] and induction of apoptosis in cancer cells.[12] The natural history of carcinogenesis and cancer provides a strong rationale for a preventive approach to the control of this disease and leads to considerations of the possibility of active pharmacological intervention, or chemoprevention, to arrest or reverse the carcinogenesis prior to invasion and metastasis.[13,14]

The inhibitory effects of tea against carcinogenesis have been attributed to the biological activities of its polyphenolics; however, the molecular mechanisms of cancer chemoprevention by tea extract are not fully elucidated. The results of some recent studies that may shed light on this important area are discussed below (see Figure 9.3).

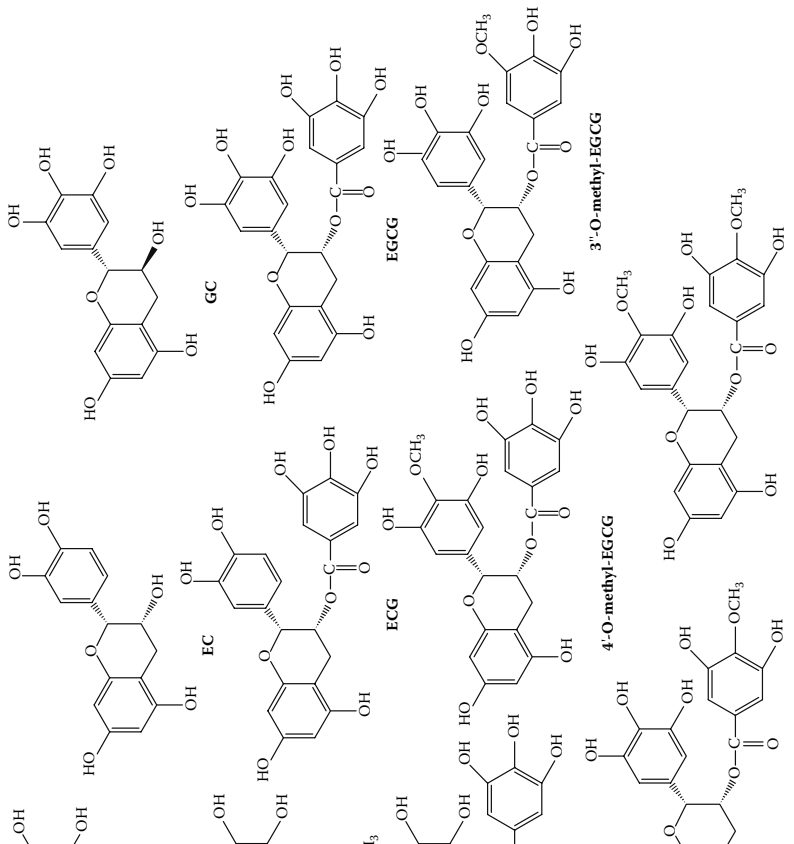

FIGURE 9.1 Chemical structures of green tea polyphenols. EC, epicatechin; GC, gallocatechin; EGC, epigallocatechin; ECG, epicatechin 3-gallate; EGCG, epigallocatechin 3-gallate.

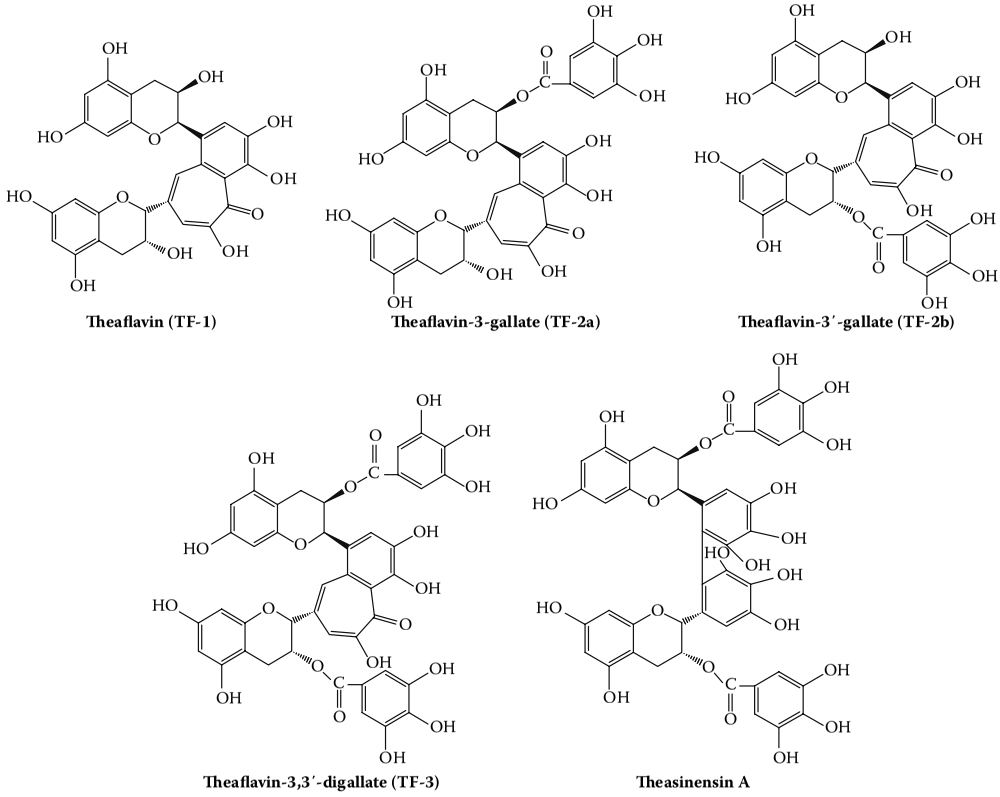

FIGURE 9.2 Chemical structures of black tea polyphenols (TF-1, TF-2a, TF-2b, and TF-3). Theasinensin A is oolong tea polyphenol.

9.3.1 Antioxidative Effects and Scavenging of Reactive Oxygen Species

Tea polyphenols show profound antioxidative effects in various systems; they are strong scavengers of superoxide, hydrogen peroxide, hydroxyl radicals, nitric oxide, and peroxynitrite, the products of various chemical reactions and biological systems.

Modulation of urinary excretion of green tea polyphenols (GTPs) and oxidative DNA damage biomarker 8-hydroxydeoxyguanosine (8-OHdG) was assessed in urine samples collected from a randomized, double-blinded, placebo-controlled phase IIa chemoprevention trial with GTPs in 124 individuals.[15] These individuals were seropositive for both HBsAg and aflatoxin-albumin adducts, and took GTP capsules daily at doses of 500 or 1000 mg or a placebo for 3 months. The baseline levels of 8-OHdG and GTP components among the three groups showed homogeneity ($p > 0.70$), and a nonsignificant fluctuation was observed in the placebo groups over the 3 months ($p > 0.30$). In GTP-treated groups, epigallocatechin (EGC) and epicatechin (EC) levels displayed significant and dose-dependent increases in both the 500 mg group and 1000 mg group ($p < 0.05$). The 8-OHdG levels did not differ among the three groups at the 1 month collection, with medians of 1.83, 2.08, and 1.86 ng/mg creatinine for placebo, 500 mg, and 1000 mg groups, respectively ($p = 0.999$). At the end of 3 months' intervention, 8-OHdG levels decreased significantly in both GTP-treated groups, with medians of 2.02, 1.03, and 1.15 ng/mg creatinine for placebo, 500 mg, and 1000 mg groups, respectively ($p = 0.007$). The results suggest that urinary excretion of EGC and EC can serve as a practical biomarker for green tea consumption in the human population, and chemoprevention with GTPs is effective in diminishing oxidative DNA damage.[15]

Theaflavins and EGCG inhibit xanthine oxidase (XO) to produce uric acid and also act as scavengers of superoxide.[8] Theaflavin-3,3'-digallate (TF-3; figure 9.2) acts as a competitive inhibitor and is the most potent inhibitor of XO among the compounds examined; it also inhibited superoxide production in HL-60 cells. It may be concluded that the antioxidative activity of tea polyphenols is due not only to their ability to scavenge superoxides but also to their ability to block XO and associated oxidative signal transducers.[8]

9.3.2 Suppression of Tumor Proliferation through Mitogenic Signaling Blockade

Tea polyphenols are known to inhibit a broad range of enzymes associated with cell proliferation and tumor progression. Liang et al.[16] have investigated the effects of EGCG on the proliferation of human epidermoid cell line A431. In experiments using a tritiated thymidine incorporation assay, EGCG was found to significantly inhibit the DNA synthesis of A431 cells, to strongly inhibit the protein tyrosine kinase activities of EGF-R, PDGF-R, and FGF-R *in vitro*, and to exhibit the IC_{50} value of 0.5–1 µg/ml. In contrast, EGCG scarcely inhibited the protein kinase activities in $pp60^{src}$, PKC, and PKA.

9.3.3 Inhibition of MAPK Signaling

Overexpression of transcription factors AP-1 and NF-κB has been identified as a key feature in some carcinogenic pathways, including UVB-induced skin tumorigenesis; this enhanced activity can result from activation of one or more mitogen-activated protein kinase (MAPK) pathways.[17] EGCG and theaflavins have both been shown to inhibit TPA and EGF-induced transformation of JB6 mouse epidermal cells in a dose-dependent manner. This inhibition was shown to correlate with decreased JNK activation, leading to an inhibition of AP-1 binding to its recognition site.

EGCG and TF-3 affect numerous events in the Ras-MAP kinase signaling pathway.[18] Treatment of 30.7b Ras 12 Ras–transformed mouse epidermal cells with 20 μM of either of these polyphenols resulted in decreased levels of phosphorylated Erk1/2 and MEK-1/2. EGCG inhibits the association between Raf-1 and MEK-1, while TF-3, but not EGCG, enhances degradation of Raf-1. In addition to inhibiting the phosphorylation of Erk1/2, TF-3 and EGCG can also directly inhibit the kinase activity of this protein by competing for access to the enzyme active site with its substrate, ELK-1.[18]

Exposure of normal human epidermal keratinocytes (NHEKs) to UVB radiation induces intracellular release of hydrogen peroxide (oxidative stress) and phosphorylation of MAPK cell signaling pathways. Pretreatment of NHEK with EGCG inhibits UVB-induced hydrogen peroxide production and its mediated phosphorylation of MAPK signaling pathways.[19] It has been demonstrated that tea polyphenols inhibit PKC, MAPK, and AP-1 activities in NIH 3T3 cells.[20]

9.3.4 Inhibition of PI3K Pathway

The inhibitory effects of tea polyphenols on UVB-induced phosphatidylinositol-3-kinase (PI3K) activation have been demonstrated in mouse epidermal JB6 C1 41 cells.[21] Pretreatment of cells with EGCG and TF-3 inhibited UVB-induced PI3K activation. Furthermore, UVB-induced activation of the PI3K downstream effectors, Akt and ribosomal p70S6 kinase (p^{70}S6-K), were also attenuated by these tea polyphenols. In addition to LY294002, a PI3K inhibitor, pretreatment with MAP-ERK kinase 1 inhibitor, U0126, or a specific p38 kinase inhibitor, SB202190, blocked UVB-induced activation of both Akt and p^{70}S6-K. It is noteworthy that UVB-induced p^{70}S6-K activation was directly blocked by the addition of EGCG or TF-3, whereas these polyphenols showed only a weak inhibition on UVB-induced Akt activation.[21]

9.3.5 Inhibition of Cell Cycle Progression

The effects of EGCG and other catechins on cell cycle progression have been reported.[22] Studies using DNA flow cytometric analysis indicated that EGCG was able to block cell cycle progression at the G1 phase in asynchronous MCF-7 cells; over a 24 h exposure to EGCG, the Rb protein changed from the hyper- to its hypophosphorylated form, and G1 arrest developed. Under the same conditions, protein expressions of cyclins D1 and E were both reduced slightly. Immunocomplex kinase experiments showed that EGCG inhibited the activities of cyclin-dependent kinase 2 (Cdk-2) and 4 (Cdk-4) in the cell free system in a dose-dependent manner; when

the cells were exposed to EGCG (30 μM) over 24 h, a gradual loss of both Cdk2 and Cdk4 kinase activities was observed.

EGCG also induced expression of the Cdk inhibitor p21, an effect that correlated with an increase in p53 levels; the level of p21 mRNA also increased under the same conditions. Within 6 h of EGCG treatment, the expression of the Cdk inhibitor p27 protein was increased. These results suggest that EGCG exerts its growth-inhibitory effects either through modulation of the activities of several key G1 regulatory proteins such as Cdk2 and Cdk4 or via induction of Cdk inhibitors p21 and p27.

9.3.6 Suppression of iNOS Signaling

The effects of tea polyphenols on inducible nitric oxide synthase (iNOS) in thioglycolate-elicited and LPS-activated peritoneal macrophages have been studied.[9,11] Gallic acid, EGC, EGCG, TF-1, TF-2, and TF-3 were found to inhibit nitrite production, iNOS protein, and mRNA in activated macrophages. Reverse transcription polymerase chain reaction (RT-PCR) and Western and Northern blot analyses demonstrated that, compared with controls, significantly reduced 130 kDa protein and 4.5 kb mRNA levels of iNOS were expressed in LPS-activated macrophages treated with EGCG or theaflavins. Electrophoretic mobility shift assay indicated that EGCG blocked the activation of NF-κB, a transcription factor necessary for iNOS induction; this flavonoid and the theaflavins also blocked the disappearance of inhibitor IκB from the cytosolic fraction. These results suggest a mechanism of action involving the reduction in expression of iNOS mRNA, possibly occurring via prevention of NF-κB binding to the iNOS promoter, thereby inhibiting the induction of iNOS transcription.[11]

Expression of vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1) is known to be elevated at sites of inflammation. Studies have been conducted into the effects of EGCG and TF-3 on the expression of these adhesion molecules induced by interleukin-1β (IL-1β) in cultured human umbilical vein endothelial cells (HUVECs).[23] Both compounds significantly inhibited IL-1β-induced protein expression of VCAM and ICAM in dose-dependent manners and were associated with reduced adhesion of leukocytes to HUVECs. The m-RNA level of VCAM-1 was also inhibited by these tea polyphenolics, as was the NF-κB-dependent transcriptional activity induced by IL-1β. It is concluded that these molecules exhibit anti-inflammatory and anti-invasion properties, probably via a route involving blockage of IκB kinase.

9.3.7 Suppression of NF-κB Activation through Downregulating IκB Kinase

The inhibition of IκB kinase (IKK) activity in LPS-activated murine macrophages (RAW 264.7 cell line) by various polyphenols, including EGCG and theaflavins, has been described by Pan et al.[24] TF-3 inhibited IKK activity more strongly than other polyphenols; it strongly inhibited both IKK1 and IKK2 activities, and prevented degradation of IκBα and IκBβ in activated macrophage cells. These data suggest that the inhibition of IKK activity by TF-3 and other tea polyphenols could occur

by direct effect on IKKs, or on upstream events in the signal transduction pathway. Furthermore, TF-3 blocked phosphorylation of IκB from the cytosolic fraction, and inhibited both NF-κB activity and increases in iNOS levels in activated macrophages. TF-3 and other polyphenols in tea may therefore exert their anti-inflammatory and cancer chemoprevention actions by suppressing the activation of NF-κB through inhibition of IKK activity.[24]

9.3.8 Inhibition of Proteasome Activity

EGCG selectively inhibits the activity of topoisomerase I but not topoisomerase II in human colon cancer cell lines[25]; the doses necessary for this inhibition (10–17 μM) are lower than those required for inhibition of cell growth (IC_{50} = 10–90 μM). EGCG has been shown to inhibit the chymotryptic activity of the 20s proteasome in leukemic, breast cancer, and prostate cancer cell lines,[26] leading to accumulation of $p27^{kip1}$ and IκB, and subsequent cell cycle arrest and inhibition of NF-κB activity, respectively.

9.3.9 Inhibition of Matrix Metalloproteinase (MMP)

MMPs play important roles in the development and metastatic spread of cancer. One of the earliest events in the metastatic spread of cancer is the invasion through the basement membrane and proteolytic degradation of extracellular matrix proteins such as collagens, laminin, elastin, and fibronectin, among others, and nonmatrix proteins. MMPs are important regulators of tumor growth, both at the primary site and in distant metastases. Given the clear implications of MMPs in many human cancers, MMPs remain important targets of cancer therapy. The beneficial effects of dietary botanicals, such as tea polyphenols and grape seed proanthocyanidins, in chemoprevention of cancer with particular emphasis on the involvement of MMPs in prostate cancer has been emphasized.[27]

It has been demonstrated that EGCG can inhibit the matrix metalloproteinase activity at a relatively low dose.[28] EGCG inhibits the activity of secreted MMP-2 and MMP-9 at concentrations of 8–13 μM. Meanwhile, 1 μM EGCG increased the expression of TIMP-1 and TIMP-2, proteins that inhibit the activity of activated MMPs. Because such concentrations of tea polyphenols are physiologically significant, this is an attractive mechanism for the observed anti-invasive and anti-angiogenic activities of EGCG[29] and other tea polyphenols *in vivo*. Recently, similar observations of the inhibitory effects of tea polyphenols on the activities of MMPs have been made; TF-3 appears to be the most active of the compounds examined in this respect (unpublished results).

9.3.10 Induction of Apoptosis

Certain green tea catechins and black tea theaflavins are very potent inhibitors (Ki in the nanomolar range) of the antiapoptotic Bcl-2 family proteins, Bcl-XL and Bcl-2. These data suggest a strong link between the anticancer activities of these tea polyphenols and their inhibition of a crucial antiapoptotic pathway, which is implicated in the development of many human malignancies.[30,31]

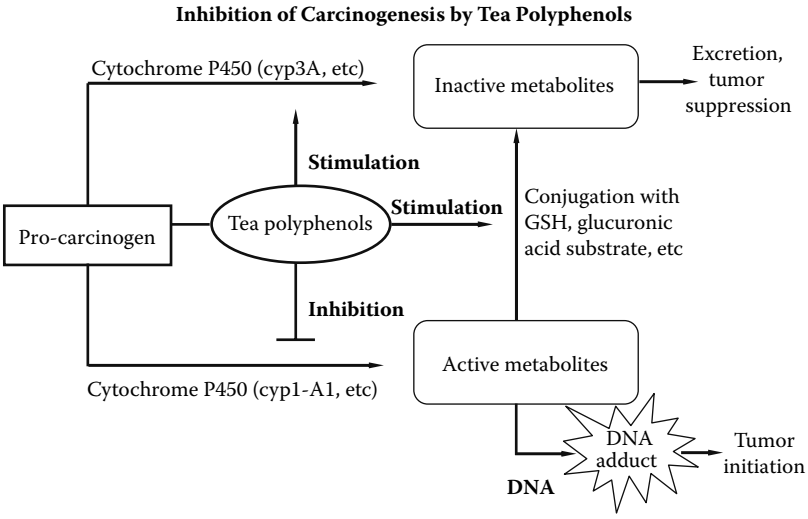

FIGURE 9.3 Biochemical mechanisms of anticarcinogenesis by tea polyphenols. Metabolic activation of chemical carcinogens is by cytochrome p450 mixed-function oxidases, such as cyp-1-A-1. Metabolic inactivation of chemical carcinogens is by cytochrome p450–conjugating enzymes, including glutathione transferase, glucurosyl transferase, and methyltransferase.

One of the most promising strategies for cancer prevention is chemoprevention by daily used food and beverages. Black tea, the most widely consumed beverage, is a good source of compounds with antioxidative, antimicrobial, antimutagenic, and anticarcinogenic properties.[32] Lung cancer is the most common cause of cancer deaths in both men and women worldwide. Therefore, chemopreventive intervention using black tea and its active components may be viable means to reduce lung cancer death.[32]

Theasinensin A, theaflavin, and theaflavin-3-gallate (TF-1 and TF-2a,b) displayed strong growth-inhibitory effects against human histolytic lymphoma U937 (IC_{50} = 12 μM), but were less effective against human acute T-cell leukemia Jurkat, whereas TF-3 and EGCG had lower activities.[24] The mechanisms by which tea polyphenols induce apoptosis were further studied by annexin V apoptosis assay, DNA fragmentation, and caspase activation. Treatment with tea polyphenols caused rapid induction in activity of caspase-3, but not caspase-1, and enhanced proteolytic cleavage of poly(ADP-ribose)-polymerase (PARP). Pretreatment with a potent caspase-3 inhibitor, Z-Asp-Glu-Dal-Asp-fluoromethyl ketone, inhibited theasinensin A–induced DNA fragmemtation. Experiments using flow cytometry showed that theasinensin A induced a loss of mitochondrial potential, elevation of ROS production, release of mitochondrial cytochrome C into the cytosol, and subsequent induction of caspase-9 activity. Further data indicate that theasinensin A is effective in inducing the degradation of DFF-45 (an inhibitor binding to DNAase), which allows caspase-activated DNAase to enter the nucleus and degrade chromosomal DNA. As a consequence, it has been suggested that induction of apoptosis by theasinensin A and other tea polyphenols affords a pivotal mechanism for their cancer chemopre-

ventive function[12]; a commentary on the cancer chemoprevention by tea polyphenols through blockage of mitotic signal transduction has been critically elaborated by Lin et al. [9] and Lin and Liang.[33]

9.3.11 Suppression of Fatty Acid Synthase (FAS)

Results have recently been published of a long-term feeding trial examining the effect of green tea leaves on levels of cholesterol, lipid, antioxidant, and phase II enzymes in Wistar rats.[34] These indicate that such feeding can reduce total cholesterol, triacylglycerol, and low-density lipoprotein (LDL) cholesterol, and enhance activities of superoxide dismutase (SOD) in serum and glutathione S-transferase (GST) and catalase in the rat liver.[34] At the fifteenth week, the average body weights of experimental and control groups were 449 and 510 g, respectively, indicating that oral feeding of green tea leaves resulted in a significant (12%) decrease in body weight ($p < 0.05$). The dose of green tea leaves used in the study did not reduce diet or water consumption throughout the feeding regimen, and survival ratios of both groups were 100% (12/12).[34]

The key enzyme for lipogenesis fatty acid synthase (FAS) has been shown to be significantly suppressed by tea extracts and tea polyphenols, including EGCG and TF-3 in human breast carcinoma cells; expression of FAS was enhanced by EGF and inhibited by the presence of tea polyphenols. These experimental data indicate that suppression of FAS may result from downregulation of the EGF receptor/PI3K/Akt signal transduction pathway (unpublished results).

9.4 GENERAL REMARKS ON THE MECHANISMS OF CANCER CHEMOPREVENTION

It has been recognized that deregulated proliferation and inhibition of apoptosis lie at the heart of all tumor development, and these loci provide two obvious targets for general therapeutic and chemopreventive interventions. Clearly there are numerous mechanisms through which these two defects can occur, and the success of targeted therapy and chemoprevention will depend to a very considerable extent on the molecular fingerprinting of individual tumors.[35]

Most receptor tyrosine kinases (RTKs) transduce key intracellular signals that trigger cellular events, such as mitosis and cytoskeletal rearrangement, and serve to orchestrate physiological processes, such as development, wound repair, and oncogenesis. Ligand binding of RTKs mediates these responses by activating a variety of intracellular signaling pathways through an intrinsic kinase activity. It appears that these diverse signaling pathways, activated by growth factor receptors, induce broadly overlapping, rather than independent, sets of genes.[36] RTKs are important regulators of intracellular signal transduction pathways mediating development and multicellular communication in metazoans. Their activity is normally tightly controlled and regulated, and perturbation of RTK signaling by mutation and other genetic alterations leads to deregulated kinase activity and malignant transformation.[37]

Several lines of evidence have demonstrated that signal transduction events leading to the activation of the MAPK pathways, including ERK, JNK, and P38, NF-κB pathway, and JAK-STAT pathway can result in cell proliferative, survival, differentiating, and apoptotic responses. Figure 9.4 illustrates the key mechanisms that lead to inhibition of survival gene expression (c-jun, c-fos, c-myc, etc.) and activation of apoptotic signal pathways (caspase 8 and caspase 9 cascades). Two important signaling events, namely, the MAPK, NIK (NF-κB-inducing kinase) and caspase cascades (ICE/ced 3 family proteases) pathways, have been highlighted. Most tea polyphenols suppress the MAPK and NIK pathways[24,38] but activate the caspase cascade pathways[11]; such a combination of effects will potentially lead to apoptotic response in the target cells.

Most tea polyphenols with cancer chemopreventive activities possess antioxidant activity[8,39]; it should be emphasized that in addition to acting as ROS scavengers, these compounds can act through multiple mechanisms to modulate the functions of

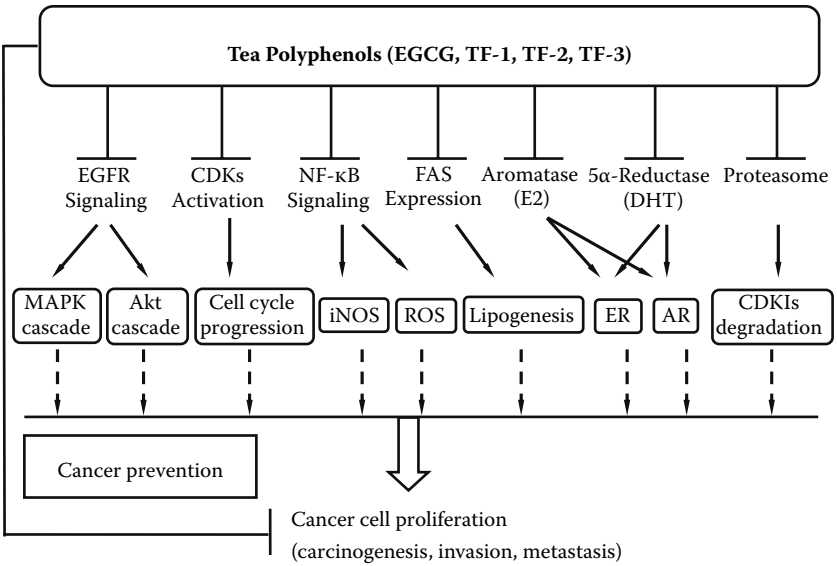

FIGURE 9.4 Proposed multiple biochemical and molecular mechanisms of cancer chemoprevention by tea polyphenols. Several lines of results indicate that antioxidant effects and signal blockades have played important roles in the modulation of cancer chemoprevention by tea and tea polyphenolics. Cell proliferation and differentiation are strictly regulated by a programmed network of intracellular signal transduction pathways through various transducers, including intrinsic factors such as receptor proteins, EGFR, RTK, PKA, PKB, PKC, PI3K, Akt, MAPK, NF-κB, AP-1, c-jun, c-fos, c-myc, iNOS, FAS, and ROS, and extrinsic factors such as cytokines, growth factors, androgen receptor, estrogen receptor, tumor promoters, TNF, and LPS. Our recent studies have demonstrated that tea polyphenols can suppress carcinogenesis through inhibiting aromatase[45] and 5α-reductase[46] activities. Furthermore, our recent studies have shown that tea polyphenols might modulate tumor development by inhibiting proteasomal activities.[47] The illegitimate regulation or hyperfunction of such signaling processes may lead to induction of carcinogenesis or inflammation. Tea polyphenols were found to suppress the hyperfunction of signaling in various systems that may block carcinogenesis and afford cancer chemoprevention.[44]

receptors, effectors, protein kinases, protein phosphatases, and protein substrates in the mitogenic and differentiating signaling involved in tumor promotion.

Although most existing cancer drugs are antimitotic, they act by crudely interfering with the basic machinery of DNA synthesis and cell division rather than by targeting the specific lesions responsible for regulated tumor growth. It is now also clear that the surprising selectivity of such crude agents is mainly a result of increased sensitivity to apoptosis afforded to tumor cells by their oncogenic lesions.[40,41] Drugs designed to specifically inhibit growth-deregulating lesions are currently being tested in clinical trials, and include inhibitors of RTKs, Ras, downstream signaling kinases such as the MAPK and Akt pathways, and CDKs.[42]

9.5 CONCLUSIONS

Green tea is perhaps the best known and most studied for its effects on cancer chemoprevention. However, the results of epidemiological studies in humans have been inconsistent; some studies have shown reduced cancer incidence and recurrence associated with green tea consumption, whereas others have failed to show an effect.[29] On the other hand, studies in animal models of carcinogenesis are far more convincing and have clearly demonstrated the preventive effects of green tea and EGCG against tumorigenesis in the breast, prostate, lung, and skin.[29]

Studies in animal models have demonstrated that tea polyphenols can inhibit carcinogenesis at all stages, including initiation, promotion, and progression.[42] This multifaceted inhibition of carcinogenesis process is attributed to a combination of antioxidative, antiproliferative, and proapoptotic effects of these polyphenols. Tea polyphenols have also been shown to inhibit the processes of angiogenesis, tumor metastasis, and invasion in animal models.[43]

Massive experimental findings have demonstrated that biochemical mechanisms of anticarcinogenesis of tea polyphenols may be due to the inhibition of carcinogen metabolic activation through suppressing the expression of cytochrome p450 (cyp-1-A-1, etc.), as well as the activation of carcinogen metabolic inactivation through enhancing the expression of cytochrome p450 (cyp-3-A, etc.),[42,44] as illustrated in figure 9.3.

It has been demonstrated that both green and black teas are equally effective in cancer chemoprevention, and the biological functions of both teas have been attributed to their polyphenols, including catechins and theaflavins, respectively. Tea polyphenols exhibit a wide variety of biological properties, including antioxidative effects, inhibition of extracellular mitotic signals through blocking the growth receptor signalings, inhibition of cell cycle at G1 phase through cyclin-dependent kinases suppression, suppression of inducible nitric oxide synthase through inhibiting the activation of IκB kinase (IKK) and NF-κB, and induction of apoptosis in cancer cells through releasing cyctochrome c and activating caspase cascades. Recently, tea polyphenols, including catechins (ECG, EGCG) and theaflavins (TF-3), have been shown to inhibit IL-lβ-induced IKK activity, and thus the nuclear translocation of NF-κB, leading to the suppression of vascular cell adhesion molecule-1 (VCAM-1) and intracellular adhesion molecule-1 (ICAM-1). These findings have provided additional mechanisms for the anti-inflammatory effect of tea polyphenols. It is significant that tea polyphenols suppress the fatty acid synthase (FAS) in human breast carcinoma

MCF-7 cells, since several FAS inhibitors have been shown to be potent antitumor agents and FAS is the key enzyme for controlling lipogenesis and body weight gain. Recent results indicate that tea and tea polyphenols may induce hypolipidemic and antiobesity effects through suppressing FAS expression.

In summary, cancer chemopreventive agents can inhibit tumor growth through arresting cell cycle and inducing cellular apoptosis. During the past few years, experimental results have demonstrated that cancer chemoprevention by tea polyphenols can be achieved by signal transduction blockade.[42,44] Discovering novel therapeutics and chemopreventive agents with clinical utility continues to be the focus of biochemical and pharmacological scientists working in the signal transduction therapy. Developing compounds designed to manipulate kinase pathways and signal events through both inhibitory and stimulatory methods for treating cancer and other diseases offers a promising trend for biomedical research.

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10 Effect of Tea and Tea Constituents on Inflammation

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CONTENTS

10.1 INTRODUCTION

Tea, the second largest consumed beverage in the world after water, is made from the dried and processed leaves of the plant *Camellia sinensis*.[1,2] Its popularity can be attributed to the sensory qualities, namely, the taste and aroma. More recently, the health-beneficial effects of tea have also attracted consumers toward this traditional beverage. These qualities are a function of the chemical characteristics of tea that vary depending on several factors, such as the climate, manufacturing process, and age of the leaves.[3] The biological activity of tea has been studied extensively over the past several years. Tea has been shown to possess several health benefits, such as inhibition of mutagenesis, antioxidative, anticarcinogenic, anti-inflammatory, antihypertensive, and hypocholestrolemic properties. Although several articles have been published reviewing the various health benefits of tea,[4–6] few have discussed its anti-inflammatory activity.

The anti-inflammatory and anticarcinogenic activities of tea have been evaluated extensively using various *in vivo* and *in vitro* models. The following section describes the basics of inflammation, the link between inflammation and cancer, and the effect of tea on inflammatory disorders.

10.2 INFLAMMATION

Inflammation may be defined as the reaction or response of the body to tissue injury or damage, and is commonly characterized by four symptoms: redness, swelling, pain, and heat.[7,8] Cell or tissue damage may be caused by several agents, such as

heat, chemicals, radiation, microbial infection, and trauma, as well as certain immunological processes. To counteract the tissue damage, the body has two types of defense systems, the primary defense system (immediate, such as inflammation) and the secondary defense system (controlled by the immunological system).[9] During this process, temporary vasoconstriction of blood vessels takes place, followed by local vasodilation and increased capillary permeability, leading to redness, edema, and heat. Simultaneously, leukocytes migrate to the site of injury through the process of chemotaxis to attack the antigen. These processes are referred to as acute inflammation, during which various mediators, reactive oxygen species (ROS) and reactive nitrogen species (RNS), are released to help control the inflammatory process.[10–12] However, if the acute inflammation persists over an extended period of time, due to dysregulation of various events, it can lead to the development of chronic inflammation. The basic mediators of inflammation and their functions are discussed in the following sections.

The cytokines (peptide mediators of inflammation) are important intracellular messengers that play an important role in the inflammatory process.[13] They have a short duration of action and perform several diverse functions, such as growth regulation, cell division, inflammation, and immunity, by interacting with specific receptors present in different cells.[14,15] Cytokines can be of different classes and possess both pro- and anti-inflammatory properties. The interleukins (ILs) are cytokines produced by leukocytes, which are important in the inflammatory process. The important proinflammatory cytokines include tumor necrosis factor α (TNFα), interleukin-1 (IL-1), and interleukin-6 (IL-6).

Eicosanoids are the lipid mediators of inflammation that are derived from fatty acids such as arachidonic acid (AA). AA can undergo metabolism through several pathways to generate a wide variety of lipid mediators, such as prostaglandins (by Cox pathway) and leukotrienes (by Lox pathway), which serve as important intra- and intercellular messengers.[16,17]

The Cox enzyme has been known to occur in three isoforms: Cox-1, -2, and -3. Cox-1 is a ubiquitous enzyme and expressed constitutively in the endothelium, kidneys, vascular smooth muscle, and gastrointestinal tract. The Cox-2 enzyme is inducible, as it is upregulated during the process of inflammation by other inflammatory mediators.[18] Lipoxygenases (Lox) are a group of iron-containing dioxygenases that act by incorporating oxygen molecules into polyunsaturated fatty acids (PUFAs) at different positions to form various metabolites.[19] They exist in several isoforms (such as 5-, 12-, and 15-Lox), giving rise to myriad products, such as leukotrienes (LT), with different functions. Leukotrienes are known to play important roles in inflammatory diseases such as asthma and psoriasis.[20] LTB_4 is the most well-studied metabolite of the Lox pathway and has been shown to be involved in the activation of inflammatory cells and assist in the migration of leukocytes to the site of inflammation. The Cox and Lox pathways of arachidonic acid metabolism together produce a wide range of mediators that perform several critical functions in the body. These include their role in inflammation, blood clotting, maintenance of mucosal lining in the stomach, and as intracellular messengers.[19]

In certain cases, the inflammatory process is unable to function effectively and the cells of the inflammatory response are unable to remove the inciting stimuli,

leading to the development of chronic inflammation that is characterized by sustained tissue damage, leading to cellular hyperproliferation, accumulation of inflammatory mediators, growth factors, ROS, and RNS.[21–23] These in turn can cause DNA, protein, and cell membrane damage, ultimately leading to the development of diseases such as arthritis, ulcerative colitis, inflammatory bowel disease, and cancer.[19,24–26]

The link between inflammation and cancer was first proposed in 1863 by Rudolph Virchow, who noted that cancer arises from sites of chronic inflammation.[27,28] In recent years, a strong link has been established between chronic inflammation and carcinogenesis in epidemiological, *in vivo*, and *in vitro* studies. It has been noted that the inflammatory mediators discussed above, ROS and RNS, are involved in the development of carcinogenesis by promoting cell growth, differentiation, metastasis, and neoplastic progression.[10] In recent years it has been established that about 15% of cancers occurring worldwide are caused by chronic inflammation. Some of these include gastric cancer from *Helicobacter pylori*, hepatocellular carcinoma from hepatitis C, colorectal cancer from inflammatory bowel disease, lung cancer due to prolonged cigarette smoking, esophageal cancer from reflux esophagitis, and mesothelioma from exposure to asbestos.[25,27,29]

Arachidonic acid metabolites have received tremendous attention for their role in carcinogenesis. A link among the enzymes, metabolites, and receptors involved in the Cox and Lox pathways has been established to inflammation and a variety of cancers. Aberrant arachidonic acid metabolism has been shown to play a major role in the development and progression of several cancers.[30] Both the Cox and Lox pathways have been linked to cell proliferation, inhibition of apoptosis, and promotion of angiogenesis.[23] In addition, several clinical, *in vitro*, and *in vivo* studies have been conducted to demonstrate the chemopreventive activity of several Cox-2 and 5-Lox inhibitors on several models of cancer.[23,30–33]

Peptide mediators of inflammation, such as the cytokines and chemokines, have also been shown to play an important role in the progression of cancer and have been shown to be expressed in several cancers, such as breast, prostate, ovarian, and bladder.[27] They can lead to tumor promotion either by stimulating cell growth and differentiation or by inhibiting apoptosis.[30] Cytokines such as tumor necrosis factor, IL-6, and IL-8 have been shown to promote tumor growth by activating transcription factors such as *ras* and NF-κB through tumor inflammation and vasculogenesis, causing DNA damage due to production of ROS/RNS, releasing growth factors to promote tumor growth, and controlling tumor cell migration and leukocyte infiltration, among many others.[27,34–36]

The ROS/RNS that are released during inflammation (such as hydrogen peroxide, nitric oxide, and superoxide anion) have also been shown to play a major role in the process of carcinogenesis. These free radicals have been shown to damage proteins, DNA, RNA, and lipids, leading to mutations in several key genes involved in carcinogenesis.[25,37] They have been shown to be involved in cell growth and tumor promotion by activating certain signal transduction pathways, such as c-MYC, c-FOS, NF-κB, and mitogen-activated protein kinases (MAPKs).[25]

On the other hand, certain free radicals such as hydrogen peroxide and superoxide anion are known to induce apoptosis through the activation of certain signal transduction pathways, as mentioned earlier.[10,12,22] It has been hypothesized that

ROS such as hydroxyl radical and nitric oxide can induce pores in the mitochondria leading to cytochrome c release and eventually apoptosis.[22] Nitric oxide has been shown to produce conflicting effects on apoptosis. In certain cells it has been shown to inhibit apoptosis, while in others it has been shown to induce the same (such as cancer, epithelial, and endothelial cells). This inhibitory effect has been attributed to the capacity of nitrate to nitrosylate caspases and possibly inactivate them or to increase intracellular glutathione levels.[22]

10.3 ANTI-INFLAMMATORY ACTIVITY OF TEA

Tea and its constituents have been shown to be beneficial in several inflammatory disorders. The catechins in tea, particularly epigallocatechin gallate (EGCG), have been shown to inhibit inflammation by modulating several pathways. Tea and its constituents have been shown to be effective against several inflammatory conditions, such as osteomyelitis,[38] psoriasis,[39] arthritis,[40] and inflammation of the respiratory system.[41–43]

Osteomyelitis (inflammation of the bone) is accompanied by bone lysis and infected osteoblasts are caused due to the release of inflammatory mediators such as the cytokines. Treatment with EGCG has been shown to inhibit release of certain cytokines in osteoblasts infected with *Staphylococcus aureus*, thereby proving beneficial in osteomyelitis treatment.[38]

Green tea and its constituents have been shown to protect skin from irritation caused by radiation as well as chemicals. EGCG has been shown to prevent skin damage caused by UVA radiation and 12-*O*-tetradecanoylphorbol-13-acetate (TPA) application through inhibition of NF-κB DNA binding and expression of MAP kinase and Cox-2.[44,45] Psoriasis is an inflammatory skin disease in which there is hyperproliferation and improper differentiation of epidermal keratinocytes. Green tea polyphenols have been shown to alleviate symptoms of psoriasis in mice.[39]

Arthritis (inflammation of the joints) affects nearly one in three adults in the United States and can occur in various forms, such as osteoarthritis and rheumatoid arthritis.[46–48] The most common method of treating arthritis is with the use of anti-inflammatory agents. However, they are slow acting and can cause long-term toxicity. Alternative medicine and herbal supplements are gaining popularity due to their safety and low toxicity.[46,47] Tea and its constituents have been shown to be beneficial in arthritis in both *in vivo* and *in vitro* models of arthritis.[40] In a study conducted using normal and arthritic cartilage tissue, catechins were found to be effective in ameliorating symptoms of arthritis such as collagen breakdown, and thereby may prove beneficial to arthritic patients.[49] Several studies have shown EGCG to be effective in osteoarthritis through the inhibition of various proinflammatory mediators' production (nitric oxide, PGE2) in osteoarthritic tissue.[50,51] Administration of green tea polyphenols to mice led to a decreased incidence of arthritis with an associated decrease in inflammatory mediators such as Cox-2, interferon-γ, and tumor necrosis factor.[52] In addition, an epidemiological study showed that older women who were drinking more than three cups of green tea/day had a significantly lower risk of rheumatoid arthritis than those who drank no tea.[53] These studies indicate that intake of green tea may be beneficial in patients suffering from arthritis.

Tea and its constituents have also been shown to be beneficial to the respiratory system. In a study conducted in guinea pigs, administration of cigarette smoke led to lung damage, oxidative stress, and inflammation, which were prevented by administration of black tea beverage.[41] Tea catechins have also been shown to be effective in ameliorating airway inflammation by inhibiting the production of associated inflammatory mediators in nasal fibroblasts and bronchial epithelial cells. In this study of the catechins tested, EGCG and epicatechin gallate (ECG) were the most effective.[43] EGCG has also been shown to be beneficial in a murine asthma model through inhibiting inflammatory cell migration and ROS production.[42]

The protective effect of tea and its constituents in inflammation of the gastrointestinal tract has also been demonstrated. *Helicobacter pylori* is a bacteria that causes inflammation of the stomach, development of ulcers, and eventually, if left untreated, the development of gastric cancer. This bacterium has been known to act through several pathways to exert its deleterious effects. *H. pylori* infection stimulates the glycosylation of toll-like receptor (TLR)-4, leading to the release of inflammatory mediators. EGCG has been shown to inhibit this glycosylation, thereby leading to a decreased inflammatory response and levels of inflammatory mediators.[54] In addition, green tea mixtures prevent the migration of *H. pylori* to the gastric epithelium, thereby preventing gastric damage. In addition, EGCG has been shown to inhibit DNA damage caused by *H. pylori* infection in gastric epithelial cells.[55]

Tea and its constituents have been shown to be effective against inflammatory bowel disease by inhibiting the expression of inflammatory genes such as TNFα, IFNγ, iNOS, and NF-κB, thereby reducing the production of associated inflammatory mediators.[56] EGCG has also been shown to be effective in inhibiting the production of chemokines (IL-8) and PGE2 in rat colonic cells.[57]

EGCG possesses beneficial effects in cardiovascular diseases in which inflammation and oxidative stress are the primary causes of concern. EGCG has been shown to inhibit DNA damage, LDL oxidation, and nitric oxide production. It has also been shown to scavenge free radicals, decrease the production of inflammatory mediators (such as cytokines and eicosanoids), and modulate inflammatory genes (such as NF-κB and MMP).[58,59]

The anti-inflammatory effect of tea extract has been evaluated with reference to the primary symptoms of inflammation. In one such study, tea extracts inhibited paw inflammation induced by agents such as carrageenin and prostaglandin. There was also an associated decrease in exudative inflammation, granuloma formation, and inhibition of Cox and Lox pathways.[60,61] EGCG has also been shown to suppress neutrophil infiltration by direct action on neutrophils with an associated decrease in cytokine production both *in vivo* and *in vitro*.[62] Gallic acid (a constituent of tea) has been shown to inhibit histamine release in mast cells, thereby preventing inflammatory allergic disorders.[63]

We have evaluated the effect of black tea theaflavin derivatives on 12-*O*-tetradecanoylphorbol-13-acetate induced skin inflammation in mice. The anti-inflammatory activities of theaflavin, theaflavin monogallates, and theaflavin digallate have been evaluated using this model. The effects of these compounds on ear edema (measured as ear punch weight) and levels of inflammatory mediators (cytokines and eicosanoids) have been evaluated.[64,65]

For our experimental approach, both ears of the mice were treated with acetone or the test compound in acetone 20 minutes prior to the application of TPA in acetone. At the end of the treatment period, the mice were sacrificed by cervical dislocation and the ears punched. The ear punches were then weighed, homogenized with phosphate-buffered saline, and tested for the levels of various inflammatory biomarkers. The results of these experiments are shown in tables 10.1 to 10.3.

All the compounds tested (theaflavin mixture, theaflavin monogallate, and theaflavin-3,3'-digallate) were effective in inhibiting ear edema and levels of inflammatory mediators in a dose-dependent manner. Theaflavin-related compounds were able to inhibit both the cytokines (peptide mediators of inflammation) and eicosanoids (lipid mediators of inflammation). [66]

Thus, black tea–related compounds also possess significant anti-inflammatory properties. However, the mechanisms of action of these compounds need to be further evaluated.[67–69]

Several studies have been conducted on the anti-inflammatory and anticarcinogenic activities of both green tea and black tea, as discussed in this chapter. However, the effect that is observed *in vitro* and in certain *in vivo* experiments is not reproducible in the human situation. This could be due to several confounding factors involved in epidemiological studies, or to the poor bioavailability of catechins. More detailed mechanistic studies are warranted to understand the effects of tea catechins *in vivo* so that they can be made applicable to the human situation.[70]

TABLE 10.1
Effect of theaflavin mixture on TPA-induced ear inflammation and levels of inflammatory mediators in female CD-1 mice

Treatment	Weight of ear punch (mg)	IL-1ß concentration (pg/mg tissue)	IL-6 concentration (pg/mg tissue)	PGE_2 concentration (pg/mg tissue)	LTB_4 concentration (pg/mg tissue)
Acetonene	8.10 ± 0.30	0.95 ± 0.15	0.34 ± 0.21	1000 ± 75	1.9 ± 0.17
TPA (0.8 nmol)	14.10 ± 0.48	88.50 ± 4.00	3.95 ± 0.02	2400 ± 616	9.8 ± 0.21
Theaflavins (0.25 µmol) + TPA	10.90 ± 0.65*	12.80 ± 1.05*	2.40 ± 0.03*	2100 ± 260*	2.53 ± 0.21*
Theaflavins (0.5 µmol) + TPA	9.78 ± 0.50*	7.10 ± 2.05*	1.95 ± 0.04*	833 ± 144*	2.12 ± 0.18*
Theaflavins (1 µmol) + TPA	8.31 ± 0.35*	1.92 ± 0.15*	0.40 ± 0.03*	1277 ± 81*	1.18 ± 0.04*

Note: Female CD-1 mice (7–8 weeks old, 6 mice per group) were treated topically with acetone or theaflavin mixture in acetone 20 minutes prior to application of TPA (0.8 nmol). The mice were sacrificed after 5 hours. Ear punches (6 mm in diameter) were weighed separately. The ear punches from each group were then combined and homogenized. The tissue homogenate was tested for the level of interleukin-6 (IL-6) using ELISA. Data are expressed as mean ± standard error from three separate determinations. Values with asterisks (*) are significantly different ($p < 0.05$) from positive control (TPA) group 2, as determined by Student's *t*-test.

TABLE 10.2
Effect of theaflavin monogallate (TF-2) on TPA-induced ear inflammation and levels of inflammatory mediators in female CD-1 mice

Treatment	Weight of ear punch (mg tissue)	IL-6 concentration (pg/mg tissue)	LTB_4 concentration (pg/mg tissue)
Acetone	6.71 ± 0.20	1.15 ± 0.11	5.9 ± 0.40
TPA (0.8 nmol)	14.26 ± 0.65	8.61 ± 0.35	20 ± 1.13
TF-2 (0.25 μmol) + TPA	11.04 ± 0.58*	3.94 ± 0.15*	16.8 ± 1.82*
TF-2 (0.5 μmol) + TPA	10.09 ± 0.65*	2.82 ± 0.1*	13.9 ± 1.20*

Note: Female CD-1 mice (7–8 weeks old, 6 mice per group) were treated topically with acetone or theaflavin monogallate in acetone 20 minutes prior to application of TPA (0.8 nmol). The mice were sacrificed after 5 hours. Ear punches (6 mm in diameter) were weighed separately. The ear punches from each group were then combined and homogenized. The tissue homogenate was tested for the level of interleukin-6 (IL-6) using ELISA. Data are expressed as mean ± standard error from three separate determinations. Values with asterisks (*) are significantly different ($p < 0.05$) from positive control (TPA) group 2, as determined by the Student's *t*-test.

TABLE 10.3
Effect of theaflavin-3,3′-digallate (TF-3) on TPA-induced ear inflammation and levels of inflammatory mediators in female CD-1 mice

Treatment	Weight of ear punch (mg)	IL-1ß concentration (pg/mg tissue)	PGE_2 concentration (pg/mg tissue)	LTB_4 concentration (pg/mg tissue)
Acetonene	7.85 ± 0.21	1.785 ± 0.05	3223.42 ± 550.94	2.105 ± 0.30
TPA (0.8 nmol)	19.63 ± 0.6	359.58 ± 5.11	5384.663 ± 366.37	72.47 ± 7.80
TF-3 (0.25 µmol) + TPA	19.49 ± 1.00	281.12 ± 6.19*	4140.127 ± 424.85	12.84 ± 0.78*
TF-3 (0.5 µmol) + TPA	16.92 ± 0.55*	175.54 ± 20.71*	3212.847 ± 445.59*	9.96 ± 1.94*

Note: Female CD-1 mice (7–8 weeks old, 6 mice per group) were treated topically with acetone or theaflavin-3,3'-digallate (TF-3) in acetone 20 minutes prior to application of TPA (0.8 nmol). The mice were sacrificed after 5 hours. Ear punches (6 mm in diameter) were weighed separately. The ear punches from each group were then combined and homogenized. The tissue homogenate was tested for the level of interleukin-6 (IL-6) using ELISA. Data are expressed as mean ± standard error from three separate determinations. Values with asterisks (*) are significantly different ($p < 0.05$) from positive control (TPA) group 2, as determined by the Student's *t*-test.

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11 Inhibition of Breast Cancer Cell Proliferation by Theaflavins and Epigallocatechin 3-gallate through Suppressing Proteasomal Activities

Jen-Kun Lin, Yu-Wen Chen, and Shoei-Yn Lin-Shiau

CONTENTS

11.1 INTRODUCTION

The plant-based diets, rich in vegetables and fruits, protect against different cancers as evidenced in multiple epidemiological studies.[1] The identification of the specific and protective constituents of these foods may lead to additional means of disease prevention, such as the use of chemopreventive agents in high-risk individuals and the engineering of "designer foods" enriched in protective compounds. The effective compounds, polyphenols, for example, have been considered as the responsible agents for such observations, and since then, much research has been conducted on their potential anticancer effects.

Polyphenols are the most common and widely distributed group of plant chemicals present in fruits, vegetables, and teas, among other sources. Recent studies have provided growing evidence for the beneficial actions of polyphenols on multiple carcinogenic pathways, including inhibition of carcinogen bioactivation, regulation of cell signaling and cell cycle, antiangiogenesis, scavenging oxidative stress, and anti-inflammation.[2] Several polyphenolic compounds are known as cancer chemopreventive agents, such as black tea and green tea polyphenols,[3] tannins, tannic acid,[4] ellagic acid,[5] corilagin, and geraniin.[6,7] In addition, flavonoids, a class of natural polyphenolic compounds, display a variety of biological activities, including tumor growth inhibition and cancer chemoprevention in various animal models.[8,9] For example, genistein,[10] quercetin,[11] apigenin,[12] rutin,[13] and genistin[14] have growth inhibitory activities on various cancer cell lines.

Tea (*Camellia sinensis*) is the most consumed beverage worldwide. Recently, the cancer chemopreventive actions of tea have been extensively investigated. Previous epidemiological studies suggest that tea consumption may have a protective effect against human cancer.[15,16] Among a number of bioactive compounds of tea, the abundant tea polyphenols are thought to contribute to the beneficial effects on cancer chemoprevention. According to the fermentation procedure employed in the manufacture of tea, major teas can be classified into black tea and green tea. The inhibitory effects of black tea bioactive polyphenols, including theaflavin (TF1), theaflavin-3-gallate (TF2a), theaflavin-3'-gallate (TF2b), and theaflavin-3,3'-gallate (TF3), and the green tea major polyphenol (–)-epigallocatechin-3-gallate (EGCG) on carcinogenesis and tumor growth have been elaborated recently.[17–19] These studies indicate that the action mechanisms of cancer prevention by tea polyphenols are through modulating signal transduction pathways to inhibit certain cancer-related proteins that are involved in tumor survival and metastasis, or regulate DNA replication and transformation.[18,19] However, more undefined mechanisms or molecules involved in chemoprevention by tea polyphenols might exist that need further studies.

The ubiquitin-proteasome pathway is a central route for the selective degradation of intracellular short-lived proteins' proteolysis, an essential metabolic process

that also plays an important role in the regulation of many physiological processes, as well as in the development of a number of human diseases.[20,21] The 26S proteasome, the main part of this pathway, is a large multisubunit complex (2.4 MDa) that comprises 20S catalytic core and two 19S regulatory caps that contain the ubiquitin recognition sites and ATPases for protein unwinding.[22–24] There are three major proteasomal activities that locate at central β-rings and differ in their specificities: chymotrypsin-like, trypsin-like, and peptidyl glutamyl peptide hydrolase (PGPH) activities.[25] The proteasome functions are involved in allowing tumor cell cycle progression and protecting tumor cells against apoptosis. For example, degradation of proteasome substrates, such as the tumor supressors p53 and $p27^{Kip1}$, an inhibitor of cyclin-dependent kinases, can promote tumorigenesis.[21] The evidence has shown that inhibition of the chymotrypsin-like site of the proteasome or its inactivation by mutation alone causes a large reduction in the rates of protein breakdown,[26,27] and the chymotrypsin-like activity of the proteasome was associated with tumor cell survival.[28] The well-established proteasome inhibitors inhibiting the chymotrypsin-like activity of the proteasome indicate that the proteasome activity is essential for tumor cell survival.[28] Most of the recent experiments using cell cultures and mouse models have reported that proteasome inhibitors induce cancer cell apoptosis, and therefore inhibit tumor growth and may be used as potential novel anticancer agents.[29,30]

Previous reports have indicated that EGCG and tannic acids both bearing ester bonds specifically inhibit the proteasomal chymotrypsin-like activity.[31,32] Recently, we have demonstrated that pentagalloylglucose (5GG), a natural hydrolyzable tannin, is a potent proteasome inhibitor and induces cell cycle arrest and apoptosis in Jurkat T cells through accumulating CDK inhibitors, $p21^{Cip1/Waf1}$ and $p27^{Kip1}$, and proapoptotic Bax protein by proteasome inhibition.[33] Interestingly, 5GG containing five galloyl groups with an ester bond linkage exhibited strong inhibitory effects on proteasome activity *in vitro* and *in vivo*. This suggested that galloyl-ester moiety might be an important structure to commit to the proteasome inhibition. The present study was performed to test this assumption, using structurally similar tea polyphenols, theaflavins, and EGCG to examine their inhibitory effects on the proteasomal activities. We found that the ester bond–containing polyphenols potentially and selectively inhibited the proteasomal chymotrypsin-like activity *in vitro*. Among selected polyphenols, theaflavins showed significant inhibitory effects on 20S or 26S proteasome that resulted in downregulation of tumor cell growth. Furthermore, we evaluated the relationship of the structures of the galloyl polyphenols and their inhibitory efficacies on proteasome activity.

11.2 INHIBITION OF TEA POLYPHENOLS ON PROTEASOMAL ACTIVITIES

11.2.1 Tea Polyphenols and Gallates

Tea polyphenol (–)-epigallocatechin-3-gallate (EGCG) was purified as described previously, and its purity is approximately 97%. TF1 (theaflavin), TF2a (theaflavin-3-gallate), TF2b (theaflavin-3'-gallate), and TF3 (theaflavin-3,3'-gallate) were isolated from black tea, as described previously.[34] Apigenin, quercetin, genistein, genistin,

rutin, tannic acid, ellagic acid, gallic acid, and n-propyl gallate were purchased from Sigma. Corilagin and geraniin were isolated as described previously.[35]

11.2.2 20S and 26S Proteasomes

Purified 20S proteasome (*Methanosarcina thermophila*, recombinant, *Escherichia coli*) was purchased from Calbiochem. Purified eukaryotic 20S proteasome (from rabbit) and eukaryotic 26S proteasome (from rabbit) were purchased from Sigma. Fluorogenic peptide substrates, Suc-Leu-Leu-Val-Tyr-AMC (for the proteasomal chymotrypsin-like activity) and benzyloxycarbonyl, (*Z*)-Leu-Leu-Glu-AMC (for the proteasomal PGPH activity), were also obtained from Calbiochem, and *Z*-Gly-Gly-Arg-AMC (for the proteasomal trypsin-like activity) was purchased from Bachem (King of Prussia, PA).

11.2.3 Cell Culture and Cell Extract Preparation

Human leukemia Jurkat T cells and human histiocytic leukemia U937 cells were cultured in RPMI 1640 medium supplemented with 10% (v/v) fetal calf serum, or 15% (v/v) fetal calf serum in the case of promyelocytic leukemia HL60 cells, 100 units/ml of penicillin, and 100 μg/ml of streptomycin. Human breast cancer MCF7 cells were grown in Dulbecco's modified Eagle's medium containing 10% (v/v) fetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. All cells were maintained in a humidified atmosphere of 5% CO_2 at 37°C. Cells were harvested, washed with cold phosphate-buffered saline twice, and homogenized in a lysis buffer (50 mM Tris-HCl [pH 8.0], 5 mM EDTA, 150 mM NaCl, 0.5% [v/v] Nonidet P-40, 0.5 mM phenylmethylsulfonyl fluoride, and 0.5 mM dithiothreitol) for 30 min at 4°C. Afterward, the lysates were centrifuged at 12,000*g* for 30 min, and the supernatants were collected as whole cell extracts.

11.2.4 Assay for Purified 20S and 26S Proteasome Activity

The chymotrypsin-like activity of purified 20S proteasome was measured as follows: 2 μg of purified recombinant 20S proteasome or 0.5 μg of eukaryotic 20S proteasome or 1 μl of eukaryotic 26S proteasome was incubated with 20 μM fluorogenic peptide substrate, Suc-Leu-Leu-Val-Tyr-AMC (for the proteasomal chymotrypsin-like activity), for 30 min at 37°C in 200 μl of assay buffer (20 mM Tris-HCl, pH 8.0) with or without the polyphenols (5 μM). After incubation, the reaction mixture was diluted with 200 μl assay buffer followed by a measurement of the hydrolyzed 7-amido-4-methyl-coumarin (AMC) groups using a VersaFluor™ Fluorometer with an excitation filter of 380 nm and an emission filter of 460 nm (Bio-Rad).

11.2.5 Assay for the Proteasome Activity in Whole Cell Extracts

A whole cell extract (10 μg) of Jurkat T cells or HL60 cells or MCF-7 cells or U937 cells was incubated for 90 min at 37°C with 20 μM fluorogenic peptide substrate, Suc-Leu-Leu-Val-Tyr-AMC for chymotrypsin-like or Z-Leu-Leu-Glu-AMC for PGPH or Z-Gly-Gly-Arg-AMC for trypsin-like activities, in 200 μL of the assay

buffer with or without a series of concentrations of tea polyphenols. The hydrolyzed AMCs were measured as described earlier.

11.2.6 Luminescence ATP Detection Assay

Cells were seeded at a density of 1×10^5 cells/ml into 96-well plates and incubated at 37°C. After overnight growth, cells were pretreated with a series of concentrations of tea polyphenols. Following 24 h of incubation with these tea polyphenols, the cell viability was assayed with a luminescent ATP detection assay kit (Packard BioScience B.V.), which is the alternative to colorimetric, fluorometric, and radioisotopic assays for the quantitative evaluation of proliferation of cultured mammalian cells. Briefly, 50 µl of cell lysate was used to assay the production of luminescent light caused by the reaction of ATP with added luciferase and D-luciferin. Luminescence was measured on a Top Counter Microplate Scintillation and Luminescence counter (Packard 9912V1, Meriden, CT) in single-photon counting mode for 0.1 min/well, following 2 min of adaptation in the dark.

11.3 INHIBITION OF PROTEASOMAL ACTIVITIES IN CANCER CELLS BY TEA POLYPHENOLS

11.3.1 Growth Inhibitory Effects of the Tea Polyphenols on Cancer Cells

The structures of the theaflavins and EGCG are illustrated in figure 11.1. The bioactive effects of these tea polyphenols on different kinds of tumor cell growth, namely, U937 cells, Jurkat T cells, HL60 cells, and MCF7 cells, were investigated. After 24 h of treatment with theaflavins, the growth of cancer cells was determined by bioluminescent detection of cellular ATP content. ATP is a marker for cell viability because of its presence in all metabolically active cells, and its concentration declines very rapidly when the cells undergo necrosis or apoptosis. As shown in figure 11.2, the inhibitory activity of tea polyphenols on cell growth was dose dependent in all cell lines, but with different patterns, suggesting that theaflavins exhibited potent inhibitory effects on tumor cell growth. Totally, the leukemic cell lines Jurkat T, U937, and HL60 were more sensitive to tea polyphenols than breast cancer cell line MCF7. Individually, theaflavins displayed a stronger inhibitory activity in all cell lines than EGCG. However, MCF7 cells seemed to be more resistant to tea polyphenols (an estimated IC_{50} of 23–30 µM) than other cells. Meanwhile, TF3 showed more effective inhibitory activity on the growth of Jurkat T cells and U937 cells with estimated IC_{50} of 12 and 6 µM, respectively. The growth inhibitory effect of TF2a appeared to be similar to that of TF2b in four cell lines.

11.3.2 Comparison of the Inhibitory Effect of the Black Tea Polyphenols on Purified Proteasome Activity Derived from Different Sources

To test whether theaflavins could inhibit the chymotrypsin-like activity of the purified *proteasomes*, such as the recombinant 20S proteasome from *M. thermophila*, rabbit

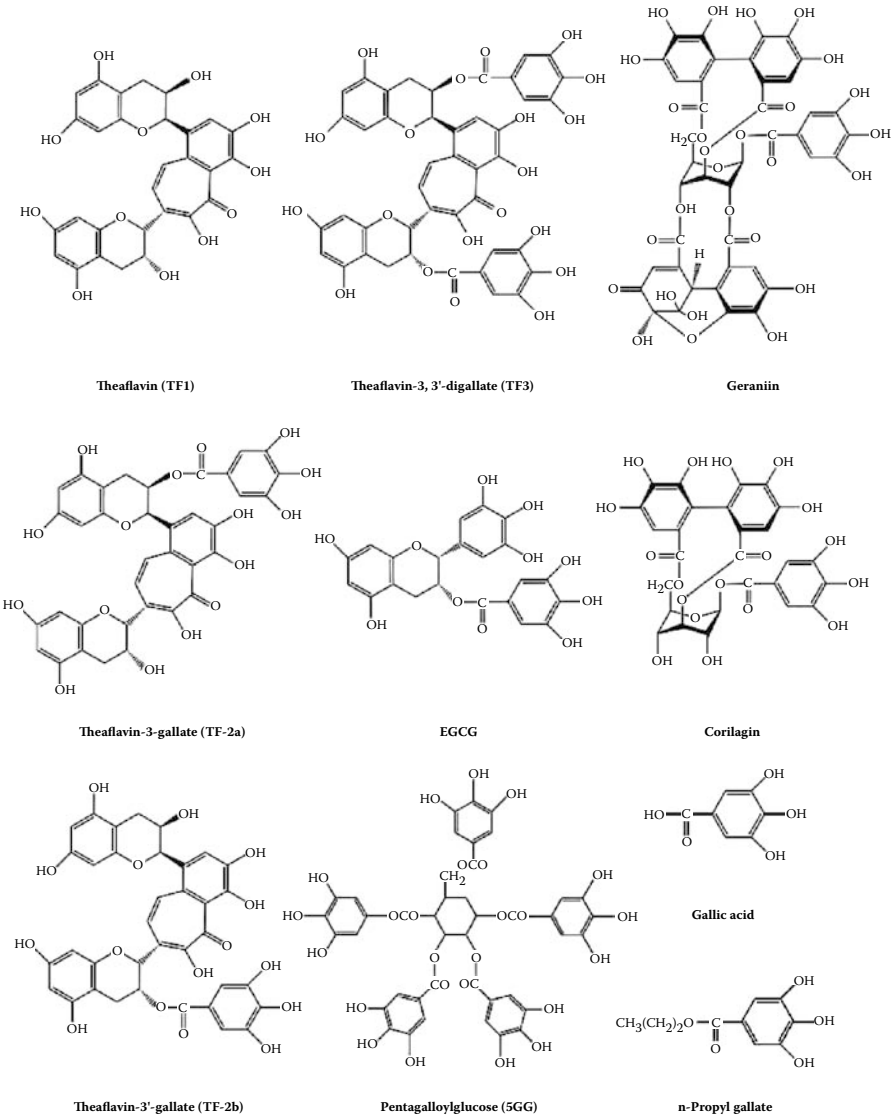


FIGURE 11.1 Chemical structures of selected polyphenols.

20S proteasome, and rabbit 26S proteasome, we performed a cell-free proteasome activity assay. Figure 11.3 shows that the chymotrypsin-like activities of purified 20S proteasome and 26S proteasome derived from prokaryotes or eukaryotes were more efficiently and significantly inhibited by theaflavins, especially TF3, compared to EGCG, which has been considered a proteasome inhibitor for chymotrypsin-like activity.[31] The tea polyphenols showed more potent inhibitory effects on prokaryotic recombinant 20S proteasomes where postmodifications are absent than on eukaryotic proteasomes. In addition, the inhibition of eukaryotic 26S proteasomes by tea

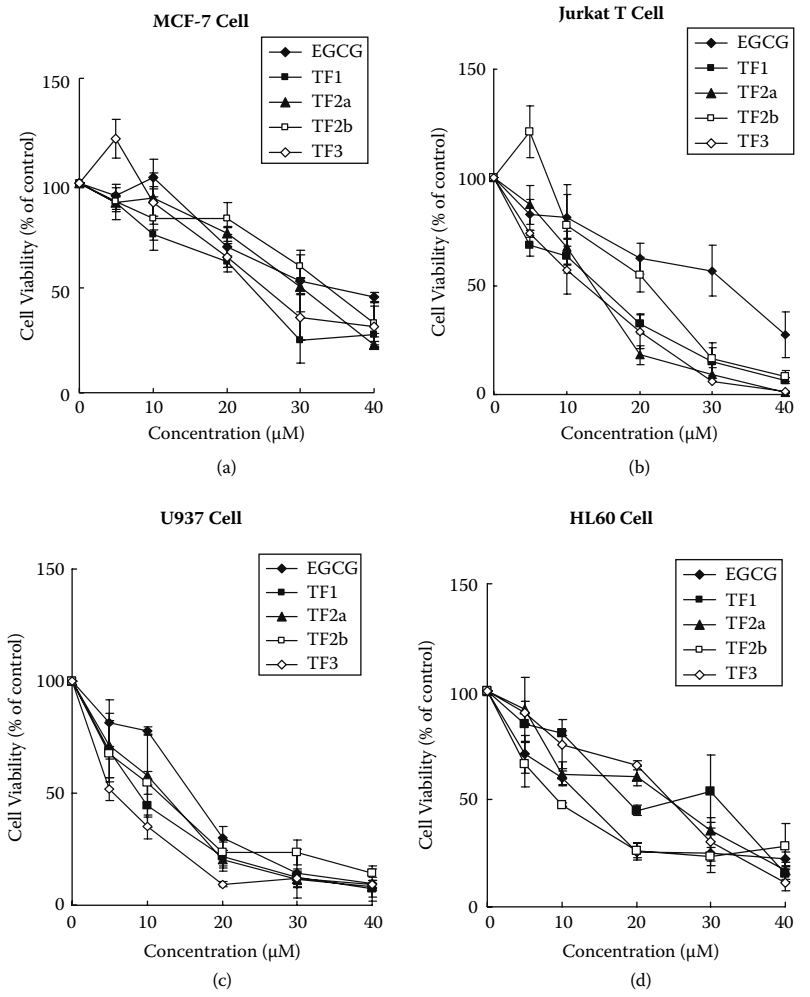


FIGURE 11.2 The effect of the tea polyphenols on the viability of different cell lines. 1 × 10^4 cells were seeded in 96-well plates and treated with various doses of tea polyphenols. After 24 h incubation, cell viability was determined by ATPLite-M cytotoxicity assay. Results were obtained from three independent experiments as mean ± SD.

polyphenols was similar to that of eukaryotic 20S proteasomes containing no 19S regulatory subunits. Thus, theaflavins as well as EGCG could efficiently inhibit both 20S and 26S proteasomes in eukaryotic cells. The data suggest that theaflavins are potential and specific proteasome inhibitors.

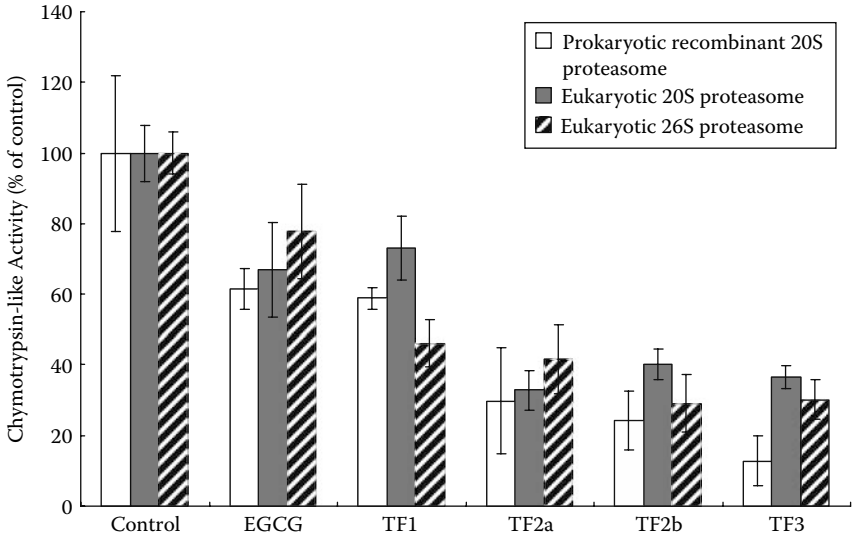


FIGURE 11.3 Comparison of the inhibitory effect of tea polyphenols on chymotrypsin-like activity of the purified proteasomes derived from different sources. The chymotrypsin-like activity of the purified proteasomes: recombinant prokaryotic 20S proteasome, eukaryotic 20S proteasome, and eukaryotic 26S proteasome were analyzed by incubation with the fluogenic substrate Suc-Leu-Leu-Val-Tyr-AMC (for chymotrypsin-like activity of the proteasome) in the presence of the tea polyphenols (5 μM) for 30 min and detected free AMC groups. Assays were performed in triplicate, and the results of representative experiments are shown as mean ± SD.

11.3.3 Inhibition of the Proteasomal Activities in Tumor Cell Extracts by the Black Tea Polyphenols

The inhibitory effects of theaflavins on chymotrypsin-like activity of the proteasomes in different tumor cell extracts were also compared. Protein extracts were prepared from different human tumor cell lines: human breast cancer MCF7 cells and human leukemia Jurkat T cells, U937 cells, and HL60 cells. As table 11.1 shows, proteasome activities in Jurkat T and U937 cell extracts were more potently inhibited by theaflavins than those in HL60 and MCF7 cell extracts. However, most of the tea polyphenols displayed different degrees of inhibitory effects on proteasome activity in tumor cell lines examined. The relative descending order of sensitivity to tea polyphenols was U937 > Jurkat T > MCF7 > HL60. TF3 exhibited the most potent inhibitory effect on the proteasomal chymotrypsin-like activity in these tumor cell extracts, and that was consistent with the previous result (figure 11.3). TF3 (at 5 μM) inhibited approximately 80% of the proteasomal chymotrypsin-like activity in U937 cell extracts, whereas EGCG had a slight inhibitory effect. Furthermore, the concentrations of theaflavins needed to inhibit the 26S proteasome activity in tumor cell extracts were higher than those used to inhibit purified 20S proteasome, suggesting that certain enzymes present in cell extracts may degrade theaflavins.

TABLE 11.1
Inhibition of chymotrypsin-like activity of the proteasome in different cell extracts by tea polyphenols

Tea polyphenols	Chymotrypsin-like activity of the proteasome (% of control)[b] Cancer cell lines[a]			
	MCF7	Jurkat T	U937	HL60
EGCG	77.0 ± 5.0	81.8 ± 6.2	61.4 ± 2.2	83.8 ± 3.8
TF1	57.4 ± 3.5	63.2 ± 0.5	45.6 ± 0	73.6 ± 4.9
TF2a	55.3 ± 2.6	38.4 ± 3.3	24.5 ± 8.2	59.0 ± 7.1
TF2b	65.3 ± 8.2	59.0 ± 5.4	37.3 ± 3.7	78.2 ± 7.0
TF3	46.2 ± 4.2	31.8 ± 4.0	18.1 ± 6.3	30.6 ± 2.0

[a] Cell extracts from cancer cell lines (10 μg/reaction) were incubated with fluorogenic peptide substrates for 90 min in the presence of 5 μM tea polyphenols followed by the measurement of free AMC groups.

[b] Assays were performed in triplicate, and the results of representative experiments are shown as mean ± SD.

The concentration of theaflavins may affect the inhibitory ability on chymotrypsin-like proteasome activity in tumor cell extract; thus, addition of tea polyphenols to MCF7 cell extracts potently inhibited the chymotrypsin-like activity in a dose-dependent manner (figure 11.4). These data indicated that the inhibitions of chymotrypsin-like activity by theaflavins were more effective than those by green tea polyphenol, EGCG, in MCF7 extracts. The IC_{50} of theaflavins inhibiting the proteasomal chymotrypsin-like activity was less than 10 μM except for TF1 (an estimated IC_{50} of 20 μM), whereas EGCG had less effect ($IC_{50} > 40$ μM) than theaflavins. The descending order of the inhibitory effects on proteasomal chymotrypsin-like activity by tea polyphenols was TF3 > TF2a > TF2b > TF1 > EGCG. Additionally, tea polyphenols also inhibited another proteasomal activity, the PGPH activity, but not the proteasomal trypsin-like activity (table 11.2). It appears that tea polyphenols selectively inhibited the proteasomal chymotrypsin-like and PGPH (peptidyl glutamyl peptide hydrolase) activities.

11.3.4 Inhibition of the Chymotrypsin-like Activity of Purified Proteasomes by Galloyl-Ester–Containing Polyphenols

It has been reported that ester bond–containing tea polyphenols, such as EGCG, specifically inhibit the chymotrypsin-like activity of the proteasome *in vitro* and *in vivo*.[31] 5GG (pentagalloylglucose), which comprises five gallic acids esterified to a glucose core, could exhibit antiproliferation activity by suppressing chymotrypsin-like proteasomal activity in Jurkat T cells.[33] To further understand the relationship between the ester bond moiety on theaflavins and consequent proteasome inhibition, the effects of well-studied polyphenols that are involved in carcinogenesis or chemoprevention on proteasomal activities were compared. These selected polyphenols were classified into three types: flavonoids, tannins, and tea polyphenols. Among

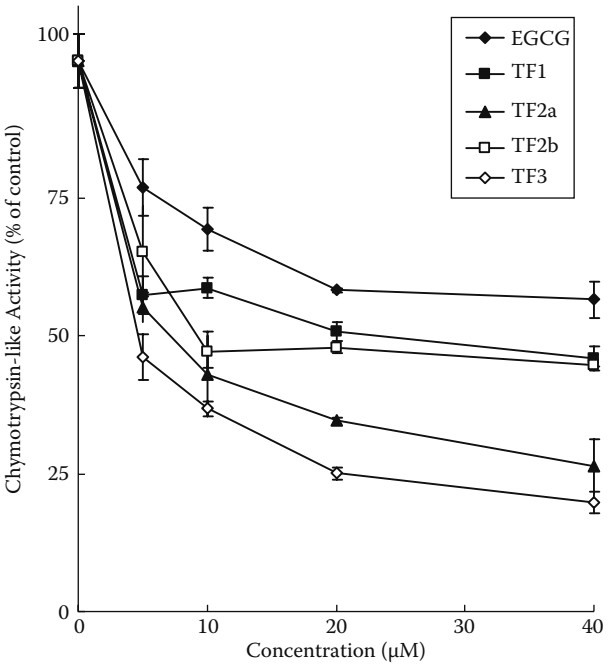


FIGURE 11.4 Inhibition of chymotrypsin-like activity of the proteasome by tea polyphenols in MCF7 cell extracts. MCF7 cell extracts (10 μg/reaction) were incubated with fluorogenic peptide substrate (Suc-Leu-Leu-Val-Tyr-AMC) for 90 min in the presence of a series of concentrations of tea polyphenols, followed by the measurement of free AMC groups. Assays were performed in triplicate, and the results of a representative experiment are shown as mean ± SD.

TABLE 11.2
Inhibitory effects of the tea polyphenols on the proteasomal activities in MCF7 cell extracts

Tea polyphenols	Activities of proteasome (% of control)[a,b]		
	PGPH	Chymotrypsin-like	Trypsin-like
EGCG	74.4 ± 7.9	69.4 ± 3.9	102.1 ± 3.6
TF1	68.7 ± 7.0	58.8 ± 1.9	91.8 ± 3.1
TF2a	33.1 ± 3.0	43.1 ± 7.7	82.0 ± 1.4
TF2b	38 ± 8.0	47.1 ± 3.0	84.7 ± 2.8
TF3	15.9 ± 2.2	36.9 ± 1.3	93.2 ± 2.0

[a] MCF7 cell extracts (10 μg/reaction) were incubated for 90 min with various fluorogenic peptide substrates for the proteasomal PGPH, chymotrypsin-like, trypsin-like activity with 10 μM tea polyphenols, and detected free AMC groups.

[b] Assays were performed in triplicate, and the results of representative experiments are shown as mean ± SD.

them, attention was paid to similar ester bonds present in several polyphenols, including tannic acid, corilagin, geraniin, 5GG, EGCG, TF2a, TF2b, and TF3. The above-mentioned polyphenols exhibited a stronger inhibitory effect on the chymotrypsin-like activity of recombinant 20S proteasome (table 11.3) than other polyphenols without ester bonds. Furthermore, 5 μM TF3 inhibited approximately 85% of proteasome activity, whereas 0.3 μM 5GG inhibited 80% of proteasome activity with an estimated IC_{50} of 73.2 nM.[33] These results also support that ester bonds are indeed responsible for highly nucleophilic susceptibility toward proteasome inhibition.[28] However, TF1 and quercetin, which do not contain ester bonds, also showed an inhibitory effect on the proteasomal chymotrypsin-like activity. This suggests that probably other unidentified structural features of these compounds may contribute to proteasome inhibition. Computational docking studies suggest that the interaction of genistein with the β5 subunit of the 20S proteasome is responsible for inhibition of proteasomal chymotrypsin-like activity.[36] Thus, we speculate that structure-activity relationships of the active polyphenols might provide the essential functional group that leads to binding to proteasome active sites and results in the inhibition of proteasomal activities.

11.3.5 The Possible Contribution of the Galloyl Group of Polyphenols on Inhibition of the Proteasome

Previous studies have demonstrated that the galloyl moiety of theaflavins and EGCG is important for growth inhibition and their antioxidant properties.[37,38] The polyphenols that were selected displayed similar chemical structures. The ester bond–bearing polyphenols that had more potent inhibitory effects on proteasomal activities as shown in table 11.3 also contained galloyl-ester structures. According to these results, the galloyl-ester structure might be one constituent involved in the inhibition of proteasome. In order to determine whether proteasome inhibition was due to the

TABLE 11.3
The effects of the polyphenols on the chymotrypsin-like activity of the recombinant 20S proteasome

Chymotrypsin-like activity of the 20S proteasome (% of control)[a,b]					
Flavonoids		**Tannins**		**Tea polyphenols**	
Rutin	77.0 ± 5.5	Tannic acid	30.0 ± 11.1	EGCG	56.6 ± 1.5
Quercetin	52.1 ± 3.1	Geraniin	42.0 ± 3.7	TF1	58.3 ± 1.6
Apigenin	92.0 ± 2.2	Corilagin	57.0 ± 2.1	TF2a	28.3 ± 9.8
Genistein	94.2 ± 0.7	Ellagic acid	104.4 ± 1.9	TF2b	27.1 ± 9.0
Genistin	94.6 ± 6.0			TF3	16.2 ± 3.8

[a] The chymotrypsin-like activity of the purified recombinant prokaryotic 20S proteasomes (2 μg) were analyzed by incubation with the fluogenic substrate Suc-LLVY-AMC (for chymotrypsin-like activity of the proteasome) in the presence of polyphenols (5 μM) for 30 min, followed by detecting free AMC groups.

[b] Assays were performed in triplicate, and the results of representative experiments are as shown mean ± SD.

galloyl-ester structure of polyphenols, the inhibitory effect of gallic acid and n-propyl gallate on proteasome activity was examined. These results revealed that gallic acid and n-propyl gallate did not exert any significant inhibitory effect on purified recombinant 20S proteasomes (figure 11.5A) or proteasomal activities in MCF7 cell extracts (figure 11.5B). However, n-propyl gallate exhibited slightly more efficient inhibition on the proteasomal chymotrypsin-like activity in a concentration-dependent manner than gallic acid (figure 11.5C). Importantly, n-propyl gallate consists of one gallate that is esterified to propanal. Thus, n-propyl gallate exhibited more inhibitory potency on proteasome activity than gallic acid. Similarly, the number of galloyl-ester moieties might enhance the inhibitory effects of polyphenols (such as TF3 and 5GG) on the chymotrypsin-like activity of the proteasome through catalytic site interactions; possible mechanisms involved will be further discussed in this chapter.

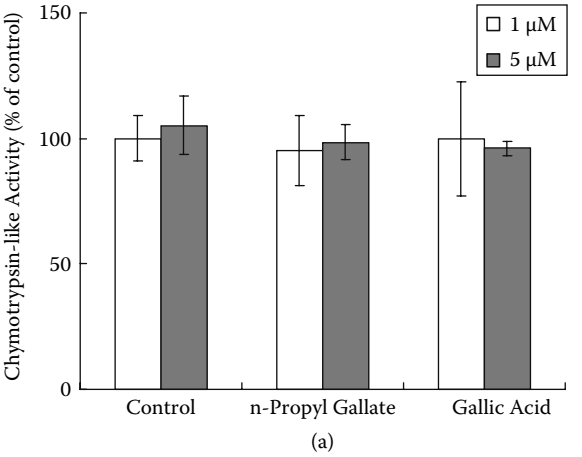
11.4 GENERAL REMARKS ON THE INHIBITION MECHANISMS OF TEA POLYPHENOLS

11.4.1 Proteasome Inhibition and Cancer Chemoprevention

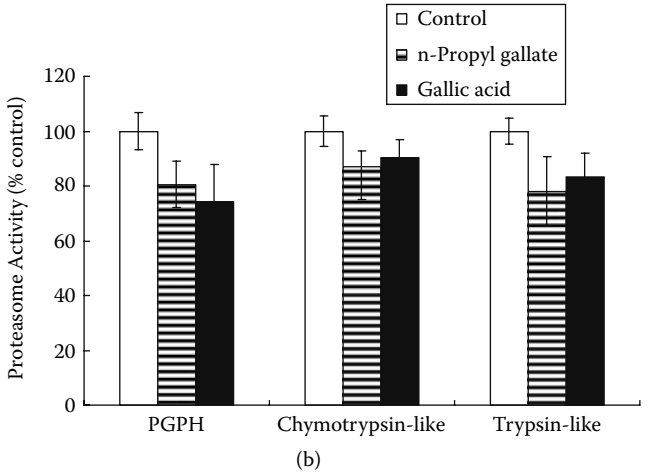
To date, the bioactive properties of tea polyphenols have come to public attention because of their chemopreventive effects. The molecular mechanisms for the effects of tea polyphenols on cancer chemoprevention have been extensively investigated by several laboratories. On the other hand, an increasing number of studies have also shown that proteasome inhibitors may be considered novel anticancer agents.[28,29,39] The theaflavins, especially TF3, were found to be able to potently inhibit tumor cell growth (figure 11.2). The leukemic cell lines showed better sensitivity to theaflavins than the solid breast cancer MCF7 cell line. TF2a and TF2b exhibited a lower inhibitory activity on cell growth, but in comparison, EGCG was even less effective.

Theaflavins efficiently and specifically inhibited the proteasomal chymotrypsin-like activity of the purified mammalian 20S and 26S proteasomes in cell-free systems (figure 11.3) as well as 26S proteasomes in tumor cell extracts (table 11.1). The purified *M. thermophila* recombinant 20S proteasome was more sensitive to theaflavins than mammalian 20S or 26S proteasomes, possibly due to prokaryotic recombinant 20S proteasome without postmodification that forms steric hindrance that prevents theaflavins from binding to the chymotrypsin-like activity subunit of the 20S proteasome. When assaying for proteasomal chymotrypsin-like activity in human cancer cell extracts, the IC_{50} values of tea polyphenols were apparently increased compared

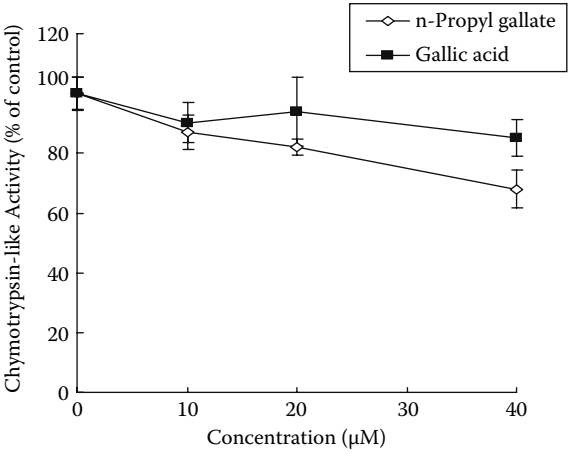
FIGURE 11.5 (opposite page) Inhibition of chymotrypsin-like activity of the proteasome by gallic acid and n-propyl gallate. (A) Inhibition of the chymotypsin-like activiy of the purified recombinant 20S proteasome (2 μg) by gallic acid or n-propyl gallate (1 or 5 μM). MCF-7 cell extracts (10 μg/reaction) were incubated for 90 min with fluorogenic peptide substrate for the proteasomal PGPH or chymotrypsin-like or trypsin-like activity with 10 μM (B) or a series of concentrations (C) of gallic acid and n-propyl gallate as indicated, followed by the measurement of free AMC groups. Assays were performed in triplicate, and the results of representative experiments are shown as mean ± SD.


Chymotrypsin-like Activity (% of control)
150
100
50
0
1 μM
5 μM
Control
n-Propyl Gallate
Gallic Acid

(a)


Proteasome Activity (% control)
120
100
80
60
40
20
0
Control
n-Propyl gallate
Gallic acid
PGPH
Chymotrypsin-like
Trypsin-like

(b)


Chymotrypsin-like Activity (% of control)
120
100
80
60
40
20
0
n-Propyl gallate
Gallic acid
0
10
20
30
40
Concentration (μM)

(c)

to those of assays on the purified proteasomal *in vitro* (table 11.1), suggesting that tea polyphenols may bind to cellular proteins and are degraded by certain enzymes in total cell lysates. Furthermore, theaflavins exhibited similar inhibitory effects on cell growth and proteasomal activities in the cell lines studied, thus suggesting that proteasome inhibition by theaflavins is the antiproteolytic component responsible for cell growth arrest, cytostasis, and apoptosis.

11.4.2 Involvement of p53 in Proteasome Inhibition

Based on previous reports, breast solid cancer MCF7 cells, which are more resistant to apoptosis caused by proteasome inhibitors than leukemic cells, may come from the induction of wild-type p53 that correlates with inhibition of proteasome and antiproliferation effect.[40] Consistent with this observation, leukemic cell lines lacking wild-type p53 certainly were more sensitive to tea polyphenols on inhibition of cell growth and the proteasomal activities than MCF7 cells containing wild-type p53 (figure 11.1 and table 11.1). Moreover, proteasome inhibition by tea polyphenols results in cell growth arrest of MCF7 cells and might induce consequent apoptosis. This result suggests that wild-type p53 plays an important role in MCF7 cells to tolerate the treatment of tea polyphenols that suppresses cell growth and downregulates proteasomal activities. However, the relationship between proteasome inhibition and suppression of cell growth by theaflavins in MCF7 cells is unclear, and this issue must be elucidated further.

11.4.3 The Role of Galloyl Structure in Proteasome Inhibition

The galloyl structure of theaflavins is important in metal chelation, antioxidant activity, and binding to cellular molecules.[41,42] It is interesting that all polyphenols that are able to inhibit proteasomal activity also contain one or more galloyl moieties (table 11.3). A recent study reported that 5GG, containing five galloyl moieties, acts as a most potent proteasome inhibitor.[33] Furthermore, n-propyl gallate had a slight inhibitory effect on proteasomal chymotrypsin-like activity in a dose-dependent fashion, but gallic acid did not (figure 11.5C). It implied that galloyl moiety in theaflavins might play an important role in docking the pocket of the catalytic site of 20S proteasome that allows substrate specificity and proteasome inhibitor binding. Lately, well-characterized proteasome inhibitors, such as MG132, linked to a pharmacophore interact with a catalytic residue of the proteasome and form a reversible or irreversible covalent adduct, resulting in the malfunction of the proteasome.[29] We presume that the galloyl-ester structure of theaflavins reacts with both the hydroxyl and amino groups of the catalytic N-terminal threonine of the proteasome, leading to the formation of a proteasome–galloyl-ester acyl adduct and loss of proteasomal catalytic activity. Interestingly, although geraniin and corilagin contain five and three ester bonds, respectively, only one galloyl-ester moiety showed equal inhibitory effects on proteasome, compared with EGCG, which also bears one galloyl-ester moiety. In addition, TF3 as well as 5GG, which consist of more than one galloyl-ester moiety, are the most active proteasome inhibitors (table 11.3). Therefore, the galloyl-ester moiety might be one of the major functional groups that interact with the proteasomal chymotrypsin-like activity site. However, TF1 and

quercetin without an ester bond or galloyl group still could inhibit proteasmal activity. It is possible that the compounds containing a flavonoid structure may sterically block the proteasome chymotrypsin-like active site and result in proteasome inhibition. On the basis of results presented, it is proposed that the galloyl-ester structure of theaflavins is the efficient pharmacophore responsible for proteasome inhibition.

11.4.4 Proposed Molecular Mechanism of Proteasome Inhibition by Tea Polyphenols

The possible molecular mechanisms of proteasome inhibition through chymotryptic site blockade by several polyphenolic compounds are depicted in figure 11.6. Based on the information on chymotryptic action of proteasomes, we have used the synthetic fluorogenic substrate Suc-Leu-Leu-Val-Tyr-AMC to assay proteasome chymotrypsin-like action. The principle of this enzyme reaction (figure 11.6A) comprises three steps: nucleophilic attack by the proteasome N-terminal threonine (step 1), formation of acyl-enzyme (step 2), and hydrolysis of acyl-enzyme (step 3). The enzyme activity was assayed by the fluorescence of the released 7-amido-4-methyl coumarin in step 2. In the presence of compounds with a galloyl group, the inhibition of proteasome activity might occur as illustrated in figure 11.6B. In step 1, compounds with a galloyl-ester group may compete with the enzyme substrate, while in step 2, the formation of acyl-enzyme adduct may lead to a transient or permanent inactivation of the proteasome. The inactivated enzyme could be reactivated when the hydrolysis of acyl-enzyme (step 3) is completed.[31] Based on the reaction scheme depicted in figure 11.6B, the R group in the galloyl compounds will affect the proteasome inhibition profoundly. TF2a and TF2b (R = flavanol with a fused seven-member ring, an ester linkage) are more active than EGCG, corilagin, and geraniin, which are equally active in proteasome inhibition. TF3 (R = flavanol with a fused seven-member ring, two ester linkages) and 5GG (R = glucose, five ester linkages) are the most active inhibitors among the compounds tested. It appears that the bulky group of R may help the subtraction of an electron in the carbonyl group and facilitate the nucleophilic attack of the threonine hydroxyl group in step 1. Furthermore, the fused seven-member ring in TF2a, TF2b, and TF3 may interact with hydrophobic residues in the chymotryptic site[28] and stabilize the structure of acyl-enzyme adduct in step 2, resulting in potent inhibition of these tea polyphenols on proteasome activity.

In view of the therapeutic potential of tea polyphenols, many researchers have tried to elucidate their action mechanisms and structure-activity relationship, which might give an insight into new drug discovery.[43] However, there is less understanding about the relationship between the structure and the anticancer activity of theaflavins. This study showed the inhibitory effect of theaflavins on proteasome activity in a brief and comprehensive manner. In conclusion, results presented suggest that proteasome inhibition is a novel mechanism that partially contributes to antiproliferation properties of theaflavins, and their inhibitory ability on the proteasomal chymotrypsin-like activity is due to the galloyl-ester and flavonoid structures. Additionally, our results also indicate that theaflavins, especially TF3, may act as potential chemopreventive agents through inhibition of proteasomal activities.

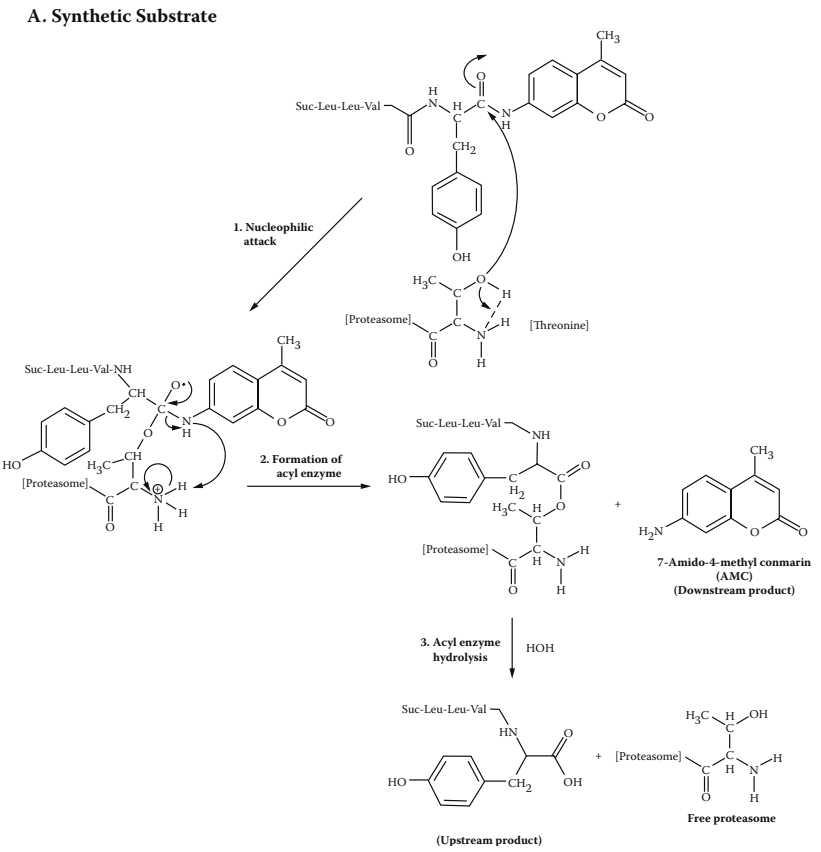


FIGURE 11.6A Reaction of proteasome chymotryptic site with synthetic substrate and inhibitors with galloyl group.

ACKNOWLEDGMENTS

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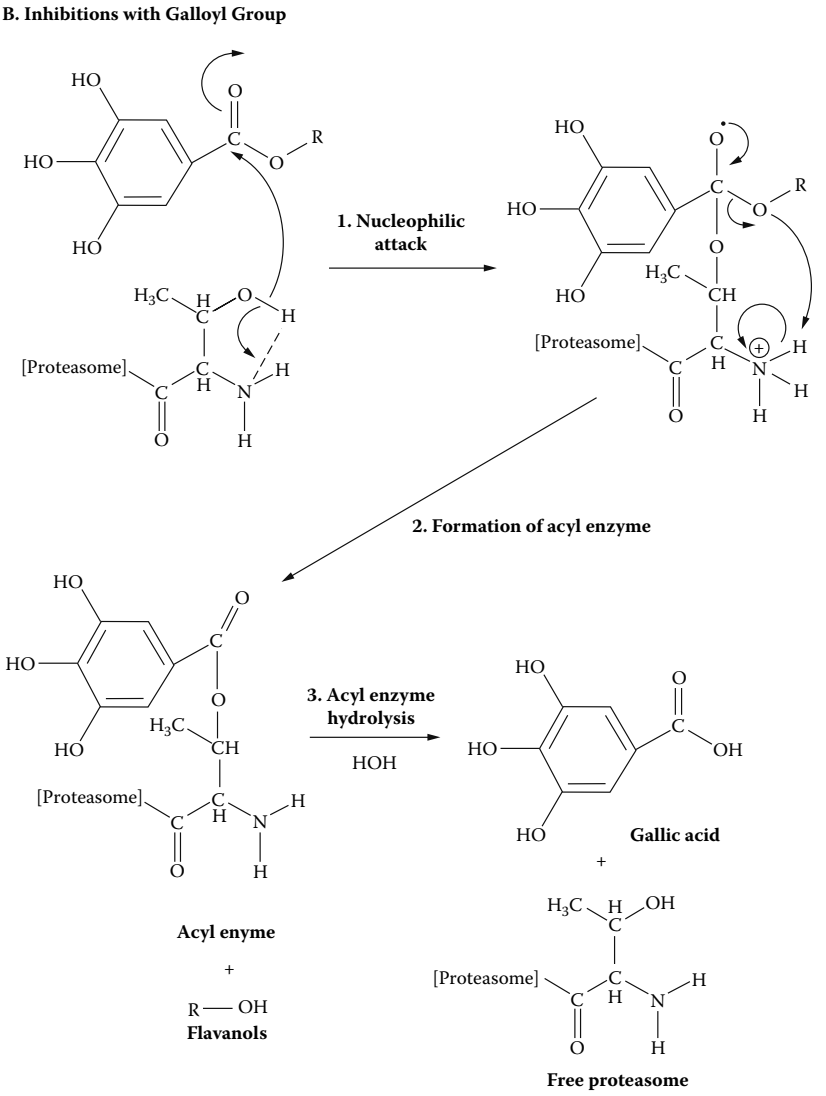

FIGURE 11.6B

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12 Suppression of Prostate Carcinogenesis by Tea Polyphenols through Inhibiting Microsomal Aromatase and 5α-Reductase

Jen-Kun Lin

CONTENTS

12.1 INTRODUCTION

Prostate carcinoma is the second leading cause of death as a result of cancer in men in the Western world.[1] Evidence shows that androgens can be risk factors for prostate cancer development.[2] Androgen action in many organs, such as prostate and skin, is dependent on the conversion of testosterone (T) by a NADPH-dependent 5α-reduc-

tase to 5α-dihydrotestosterone (DHT), which then binds to androgen receptor (AR) to exert its biological function.[3] Inhibition of 5α-reductase would limit the availability of DHT; therefore, 5α-reductase inhibitors would be useful in selective treatment of DHT-dependent diseases, such as benign prostate hyperplasia, prostate cancer, hirsutism, male pattern alopecia, and acne, without affecting testosterone-dependent testicular function, sexual behavior, and muscle growth.[3,4]

There are at least two different isoenzymes (type 1 and type 2) of 5α-reductase in mammalian organs. Their differential localization in cells[5] suggests that they may play selective roles in the development and growth of androgen-sensitive organs or tumors. Most 5α-reductase inhibitors are steroids, such as finasteride and dutasteride, or compounds with steroid-like structures. In this chapter, it is demonstrated that major constituents of black tea polyphenol theaflavins and of green tea polyphenol catechins inhibit the enzyme 5α-reductase.[4,6] The presence of 5α-reductase inhibitors in tea beverage may implicate to known geographic differences in the incidence of prostate cancer in certain populations that preferentially consume these beverages.[7]

12.2 AROMATASE AND 5α-REDUCTASE IN ESTRADIOL BIOSYNTHESIS

Aromatase is the enzyme complex responsible for the final step in estrogen biosynthesis, via the conversion of the androgens, androstenedione and testosterone, into the estrogens, estrone (E1) and estradiol (E2). Overexpression of aromatase in breast cancer cells may substantially influence breast cancer progression and maintenance. It has been proposed that aromatase overexpression is an indirect cause of breast cancer.[8] For example, in the aromatase-transgenic mouse model, aromatase overexpression in breast tissue causes premalignant lesions, such as atypical ductal hyperplasia.[9] As a consequence, great effort has been expended in devising pharmacological inhibitors of aromatase. Clinical trials have indeed now confirmed that the third-generation aromatase inhibitors, such as letrozole and anastrozole, are sufficiently powerful inhibitors of estrogen synthesis to produce undetectable levels of circulating estrogen.[10] These inhibitors have also been shown to be more effective than tamoxifen for the treatment of metastatic or locally advanced breast cancer.

Several steroids, such as androstane-3β,17β-diol (3βD), whose synthesis is not dependent on aromatase but on 5α-reductase, can stimulate estrogen receptors. The synthesis of estrogenic steroids such as 3βD was blocked by inhibition of T conversion into DHT using a 5α-reductase inhibitor finasteride, suggesting that they are generated downstream of DHT, as illustrated in figure 12.1. These findings raise the possibility that the combination of a 5α-reductase inhibitor and an aromatase inhibitor may reduce estrogenic steroids *in vivo* more completely than an aromatase inhibitor alone.[11]

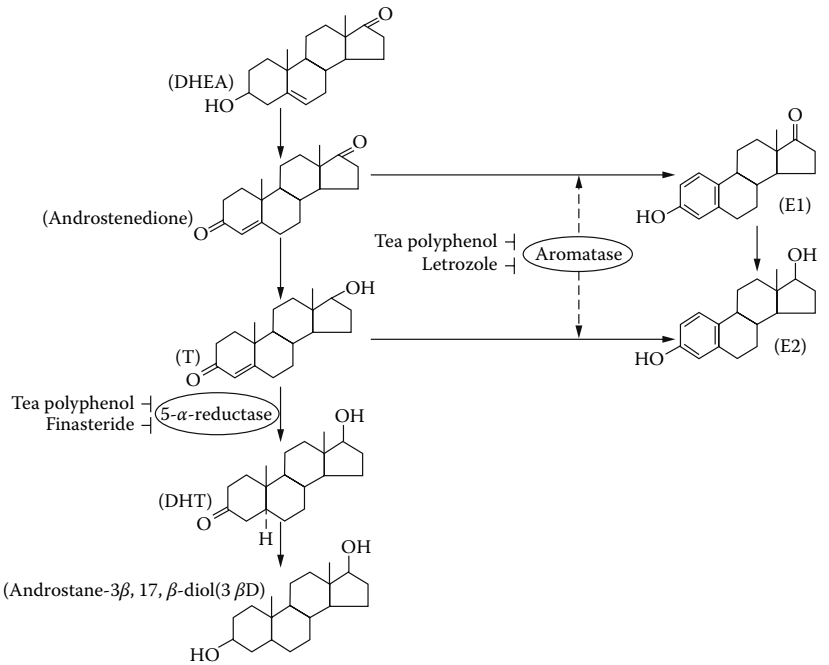

FIGURE 12.1 Biosynthesis of estrogens. Abbreviations: DHEA, dehydroepiandrosterone; E1, estrone; E2, estradiol; T, testosterone; DHT, dihydrotestosterone. Letrozole is an aromatase inhibitor, Finasteride is a 5α-reductase inhibitor. Androstane-3β,17β-diol (3βD) is an estrogenic steroid and its synthesis is blocked by 5α-reductase inhibitor, but not affected by aromatase inhibitor.[11]

12.3 INHIBITION OF AROMATASE BY TEA POLYPHENOLS

The inhibitory effects of black tea polyphenols on aromatase activities were investigated in rat ovarian and human placenta.[12] Microsomes isolated from rat ovarian and human placenta were used to determine the direct effect of black tea polyphenols and epigallocatechin gallate (EGCG) on aromatase activity. The results indicated that theaflavins, TF-1, TF-2, and TF-3, as well as EGCG induced a concentration-dependent inhibition of rat ovarian and human placental aromatase activities. The IC_{50} values of TF-1, TF-2, TF-3, and EGCG inhibiting aromatase activity of rat ovarian microsomes were 5.72, 7.33, 20.37, and 18.27 μM, respectively. Meanwhile, TF-1, TF-2, TF-3, and EGCG also inhibited the activity of aromatase obtained from human placenta, and the IC_{50} were 4.17, 3.23, 3.45, and 13.79 μM, respectively.

A previous study also shows that green tea catechins, including epicatechin gallate (ECG) and EGCG, are potential aromatase inhibitors.[13] We have demonstrated that black tea polyphenols strongly suppress aromatase activity *in vitro* and *in vivo*. They can also act as a HER2/neu tyrosine kinase inhibitor and attenuate tamoxifen-resistant breast tumors in estrogen-independent breast cancer cells. Further evalua-

tion of the molecular action of black tea polyphenols is warranted to support the role of black tea polyphenols as a chemopreventive agent in the treatment of postmenopausal women with breast cancer.[12]

12.4 INHIBITION OF 5α-REDUCTASE BY FINASTERIDE AND DUTASTERIDE

Strategies for prostate cancer chemoprevention can be broadly classified as nutritional or hormonal intervention and the use of other novel compounds. Data on various nutritional interventions have been reported primarily on vitamins A, D, and E and the trace element selenium.[14] The strongest nutritional chemoprevention evidence to date involves the Selenium and Vitamin E Cancer Prevention Trial (SELECT).[15] Other agents, such as cyclooxygenase-2 inhibitors, lycopene, soy, and green tea, are promising and in various stages of study.[14] Hormonal interventions that interfere with the growth of androgen-sensitive prostate cancer are another approach to interfering with the development of prostate cancer.

Androgen-sensitive cells convert testosterone to intracellular tetrahydrotestosterone, the most potent intraprostatic androgen. DHT plays an important role in both benign and malignant prostate pathophysiology. Intracellular inhibitors of DHT could have a role in the chemoprevention of prostate cancer. In normal and benign prostate hypertrophy (BPH) tissue, type 2-5α-reductase is the predominant form. Type 1-5α-reductase is present at low levels in BPH and appears overexpressed in many prostate cancers.[16] Finasteride primarily inhibits the type 2-5α-reductase, while dutasteride is a dual inhibitor of both 5α-reductase isoenzymes.[17] The observation that many prostate cancers express type 1-5α-reductase makes the dual-inhibitor dutasteride a reasonable agent to evaluate for chemoprevention. Both finasteride and dutasteride are U.S. Food and Drug Administration (FDA) approved for the treatment of BPH in the United States.[18]

12.5 CHEMOPREVENTION OF PROSTATE CANCER WITH FINASTERIDE

Prostate cancer is a significant cause of disease and death, making it an attractive target for chemoprevention. The association between lifetime exposure to DHT and risk of developing prostate cancer suggests that chemoprevention is possible with 5α-reductase inhibition.[19] The recently completed prostate cancer prevention trial indicates that chemoprevention is possible with the 5α-reductase inhibitor finasteride. Development of a cost-effective chemoprevention strategy for prostate cancer is evolving and is expected to have significant positive economic and public health benefits.[19]

Economic and public health impacts of finasteride chemoprevention may be improved through more selective application of this intervention. Polymorphisms in the SRD5A2 gene, which codes for type 2-5α-reductase, have functional consequences *in vitro*. The functional significance of these polymorphisms and how they might affect the risk for development of prostate cancer is currently the subject of intense study, and they may play a more important role in prognosis than carcinogenesis.[19] It is possible that other agents may prove more effective or overcome func-

tional effects of genetic variant, such as seen with SRD5A2. An international trial involving 8.000 patients investigating dutasteride (a nonselective inhibitor of both types 1- and 2-5α-reductase) is currently under way.[20]

12.6 INHIBITION OF 5α-REDUCTASE BY TEA POLYPHENOLS

Previous studies have shown that green tea catechins, including epicatechin gallate (ECG) and EGCG, are potential 5α-reductase inhibitors.[4] The *in vitro* effect of theaflavins and penta-*O*-galloyl-β-D-glucose (5GG), whose structures were related to EGCG on 5α-reductase activity, was further examined. The results showed that TF1, TF2A, TF2B, TF3, EGCG, and 5GG inhibited the 5α-reductase activity in a dose-dependent manner. TF3 was the most effective inhibitor among the polyphenols tested.[6] The IC_{50} values of these compounds producing 50% inhibition of 5α-reductase are listed in table 12.1.

12.7 INHIBITORY EFFECTS OF TEA POLYPHENOLS AND 5GG ON THE EXPRESSION OF ANDROGEN RECEPTOR PROTEIN

After testosterone (T) is transformed into dihydrotestosterone (DHT) by the enzyme 5α-reductase, DHT will bind to androgen receptor (AR) and result in a series of androgen actions. Since AR is the essential mediator for androgen action, the effects of tea polyphenols and 5GG on the expression of AR protein were determined.[6] Among the six polyphenols—namely, 5GG, TF1, TF2a, TF2b, TF3, and EGCG—5GG and TF3 significantly reduced the amount of AR protein at a dose of 40 μM. TF2a, TF2b, TF1, and EGCG had minor effects at the same concentration range.

TABLE 12.1
Inhibitory effects of polyphenols on 5α-reductase in rat liver microsome

Compound	Inhibition, IC_{50} (μM)[a]
TF3	4.9
5GG	7.8
TF2b	7.9
TF2a	20.2
EGCG	92.6
TF1	>100
Gallic acid	>100
n-Propyl gallate	>100

[a] IC_{50} in μM of tested compound producing 50% inhibition of 5α-reductase.[6]

12.8 INHIBITORY EFFECTS OF TF3 AND 5GG ON THE EXPRESSION OF ANDROGEN REGULATED GENE (PSA)

Evidence shows that alteration of 5α-reductase or AR leads to modulation of androgen-responsive genes. Prostate-specific antigen (PSA) is one of the androgen-responsible genes because the promoter of PSA gene contains functional ARE.[21] PSA is specifically produced by both prostate epithelial cells and prostate cancer and is the most commonly used serum marker for diagnosing cancers. In patients with prostate carcinomas an increase of the serum PSA level is usually observed.[22] Treatment of LNCaP prostate cancer cells with 5GG or TF3 significantly suppressed the levels of PSA in a dose-dependent manner.[6]

12.9 INHIBITORY EFFECTS OF TEA POLYPHENOLS AND 5GG ON CELL PROLIFERATION OF LNCaP PROSTATE CANCER CELLS

The LNCaP human prostate cancer cell line is well established and androgen dependent.[23] The effects of tea polyphenols and 5GG on androgen-stimulated growth of LNCaP cells were examined. LNCaP prostate cancer cells were incubated with varying concentrations of tea polyphenols or 5GG with or without testosterone for 4 days. The MTT assay was performed to measure cell viability. In the absence of tea polyphenols or 5GG, testosterone alone apparently stimulates LNCaP cell numbers by an average of 50% above the untreated control. 5GG, TF3, TF2b, or EGCG (10–40 μM) treatment resulted in a dose-dependent inhibition of LNCaP cell growth to a degree similar to that in no androgen-stimulated controls at higher concentrations of these compounds (20–40 μM) in the presence of testosterone. Compared with the six polyphenols tested in this experiment, 5GG and TF3 seem to exhibit a higher inhibitory activity on LNCaP cell growth than TF2b and EGCG. In contrast, TF1 and TF2a showed little inhibitory activity.[6]

12.10 THE MECHANISM OF PROSTATE CANCER CHEMOPREVENTION BY TEA AND TEA POLYPHENOLS

Environmental factors such as diet play an important role in modulating cancer incidence and mortality, and differences in diet may explain geographical differences in prostate cancer mortality. Since androgens regulate the growth and function of the normal prostate and prostate cancer, dietary components capable of altering this growth signaling pathway in the prostate may affect prostate cancer development and progression. Flavonoids are naturally occurring polyphenolic compounds widely distributed in fruits and vegetables. In recent years, many flavonoids have been shown to possess cancer chemopreventive effects. Green tea polyphenols have been found to inhibit prostate carcinogenesis *in vitro* and *in vivo*. EGCG, the major polyphenolic constituent of green tea, inhibits growth of several types of human prostate cancer cells by inducing cell cycle arrest and apoptosis.[24,25] EGCG also downregulates the expression of AR[26] and inhibits 5α-reductase activity.

It has been demonstrated that green tea polyphenols exhibit prostate cancer chemoprevention effects through suppressing testosterone-mediated induction of

ornithine decarboxylase, which is an important contributor of prostate cancer development.[27] Black tea polyphenols can inhibit insulin-like growth factor-1 (IGF-1)–induced signaling in DU145 prostate cancer cells.[28] We have demonstrated that 5GG and TF3, which contain several galloyl groups, inhibit prostate cancer by attenuating the function of androgen and AR.[6]

5α-Reductase inhibitors are effective for the treatment of prostate cancer. They act by inhibiting the conversion of testosterone into DHT.[18,19] Meanwhile, aromatase inhibitors are effective for the treatment of breast cancer in postmenopausal women[29]; they act by inhibiting the conversion of testosterone into 17β-estyradiol (E2), thereby preventing E2-induced activation of estrogen receptor (ER). However, ER is activated not only by estrogens but also by many other steroids, which may be generated independent of aromatase.[11] One such estrogenic steroid synthesized independently of aromatase is androstane-3β,17β-diol (3βD), a nonaromatizable steroid (figure 12.1). Because 3βD is generated downstream of DHT, an aromatase inhibitor does not block its production, whereas a 5α-reductase inhibitor might impair its synthesis by reducing DHT, the precursor of 3βD.

A large body of evidence has suggested that estrogenic hormone may be involved in prostatic malignancy. The localization of estrogen receptor-β (ERβ) in the secretory epithelium of the human prostate has raised the intriguing possibility that the action of estrogen could be mediated, at least in part, by this receptor during the process of carcinogenesis. Hence, specific interference with estrogen-activated and ERβ-mediated transcriptional activity could open new issues in the endocrine manipulation of prostate tumors.[30]

How do both estrogen and androgen stimulate the proliferation of LNCaP cells? Either type of hormone activates both ERβ and AR, and both steroid receptors are responsive for the proliferative stimulus. Cross-talk between steroid receptors was reported to regulate the nongenomic proliferation pathways of different steroids. In LNCaP cells, either E2 or the synthetic androgen R1881 induced the assembly of a ternary complex of AR, and ERβ and src stimulated the Raf-1/ERK signal transduction cascade, and triggered the prostate cancer cell entry into the S-phase.[31]

Here, it is worth noting that tea polyphenols are different from 5α-reductase inhibitors and aromatase inhibitors by possessing the inhibitory activities of both 5α-reductase and aromatase, as illustrated in figure 12.2. It is expected that tea polyphenols are effective in inhibiting both 5α-reductase and aromatase, which leads to chemoprevention of prostate and breast carcinogenesis.

In summary, the natural polyphenols TF3, EGCG, and 5GG exhibit their inhibitory effects on androgen action in prostate cancer cells by two mechanisms, including the inhibition of 5α-reductase activity (figures 12.1 and 12.2) and the suppression of AR protein levels. Therefore, tea polyphenols and 5GG may have the potential to become chemopreventive and chemotherapeutic agents for prostate tumors.

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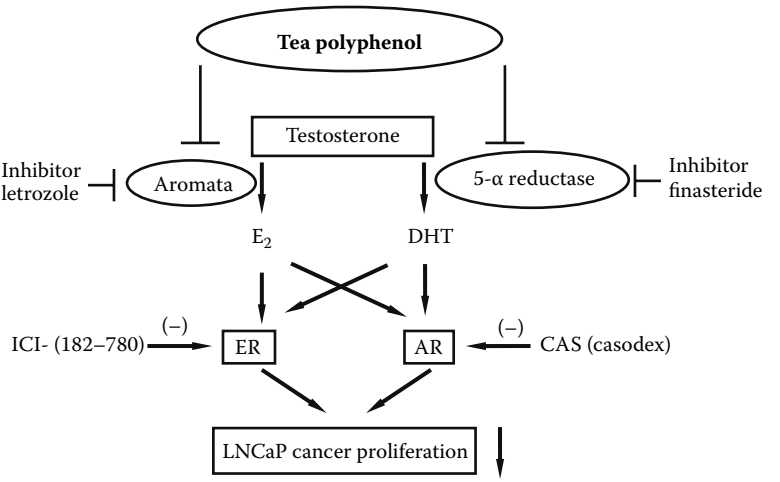

FIGURE 12.2 Suppression of prostate carcinogenesis by tea polyphenols. In a defined system, the inhibitory effects of tea polyphenols on the AR- and ERβ-mediated transcription may lead to the suppression of LNCaP prostate cancer cell proliferation. It is proposed that tea polyphenols suppress prostate cancer cell proliferation and prostate carcinogenesis through inhibiting aromatase (by affecting the E2 and ERβ system) and 5α-reductase (by affecting the DHT and AR system). It is interesting to note that the hormones E2 and DHT may interact with ERβ and AR through cross-talk mechanisms.[30] Here, ICI 182-780 is a specific inhibitor for ERβ, and CAS (casadex) is a specific inhibitor for AR.

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13 Green Tea-Induced Thermogenesis Controlling Body Weight

John Shi, Sophia Jun Xue, and Yukio Kakuda

CONTENTS

13.1 INTRODUCTION

Tea (*Camellia sinensis*, Theaceae) is one of the most popular beverages consumed throughout the world. An estimated 2.5 million metric tons of dried tea leaves are manufactured annually.[1] The majority of the tea beverages are prepared from the following three types of manufactured teas: green tea, oolong tea, and black tea. Green tea is prepared from fresh leaves and processed rapidly to prevent fermentation. Oolong tea is a partially fermented tea product with a unique flavor. Black tea is made by crushing the leaves to cause fermentation prior to the final drying step. Of all the tea consumed in the world, 78% is black tea, which is usually consumed in the United States, Europe, Africa, and India; 20% is green tea, which is commonly consumed in Asian countries, especially in China and Japan; and 2% is Oolong tea, which is produced in southern China.[2,3] Green tea infusions contain approximately 30–40% catechins and other polyphenols and flavonols. Black tea infusions contain approximately 3–9% catechins and other polyphenols and flavonols.[4,5]

Green tea has been widely consumed in China and Japan for many centuries, and hence is regarded as safe.[6] Presently, 90% of the green tea comes from China and is cultivated on approximately 4 million acres of land.[1] The belief in the medicinal powers of tea is so strong in the Orient that the Chinese actually say, "It is better to take green tea than to take medicine."[7] Compared to other processed teas, green tea has more health benefits due to minimal processing and a higher content of pharmacologically active components. Tea leaves contain more than 2,000 components, and

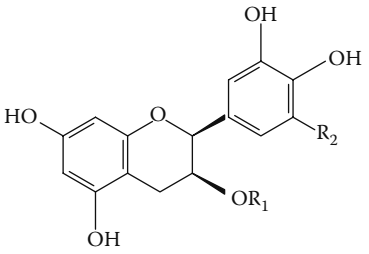


FIGURE 13.1 Major tea catechins in green tea.

catechins represent a class of compounds known as tea polyphenols. The major catechins in fresh tea leaves are epigallocatechin gallate (EGCG), epigallocatechin (EGC), epicatechin gallate (ECG), and epicatechin (EC). The catechin composition depends on the location of cultivation of the tea plant, variety of plant, season of harvest, and process conditions. Catechins are colorless, water-soluble compounds that impart bitterness and astringency to tea infusions. Almost all of the characteristics of tea, such as taste, color, and aroma, are associated with the catechins. A typical catechin composition would be 10–15% EGCG, 6–10% EGC, 2–3% ECG, and 2% EC.[8] The structures of the major catechins are shown in figure 13.1. EGCG is the most abundant catechin and has received by far the most attention in clinical studies. Flavonols such as quercetin and their glycosides are present in lower levels. In addition, caffeine, theobromine, theophylline, and phenolic acids, such as gallic acid, are also present as minor constituents. Caffeine accounts for 3–6% of the dry weight of brewed tea.

These catechins have a variety of physiological functions. EGCG is the active compound responsible for most of the health-promoting properties associated with green tea.[4] Recent scientific research has shown it to be involved with weight loss improvement. The two logical ways to treat obesity are to reduce energy intake and increase energy expenditure.[9] Total daily energy expenditure is composed of three factors: thermic effect from physical activity, resting metabolic rate, and thermic effect due to eating (diet-induced thermogenesis).[10] Thermogensis is taught to be under the control of the sympathetic nervous system (SNS), with norepinephrine (NE) controlling the biochemical pathway that leads to an increase in ATP consumption, or to an increase in mitochondrial oxidation with poor coupling of ATP synthesis, resulting in an increase in heat production.[6]

The target for pharmacological interference in the norepinephrine modulatory pathway is the enzyme catechol-*O*-methyl transferase (COMT). The main function of COMT is to eliminate the various catecholic compounds such as norepinephrine by decreasing their hydrophilicity with methylation/sulfation/glucuronidation to effectively eliminate them from the body.[11] Green tea's EGCG acts on the norepinephrine modulatory pathway and exerts a thermogenic effect by inhibiting COMT.[12,13] Therefore, when epigallocatechin gallates inhibit COMT, there is a prolonging effect of norepinephrine on the α- and β-adrenergic receptors at the postsynaptic cleft of the nerve terminal, increasing the cyclic adenosine monophosphate concentration and thermogenesis.[6]

13.2 EFFECTS OF CATECHINS (EGCG, ECG, EGC, AND EC) ON CATECHOL-*O*-METHYL TRANSFERASE

The most widely recognized property of tea catechins is their antioxidant property that results from their ability to scavenge reactive oxygen and nitrogen species and

to sequester metal ions.[14] Many epidemiological studies have shown a relationship between tea consumption and human health. Some studies have focused on the differences between green tea and black tea in terms of their ability to prevent chronic diseases. These differences were related to the differences in the content of bioactive compounds in green tea and black tea. In animal studies that investigated the inhibition of chronic disease and cancer formation by tea, the effective compounds were shown to be catechins, which are present in higher amounts in green teas than in black teas. In recent studies, the cancer chemopreventive effects of green tea were shown to be mediated by EGCG, which is the major catechin constituent of green tea. Catechins are reduced by 85% during black tea manufacturing, and only 10% can be accounted for as theaflavins and theaflavic acids. The catechins undergo several chemical modifications after absorption. These include condensation reactions, methylation, glycosylation, and sulfation.[15] The reduction of cancer risks and chronic diseases by catechins depends on the content, the composition of catechins, and their bioavailability and bioactivity.

Although there are similarities in their chemical structure, EGCG, EGC, and EC display different pharmacokinetics. According to Chen et al.,[16] EGC and EC are absorbed faster than EGCG, and EGCG is not bioavailable. The low bioavailability of EGCG is evident when consumed either in decaffeinated green tea or as pure EGCG. It seems that EGCG is better absorbed when given as a tea infusion rather than in pure form. All catechins are not equally active. Galloyl esters of catechins are more active than nongalloylated catechins, because they have lower redox potentials.[17]

There is ample evidence that EGCG from green tea stimulates thermogenesis by inhibiting COMT and prolonging the effect of norepinephrine on thermogenesis. Caffeine is another component of green tea that can exert a thermogenic effect. Caffeine inhibits phosphodiesterase, which increases the cellular amounts of cAMP and, as a result, increases thermogenesis.[7] Catechin consumption is associated with the intake of other nutrients. Some positive associations between the consumption of catechins and intake of fiber, vitamin C, and β-carotene are shown in table 13.1. Catechins also limit consumption of α-tocopherol and allow it to scavenge aqueous peroxyl radicals near the membrane surface.[18] The combined effect of green tea extract rich in catechin, and caffeine on thermogenesis will increase energy expenditure and fat oxidation in humans.

Dulloo et al.[6] reported higher MO_2 (respiration rate) values with the green tea extract containing isomolar concentrations of caffeine than with caffeine alone at 100 and 250 μM (figure 13.2A, intact tissue). This indicates that at the concentration used, caffeine alone does not increase basal interscapular brown adipose tissue (IBAT) MO_2 (millimolar concentrations are needed to stimulate IBAT respiration rates), but green tea extracts containing isomolar concentrations of caffeine produced a significant increase in IBAT MO_2 above basal levels in a dose-dependent fashion. Their results also show that the effect of green tea extract on MO_2 was considerably blunted in sympathectomized animals (figure 13.2B, Sympx). The blunting effect on BAT thermogenesis in sympathectomized animals was not attributed to postsynaptic tissue damage, but was credited to the depletion of NE stores following the chemical sympathectomy. These effects support the notion that thermogenesis is

TABLE 13.1
Urinary catecholamines (nmol) during diurnal, nocturnal, and total 24 h periods

	Placebo	Caffeine	Green tea
Diurnal			
Epinephrine	66 ± 16	49 ± 4	55 ± 7
Norepinephrine	106 ± 15	127 ± 24	146 ± 23
Dopamine	893 ± 173	946 ± 160	1086 ± 179
Nocturnal			
Epinephrine	12 ± 4	19 ± 4	15 ± 3
Norepinephrine	54 ± 5	61 ± 11	73 ± 7
Dopamine	694 ± 80	632 ± 126	803 ± 105
Total 24 h			
Epinephrine	78 ± 13	67 ± 4	70 ± 8
Norepinephrine	160 ± 14	187 ± 29	219 ± 27
Dopamine	1587 ± 187	1578 ± 165	1889 ± 241

Source: Modified from Dulloo, A., Duret, C., Rohrer, D., Girardier, L., Mensi, M., Fathi, M., Chante, P., and Vandermander, J. 1999. Efficacy of a green tea extract rich in catechin polyphenols and caffeine in increasing 24-h energy expenditure and fat oxidation in humans. *Am. J. Clin. Nutr.* 70:1040–45.

highly dependent upon the release of endogenous NE from the sympathetic nerves innervating this tissue.

Studying the effects of EGCG on the *in vitro* respiration rate of IBAT, Dulloo et al.[6] showed that catechin polyphenols contribute to the efficacy of the green tea extract in potentiating thermogenesis. The EGCG alone at 200 μM induced a small but statistically significant increase in MO_2 (40%) relative to basal values. When combined with 100 μM caffeine (100 μM caffeine has no effect on IBAT MO_2 on its own), EGCG (200 μM) enhanced the IBAT MO_2 rate 2.4-fold over basal values (figure 13.3). In all measurements, caffeine was administered at 100 μM (a low subthreshold concentration that does not increase MO_2 in its own right), and in the study shown in panel B, ephedrine was administered at 0.1 and 0.25 μM, which in its own right either did not increase basal MO_2 or significantly increased basal MO_2 between 50 and 100%. The shaded areas represent the synergistic increases in tissue thermogenesis in response to the interaction between EGCG and caffeine in the absence (panel A) or presence (panel B) of an increase in NE release (induced by ephedrine).[6] The effect of combining caffeine and EGCG was also studied under stimulated conditions, where a subthreshold concentration of ephedrine (0.1 and 0.25 μM) was used to increase NE release. In this study, 0.1 μM ephedrine concentration alone had no effect, but the addition of caffeine increased MO_2 by 84% over basal, and with E + C + 200 μM EGCG a 2.8-fold increase in MO_2 relative to basal values was observed. The 0.25 μM ephedrine concentration alone resulted in a significant stimulation of MO_2 (50–100% relative to basal values), which was further increased

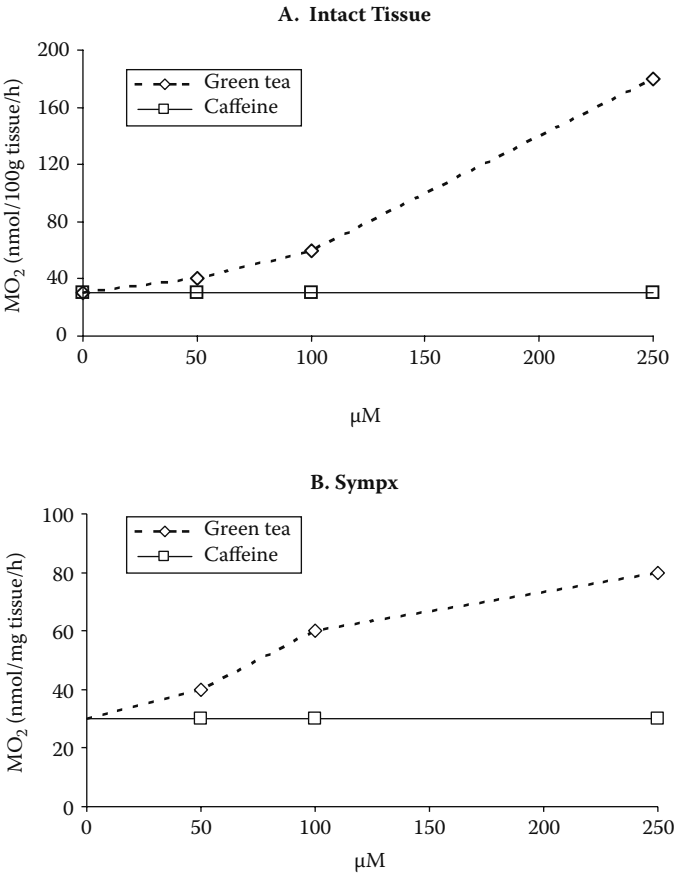


FIGURE 13.2 Respiration rates (MO_2) of interscapular brown adipose tissue (IBAT) from intact rats (A) and from rats chemically sympathectomized (B) in response to caffeine or to green tea extract containing isomolar concentrations of caffeine. (Modified from Dulloo, A. G., Seydoux, J., Girardier, L., Chantre, P., and Vandermander, J. 2000. Green tea and thermogenesis: Interactions between catechin-polyphenols, caffeine and sympathetic activity. *Int. J. Obesity* 24:252–58.)

4-fold by the addition of caffeine (100 μM) and 7.4-fold when all three components were present (E + C + 200 μM EGCG).

Since the IBAT MO_2 is higher by severalfold with an increase in NE in the synaptic cleft, it shows that EGCG cannot increase the release of NE, but had a prolonged effect and increased thermogenesis. The interaction between EGCG and caffeine released NE and increased the efficacy of green tea extract to potentiate BAT thermogenesis. EGCG degradation caused a reduction in the degradation of NE and released NE on adrenoreceptors. It can be seen that 100 μM EGCG increases MO_2 in the presence of added ephedrine (figure 13.4), where earlier it did not have an effect alone. In all measurements, EGCG was administered at 100 μM (a low subthreshold concentration that does not increase MO_2 in its own right), and ephedrine

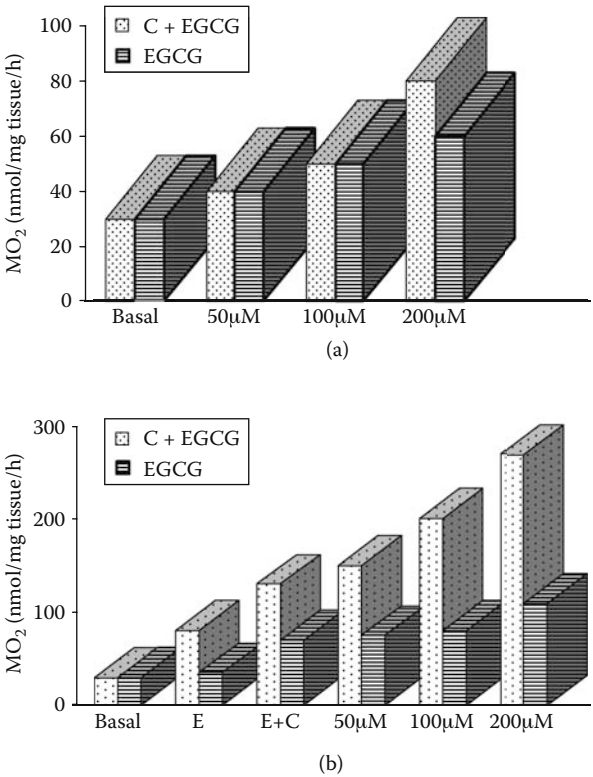


FIGURE 13.3 Respiration rates (MO_2) of IBAT in response to EGCG alone or in combination with caffeine (C) in the unstimulated state (panel A), and EGCG in the stimulated state induced by low doses of ephedrine (E) and caffeine (C) (panel B). (Modified from Dulloo, A. G., Seydoux, J., Girardier, L., Chantre, P., and Vandermander, J. 2000. Green tea and thermogenesis: Interactions between catechin-polyphenols, caffeine and sympathetic activity. *Int. J. Obesity* 24:252–58.)

was administered at 0.1 or 0.25 μM, which in its own right either did not increase basal MO_2 or significantly increased basal MO_2 by about twofold. The shaded areas represent the synergistic increase in tissue thermogenesis in response to the interaction between EGCG and ephedrine.[6] This may be due to the increased release of NE, allowing EGCG to have a prolonged effect and increase thermogenesis.

Caffeine would increase the NE-induced cAMP activation in the cell, by inhibiting phosphodiesterase-induced degradation of intracellular cAMP. The combination of caffeine and EGCG, by its inhibitory actions, increases the action of NE on thermogenesis.

A study by Dulloo et al.[9] showed that caffeine and catechins produced a thermogenic effect via sympathetic activation of NE, and that the effect of green tea extracts on the metabolic rate in humans is greater than that explained by caffeine content per se. In table 13.2, diurnal, nocturnal, and total 24 h energy expenditure values are presented for placebo, caffeine, and green tea extract. Diurnal energy expenditure

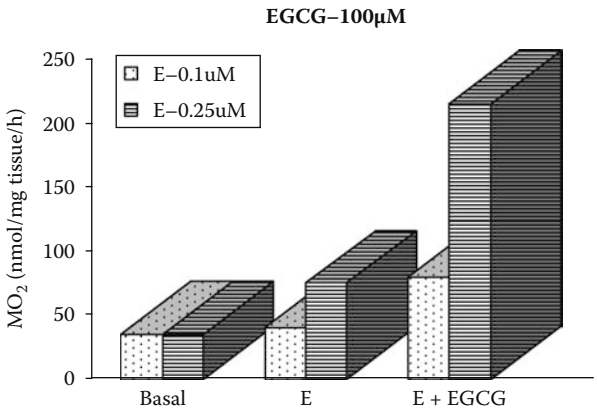


FIGURE 13.4 Respiration rates (MO_2) of IBAT in response to EGCG in the stimulated state induced by relatively low doses of ephedrine. (Modified from Dulloo, A. G., Seydoux, J., Girardier, L., Chantre, P., and Vandermander, J. 2000. Green tea and thermogenesis: Interactions between catechin-polyphenols, caffeine and sympathetic activity. *Int. J. Obesity* 24:252–58.)

TABLE 13.2
Energy expenditure (EE) (kJ) during diurnal, nocturnal, and total 24 h periods

	Placebo	Caffeine	Green tea
Diurnal EE	6463 ± 386	6547 ± 383	6754 ± 352
Nocturnal EE	3075 ± 149	3053 ± 145	3112 ± 140
Total 24 h EE	9538 ± 521	9599 ± 518	9867 ± 488

Source: Modified from Dulloo, A., Duret, C., Rohrer, D., Girardier, L., Mensi, M., Fathi, M., Chante, P., and Vandermander, J. 1999. Efficacy of a green tea extract rich in catechin polyphenols and caffeine in increasing 24-h energy expenditure and fat oxidation in humans. *Am. J. Clin. Nutr.* 70:1040–45.

was significantly higher during treatment with green tea extract than during treatment with placebo or caffeine by 4.5 and 3.2%, respectively. Also, total daily energy expenditure with green tea extract was significantly higher than that with both the placebo and caffeine by 3.5 and 2.8%, respectively. There was an increase in energy expenditure in six of the ten subjects after treatment with green tea extract, ranging from 266 to 836 kJ, and only two of the ten subjects with caffeine. These observations are consistent with the inhibiting effect of green tea on COMT, resulting in a prolonged life of norepinephrine in the sympathetic synaptic cleft. The results show that tea catechins have the potential to influence energy expenditure since dietary intake and diet composition were identical during all treatments, and the subjects maintained the same feeding and physical activity patterns during each 24 h respiratory chamber trial. The 4% increase in 24 h energy expenditure treatment with green tea extract reflects its stimulatory effect on thermogenesis. These findings

could not be explained by caffeine content alone because treatment with caffeine levels equivalent to those in the tea extract did not affect energy expenditure, respiratory quotient, or substrate oxidation. It is possible that the caffeine consumed during treatment reacts synergistically with other bioactive ingredients in the green tea to promote catecholamine-induced thermogenesis.[9]

13.3 TEA CATECHINS AND THE TREATMENT OF OBESITY

Long-term consumption of green tea or EGCG-containing extracts may help reduce the incidence of obesity, diabetes, and cardiovascular disease.[19] Chantre and Lairon,[4] studying the effects of green tea extracts on moderately obese patients, observed a steady decrease in body weight as well as waist circumference over a 12-week period (tables 13.3 and 13.4). The mean body weight was decreased by 4.60% and waist circumference by 4.48%. Even though that study used an open uncontrolled design, it was very clear that body weight decreased by 4.6% in 3 months. These results suggest that diminished sympathetic nervous system activity may contribute to the diminished energy expenditure, leading to obesity. Therefore, the regulation by green tea extracts of sympathetic nervous system activity by heat-producing norepinephrine may play an important role in weight management.[20]

In a study by Lu et al.,[19] where they provided basic information on mice, rats, and humans, EGCG methylation and suggested that EGCG may inhibit COMT-catalyzed methylation of endogenous and exogenous compounds. The possible sites of methylation and glucuronidation of EGCG were determined and are shown in figure 13.5. The study showed that EGCG potently inhibits the activities of COMT. Using EGC and 3,4-dihydroxy-L-phenylalanine as substrates, EGCG, 4"-*O*-methyl-EGCG, and 4',4"-di-*O*-methyl-EGCG were all potent inhibitors of COMT. The noncompetitive inhibition of COMT by 4"-Me-EGCG and 4',4"-di-methyl-EGCG suggests that they

TABLE 13.3
Evolution of body weight loss

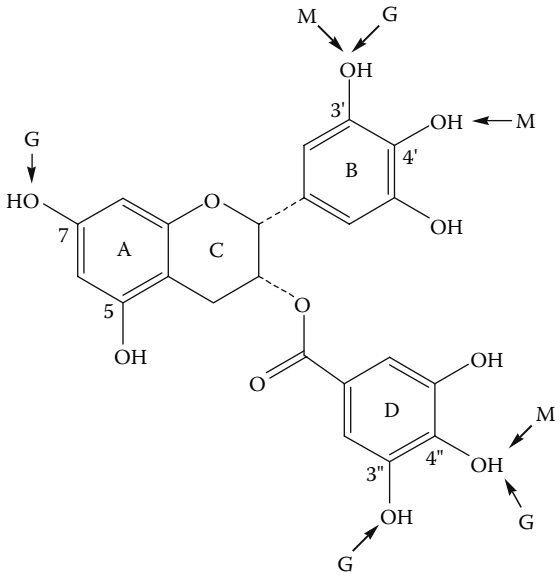
	Baseline	Week 4	Week 8	Week 12
Number of people	70	68	63	66
Mean (kg)	75.99 ± 8.85	74.64 ± 8.93	73.97 ± 9.02	72.49 ± 8.97

Source: Modified from Chantre, P. and Lairon, D. 2002. Recent findings of green tea extract AR25 (exolise) and its activity for the treatment of obesity. *Phytomedicine* 9:3–9.

TABLE 13.4
Evolution of waist circumference

	Baseline	Week 4	Week 8	Week 12
Number of people	70	68	63	66
Mean (cm)	94.41 ± 11.72	90.6 ± 10.86	89.98 ± 10.91	88.27 ± 10.8

Source: Modified from Chantre, P. and Lairon, D. 2002. Recent findings of green tea extract AR25 (exolise) and its activity for the treatment of obesity. *Phytomedicine* 9:3–9.



EGCG

FIGURE 13.5 A summary of possible sites of methylation (M) and glucuronidation (G) of EGCG.

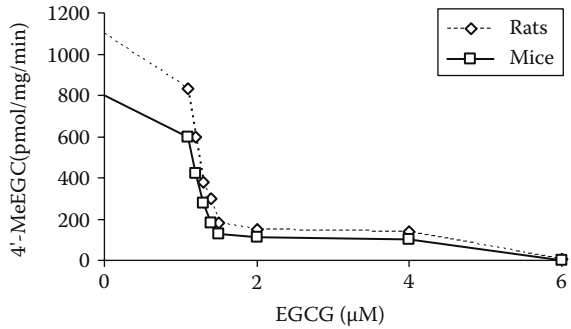


FIGURE 13.6 Concentration-dependent inhibition of COMT activity by EGCG. (Modified from Lu, H., Meng, X., and Yang, C., 2003. Enzymology of methylation of tea catechins and inhibition of catechol-o-methyl transferase by (–)-epigallocatechin gallate. *Drug Metabolism and Disposition*, 31, 572-579.)

inhibit COMT by binding to sites other than the catechin binding site. Figure 13.6 shows the concentration-dependent inhibition of COMT activity by EGCG. As the levels of EGCG increase, the COMT substrate levels decrease, implying that EGCG can inhibit the action of COMT on endogenous and exogenous compounds.

The green tea extract is distinct from sympathomimetic drugs, as these drugs are limited by their adverse cardiovascular effects, and are particularly inappropriate for obese individuals with hypertension and other cardiovascular implications. In the

Dulloo et al.[6] study, there were no side effects and no significant differences in heart rate across treatments.

Dulloo et al.[9] tested green tea extract on ten healthy men and found the 24 h energy expenditure values increased by approximately 4%. It is generally accepted that thermogenesis contributes up to 8–10% of daily energy expenditure (760–950 kJ) in a typical sedentary man. Therefore, this 4% increase (328 kJ) due to green tea extract would extrapolate to a 35–43% (75–100 kJ) increase in the thermogenesis compartment of daily energy expenditure. Tea catechins have strong affinities for proteins with a high proline content, such as caseins in milk, gelatin, and salivary proteins. The strong binding of catechins to proteins is due to the ability of catechins to bind to protein via various phenolic groups at more than one area on the proline-rich protein by virtue of their open extended structure on the one hand, and the high content of proline residues on the other. Hydrophobic interactions and hydrogen bonding result in the formation of protein-catechin complexes that affect certain enzymes and membrane activities.[21] Such interactions will reduce the absorption of tea catechins and digestibility of dietary protein. The long-term effects are significant, as a lower caloric expenditure of 75 kJ per day theoretically would result in nearly 8 lb of weight loss per year.[15] Therefore, green tea extract–stimulated thermogenesis has the potential to influence body weight by changing energy expenditure.[9] Landsberg et al.[22] also showed that NE turnover rate in IBAT of the rat resulted in an increase in sympathetic activity in response to overfeeding.

13.4 CONCLUSION AND DISCUSSION

Green tea extracts have a high content of catechins and, compared to other tea products, a much higher potential for becoming effective and practical treatments for reducing chronic diseases. Clinical studies suggest that green tea extract, rich in catechins and caffeine, is an effective potentiator of sympathetically mediated thermogenesis, and a natural substance for the management of obesity. In *in vitro* study, green tea extract was shown to exert a stimulation of thermogenesis, possibly mediated by a reduction in enzymatic degradation of NE and prolongation of action of sympathetically released NE. In clinical studies there was a significant increase of energy expenditure, lowering of body weight, decrease in waist circumference, and no change in heart rate or blood pressure after oral administration of green tea. Although there is an increasing interest in green tea's role in promoting good health, the bioavailability and biostransformation of EGCG are not well understood. Tea catechins are orally bioavailable, but the degree of absorption or retention is still under investigation. The knowledge base of the absorption, metabolism, and tissue distribution is limiting.[13,19] The kinetic parameters of EGCG methylation by human liver and the contribution of methylation to the biotransformation of EGCG remain unknown.[11]

Further studies are necessary to evaluate and document the observed weight-reducing action of green tea extract, especially in patients with a severe obesity problem.[4] Further studies on the effects of tea catechins on the metabolism of catecholic hormones and their related diseases are warranted. The potential interactions between EGCG and catecholic hormones or drugs should also be considered.[11] Further studies are required to determine the best dosage for tea consumption. Pure components in

capsules might not be the best way to deliver the active catechin components effectively, compared to drinking tea, which is more practical for human health.

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14 Fermented Tea Is More Effective Than Unfermented Tea in Suppressing Lipogenesis and Obesity

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CONTENTS

14.1 INTRODUCTION

Green tea has been found to possess a high antioxidant activity owing to its high content of catechins.[1] Processes used in the manufacture of black and pu-erh teas are known to decrease the levels of monomeric catechins to a much greater extent of polymerization that leads to the formation of theaflavins and thearubigins. The production and consumption of partially fermented oolong tea and drastically fermented pu-erh tea are confined to China.

Green, oolong, black, and pu-erh teas are processed differently during manufacturing. To produce green tea, freshly plucked tea leaves are steamed to prevent fermentation.[2] Catechins are the main compounds in green tea; they consist of

(–)-epicatechin (EC), (–)-epicatechin-3-gallate (ECG), (–)-epigallocatechin (EGC), and (–)-epigallocatechin-3-gallate (EGCG). To produce oolong, black, and pu-erh teas, the fresh tea leaves are allowed to wither, decreasing their moisture content until their weight approaches the appropriate percentage (this value is assessed by experience) of the original leaf weight. The withered leaves are then rolled and crushed, initiating the fermentation of polyphenols. This fermentation process converts catechins to theaflavins, mainly theaflavin (TF-1), theaflavin-3-gallate (TF-2a), theaflavin-3'-gallate (TF-2b), and theaflavin-3,3'-digallate (TF-3), and thearubigins, consequently decreasing the catechin content.

It is striking that excess body weight is a major health problem in most developed nations and is increasing in both prevalence and severity.[3] Obesity is a major risk factor for cardiovascular disease, diabetes, and cancer, for which the social costs are incalculable. Development of drugs to treat obesity or implementation of a dietary regimen to prevent obesity is a public health goal of the highest priority.[4] Obesity has increased at an alarming rate in recent years and is now a worldwide health problem.

It has been known for some time that tea helps to control obesity, and this is a common belief in the Chinese society, in which obese people are seldom found in long-term tea-drinking individuals.[5] Moreover, it is generally accepted that drinking tea for a long time will keep one living long to stay in good shape without becoming too fat and too heavy.

Based on biochemical and pharmacological studies, the mechanisms of action of tea in preventing obesity may be through stimulating hepatic lipid metabolism,[6] inhibiting gastric and pancreatic lipases,[7] stimulating thermogenesis,[8,9] modulating appetite,[10] promoting synergism with caffeine and theanine,[11] and finally suppressing fatty acid synthase.[4]

14.2 INHIBITION OF THE BODY WEIGHT OF RODENTS BY TEA AND TEA POLYPHENOLS

In 1998, significant hypolipidemic and growth-suppressive effects of green tea leaves in rats after 63 weeks of feeding were observed in a study.[12] Recently, further comparative studies on the hypolipidemic and growth-suppressive effects of oolong, black, pu-erh, and green tea leaves in rats were carried out.[5] The body weights of rats were examined when 3-week-old male Sprague-Dawley rats were fed the basal diet, 1.5% and 4% green, oolong, black, and pu-erh tea leaves. At 30 weeks, feeding 1.5% green tea leaves produced no reduction in body weight, but 1.5% oolong tea ($p < 0.01$), black tea ($p < 0.05$), and pu-erh tea ($p < 0.005$) remarkably decreased the body weight compared to the basal diet–fed group. In the feeding of 4% tea leaves, the rats were all remarkably reduced in body weight. At 30 weeks, the body weights of green tea–fed rats were about 6% ($p < 0.005$); oolong tea fed, 11% ($p < 0.001$); black tea fed, about 7% ($p < 0.001$); and pu-erh tea fed, about 13% ($p < 0.005$) lower than that of the basal diet–fed group.[5] It is interesting to note that the diet intake among these five groups was not much different. The experimental results indicated that the fermented teas such as oolong, black, and pu-erh tea leaves were more effective than the unfermented green tea leaves in suppressing the body weights of rats.[5]

The effects of purified tea catechins on the body weights of rats were investigated by Kao et al.[13] Intraperitoneal injection of EGCG, but not other structurally related catechins such as EC, EGC, and ECG, caused acute body weight loss in Sprague-Dawley male and female rats within 2–7 days of treatment. In male rats, the effect of EGCG on body weight was dose dependent. Female rats injected daily i.p. with 12.5 mg EGCG (ca. 92 mg/1 kg BW) lost 10% of their body weight relative to their initial weight, and 29% relative to the control weight, after 7 days of treatment.[13] Dietary supplementation with EGCG to mice significantly affected body weight development, as demonstrated in a recent study.[14] EGCG dose-dependently reduced the body weight increase observed after feeding of a high-fat diet. Control mice (male, New Zealand black) gained twice as much body weight during the 4-week treatment as animals supplemented with 1% EGCG in the diet. This was not due to a decreased food intake since neither daily food intake nor total food intake during the treatment period was changed. The reduced body weight gain induced by EGCG was exclusively due to a reduction in body fat. Lean body mass development was not affected by EGCG.[14] Similar results on the beneficial effects of tea catechins on high-fat-diet-induced obesity were observed by Murase et al.[6]

14.3 INHIBITION OF LIPOGENESIS BY TEA AND TEA POLYPHENOLS

The synthesis of fatty acids is the key step for lipogenesis, which is responsible for the complete synthesis of palmitate from acetyl-CoA in the cytosol. In the rat, the pathway is well represented in adipose tissue and liver. In most mammals, glucose is the primary substrate for lipogenesis. Inhibition of lipogenesis occurs in type I diabetes mellitus, and variations in its activity may affect the nature and extent of obesity.[15]

The inhibition of lipogenesis by oolong, black, pu-erh, and green tea leaves in rats has been demonstrated by the suppression of plasma triacylglycerol, cholesterol, and low-density lipoprotein (LDL) cholesterol in experimental animals.[5] Pu-erh tea and oolong tea could lower the levels of triacylglycerol more significantly than green tea and black tea; meanwhile, pu-erh tea and green tea were more efficient than oolong tea and black tea in lowering the level of total cholesterol.

A recent study on the first dose-response comparison of a green tea and a black tea on normal hamsters after long-term supplementation and on a hamster model of atherosclerosis was reported.[16] Both teas were equally effective in inhibiting atherosclerosis with the lower dose (0.0625% tea solution), decreasing it 26–40%, and the high dose (1.25% tea solution), decreasing it 46–63%. Atherosclerosis was inhibited by three mechanisms: hypolipidemic, antioxidant, and antifibrinolytic. There was a significant correlation between atherosclerosis and the three mechanisms.[16]

14.4 INHIBITION OF FATTY ACID SYNTHASE BY TEA POLYPHENOLS *IN VITRO*

Fatty acid synthase (FAS) is a key enzyme participating in energy metabolism *in vivo*[17] and is related to various human diseases such as obesity and cancer. Human cancer cells express high levels of FAS, and inhibition of FAS is selectively cytotoxic

to human cancer cells.[18] EGCG is an effective inhibitor of FAS from chicken liver *in vitro*.[19] Its inhibition of FAS is composed of reversible fast-binding inhibition, through which 52 μM EGCG can inhibit 50% of the activity of FAS. The marked inhibition of ketoacyl reduction shows that the inhibition is related to β-ketoacyl reductase of FAS. The observable protection of NADPH and competitive inhibition of NADPH for ketoacyl reduction indicate that EGCG may compete with NADPH for the same binding site.

14.5 SUPPRESSION OF FATTY ACID SYNTHASE BY TEA POLYPHENOLS *IN VIVO*

The *in vivo* suppression of FAS by tea and tea polyphenols has been demonstrated in the MCF-7 human breast carcinoma cells and HepG2 hepatocellular carcinoma cells.[4] FAS is overexpressed in the malignant human breast carcinoma MCF-7 cells, and its expression is further enhanced by epidermal growth factor (EGF). The EGF-induced expression of FAS was inhibited by green and black tea extracts (120 μg/ml). The expression of FAS was slightly stimulated by green, black, and oolong tea extracts at lower concentrations (30 μg/ml). The expression of FAS was also suppressed by green tea polyphenol EGCG, but not catechin, epicatechin (EC), and epigallocatechin (EGC). Furthermore, the expression of FAS was significantly suppressed by theaflavin (TF-1), theaflavin-3-gallate (TF-2a), theaflavin-3'-gallate (TF-2b), and theaflavin-3,3'-digallate (TF-3) at both protein and mRNA levels, which may lead to the inhibition of cell lipogenesis and proliferation. The phosphatidylinositol-3-kinase (PI3K) inhibitor Ly294002 (5 μM) can block completely the FAS expression. Both EGCG and TF-3 inhibit the activation of Akt and block the binding of Sp-1 to its target site. Furthermore, the induced FAS protein was significantly suppressed by transient transfection of dominant negative Akt mutant gene into the MCF-7 cells. Additional experimental results demonstrated that the EGF-induced biosynthesis of lipids, including triacylglycerol, cholesterol, and fatty acids, and cell proliferation were significantly suppressed by EGCG and TF-3.[4] These findings suggest that tea polyphenols suppress FAS expression by downregulating EGF receptor/PI3K/Akt/Sp-1 signal transduction pathways. Tea and tea polyphenols might induce hypolipidemic, antiobesity, and antiproliferative effects by suppressing FAS (figure 14.1). The molecular mechanisms of these biochemical reactions will be discussed later in this chapter.

14.6 ENHANCEMENT OF CELLULAR ENERGY EXPENDITURE BY TEA DRINKING

Maintenance of a constant body weight requires a balance between cellular energy intake (EI) and energy expenditure (EE), and even a slight imbalance in this energy equilibrium can lead to significant changes in body weight, and may eventually result in obesity.[20]

Daily consumption of tea containing 690 mg catechins for 12 weeks reduced body fat, which suggests that the ingestion of catechins might be useful in the prevention and improvement of lifestyle-related disease, mainly obesity.[21]

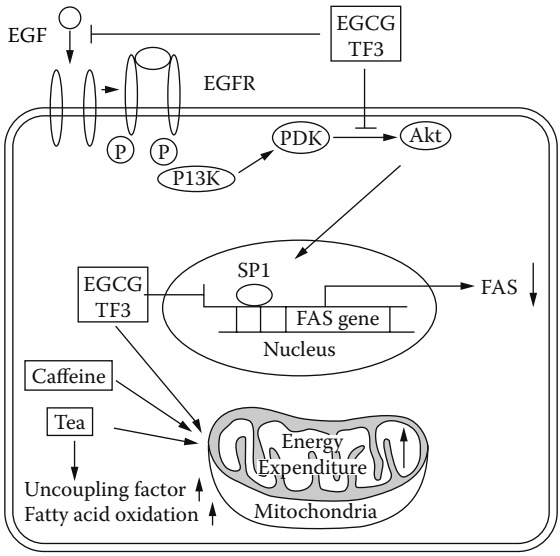

FIGURE 14.1 A possible mechanism for the hypolipidemic and antiobesity effects of tea and tea polyphenols. In the nucleus, the expression of FAS may be suppressed by tea and tea polyphenols through downregulating EGF-receptor/PI3K/Akt/Sp-1 signal transduction pathway. This mechanism may inhibit the cellular lipogenesis and tissue growth. Tea polyphenols EGCG and TF-3 inhibit the growth factor EGF binding to EGFR, block the activation of the PI3K/Akt signal pathway, then reduce the DNA-binding capacity of nuclear transcription factor Sp-1, and finally lead to downexpression of the FAS gene. Meanwhile, in the mitochondria, tea and tea polyphenols stimulate the cellular energy expenditure that may reduce body weight gain. Abbreviations: EGF, epidermal growth factor; EGFR, EGF receptor; PI3K, phosphoinositol-3-kinase; PDK, phosphatidylinositol-3,4,5-triphosphate-dependent kinase; EGCG, (–)-epigallocatechin-3-gallate; and TF-3, theaflavin-3,3'-digallate. The uncoupling factor is referred to the mitochondrial uncoupling proteins UCP-1, UCP-2, UCP-3, and other factors.

A randomized crossover design was used to compare 24 h EE of twelve men consuming each of four treatments: (1) water, (2) full-strength oolong tea (daily allotment brewed from 15 g of tea), (3) half-strength oolong tea treatment (brewed from 7.5 g tea), and (4) water containing 270 mg caffeine, equivalent to the concentration in the full-strength oolong tea treatment. Subjects refrained from consuming caffeine or flavonoids for 4 days prior to the study. Subjects received each treatment for 3 days; on the third day, EE was measured by indirect calorimetry in a room calorimeter. For the 3 days, subjects consumed a typical American diet. Energy content of the diet was tailored to each subject's needs as determined from a preliminary measure of 24 h EE by calorimetry. Relative to the water treatment, EE was significantly increased by 2.9 and 3.4% for the full-strength oolong tea and caffeinated water treatments, respectively. This increase over water alone represented an additional expenditure of 281 and 331 kJ/day for subjects treated with full-strength oolong tea and caffeinated water, respectively. In addition, fat oxidation was significantly higher (12%) when subjects consumed the full-strength oolong tea rather than

water.[22] It is concluded that oolong tea stimulated EE and fat oxidation in normal-weight males and would have some beneficial effects on an individual's ability to maintain lower body fat.

Another recent study examined the effect of oolong tea and green tea consumption on fasting EE.[23] Eleven healthy, normal-weight women were tested after drinking water and again after drinking oolong and green teas in random order. Resting EE was similar in the two groups before consumption of different beverages and remained low after water and green tea consumption, but increased significantly after oolong tea consumption. The cumulative increases in EE over resting EE after consumption of oolong tea, green tea, and water were 110.7 (26.5 kcal), 49.5 (11.8 kcal), and 11.2 kJ (2.7 kcal), respectively, over the 2 h measuring period.[23] The authors concluded that because oolong tea had less caffeine and EGCG than green tea, the rise in EE must be due to the presence in oolong tea of more polymerized polyphenols than are found in green tea.

Another study examined the effects of green tea on thermogenesis in humans.[8] Green tea has thermogenic properties and promotes fat oxidation beyond that explained by its caffeine content per se. The green tea extract may play a role in the control of body composition via sympathetic activation of thermogenesis, fat oxidation, or both.[8]

Fundamentally, there are only two ways to treat obesity: reduce EI and increase EE. Thermogenesis and fat oxidation are to a large extent under the control of the sympathetic nervous system and its neurotransmitter norepinephrine after a rational approach for obesity management.[24] In this context, there has been much interest in the potential thermogenic effects of many compounds extracted from plants, namely, caffeine from coffee and tea, ephedrine from ephedra, and capsaicin from pungent spices, largely because of their potential to modulate catecholamine release and activity.[25]

14.7 GENERAL DISCUSSION ON THE ACTION MECHANISMS OF ANTIOBESITY EFFECTS OF TEA

The effect of caffeine on metabolic rate has been well documented. A significant increase of 2–12% in metabolic rate was observed with caffeine doses of 200–300 mg.[27] It was concluded that EGCG and caffeine from the tea act synergistically to produce the thermogenic response and an increase in fat oxidation.[8] It is clear that the consumption of oolong tea stimulates both EE and fat oxidation in normal-weight man. This raises the possibility that tea consumption could have some beneficial effect on an individual's ability to maintain a lower body fat content.[22]

It seems to be a traditional Chinese belief that drinking tea promotes good health and longevity.[26] To support this belief, the scientific evidence is rapidly accumulating. Tea is one of the most frequently consumed beverages worldwide, yet very little is known about its metabolic effects in humans. Caffeine is generally regarded as the major metabolically active compound in tea. Some individuals are sensitive to caffeine and find that it induces sleeplessness or irritation to the gastrointestinal tract, but others consume it specifically because it is a mild stimulant and increases alertness and metabolic rate.[22]

The effects of the green tea catechin, EGCG, were found to be dose dependent and gender and strain independent. In addition, differential effects of green tea catechins on body weight loss, food intake restriction, decreases in accessory sexual organ weight, and decreases in blood nutrients were observed.[13] The effect of EGCG on the weight of male accessory sexual organs was due to a lowered circulating level of testosterone. Furthermore, it was demonstrated that microsomal 5α-reductase, a key enzyme to convert testosterone to its active form dihydrotestosterone, was significantly inhibited by tea polyphenols, including EGCG and theaflavins.[28]

The administration of green tea extract is reported to increase fat oxidation and EE in humans[8] and in rat brown adipose tissue,[9] and it has been assumed to be attributable to an interaction between green tea extract's high catechins and caffeine content, which influences the level of sympathetic activity. Since catechins are known to inhibit catechol-*O*-methyltransferase (the enzyme that degrades norepinephrine), and caffeine is known to inhibit the phosphodiesterase-induced degradation of cAMP, it has been proposed that these compounds synergistically prolong and augment the sympathetic stimulation of fat oxidation.[9] The molecular mechanism by which tea catechins stimulate lipid metabolism is unclear. It has become apparent that the expression of many lipid-metabolizing enzymes, including acyl-CoA oxidase and acyl-CoA dehydrogenase, is transcriptionally regulated by peroxisome proliferator-activated receptors (PPARs). It has been shown that catechins (EGCG, ECG, GCG, etc.) are not ligands for PPARα by using a transient transfection assay.[6] On the other hand, nuclear factor-κB (NF-κB) was reported to inhibit PPARα-mediated activation of a PPAR response element-driven promoter through physical interaction of PPARα with NF-κBp65.[29] Because catechin gallates inhibit the activation of NF-κB,[30] feeding with tea catechins regulates the transcription of PPAR-related genes by reducing the NF-κB activation, which may lead to upregulation of the lipid-metabolizing enzymes.

It is widely recognized that the function of the original uncoupling protein, UCP-1, is uncoupling of substrate oxidation from ATP synthesis, and that its physiological purpose is thermogenesis. UCP-2 and UCP-3 appear to function in reactive oxygen species handling and in fatty acid metabolism; uncoupling might occur secondarily.[31] It has been demonstrated that in brown adipose tissue activation of cells during exposure to cold increases substrate utilization in such a way that glucose and fatty acid oxidation detach from the orthodox coupling to ATP synthesis and result in thermogenesis. The unique mechanism of uncoupling respiration that occurs in brown adipocyte mitochondria represents an attractive strategy for promoting EE and decreasing the fat content of the body. Moreover, ectopic expression of brown fat uncoupling protein (UCP-1) in mouse skeletal muscle and induction of UCP-1 in mouse or human white adipocytes promote fatty acid oxidation and resistance to obesity.[32]

Skeletal muscle uncoupling by ectopic expression of mitochondrial UCP-1 has been shown to result in a lean phenotype in mice characterized by increased EE, resistance to diet-induced obesity, and improved glucose tolerance.[33] UCP-2 is a member of the mitochondrial transporter superfamily that uncouple proton entry in the mitochondrial matrix from ATP synthesis. Although its physiological role remains to be established, UCP-2 is considered a candidate gene for association with energy metabolism and obesity.[34] It is worth noting that the possible association of

the hypolipidemic and antiobesity effects of tea polyphenols with the expression of UCP-1, UCP-2, and UCP-3 in adipose tissue might be of great biological significance and deserve further investigation (figure 14.1).

One of the intracellular signaling pathways that are frequently activated in cancer cells is the PI3K/Akt kinase pathway.[35] This pathway has been documented as important for cell survival, cell transformation, and tumor growth. PI3K catalyzes the formation of PIP2 and IP3. Increases in phosphoinositides lead to membrane translocation of downstream effectors such as the Ser/Thr protein kinase Akt. On translocation, Akt is phosphorylated and activated by phosphatidylinositol-3,4,5-triphosphate-dependent kinase (PDK), ultimately resulting in the stimulation of cell growth and survival through transcription activation of the FAS gene.[4] Our published data indicate that the activated Akt may bind on the Sp-1 site, which leads to transcription of the FAS gene, as illustrated in figure 14.1. The suppression of FAS expression by tea polyphenols, including EGCG, TF-1, TF-2, and TF-3, has been demonstrated.[4]

FAS plays a central role in *de novo* lipogenesis in mammals by the action of its seven active sites. FAS catalyzes all the reactions in the conversion of acetyl-CoA and malonyl-CoA to palmitate. FAS concentration is sensitive to nutritional and hormonal status in lipogenic tissues such as liver and adipose tissues. The nutritional regulation of FAS occurs mainly via changes in FAS gene transcription.[36] We have also shown that the incorporation of ^{14}C-acetyl-CoA into triacylglycerol, fatty acids, and cholesterol in MCF-7 cells was significantly inhibited by EGCG and TF-3.[4] These results have provided evidence that tea polyphenols such as EGCG and TF-3 profoundly suppress FAS gene transcription through EGF receptor/PI3K/Akt/Sp-1 signal transduction pathways.

In previous studies, we have demonstrated that cancer cell proliferation is profoundly inhibited by tea polyphenols, including catechins and theaflavins, by suppression of the function of the EGF receptor,[37–39] which leads to the blockade of the MAPK/MEK/ERK/ELK signal pathway[40,41] and PI3K-dependent pathway.[42] In the present study, we have shown that FAS expression is significantly inhibited by tea polyphenols also through suppression of the function of the EGF receptor, which leads to the inhibition of the PI3K/Akt/Sp-1 signal pathway.[4] In summary, the suppression of lipogenesis through downregulating FAS expression in the nucleus and the stimulation of EE in the mitochondria could be two major mechanisms that play important roles in exerting the hypolipidemic and antiobesity effects of tea and tea polyphenols, as illustrated in figure 14.1.

14.8 CONCLUSION

The hypolipidemic and antiobesity effects in animals and humans have become a hot issue for biochemical nutrition and food research. It has been demonstrated that the body weight of rats and their plasma triacylglycerol, cholesterol, and LDL cholesterol are significantly reduced by feeding of oolong, black, pu-erh, and green tea leaves to the animals. The results have shown that the suppression of body weight of tea leaves–fed groups is in the order of: oolong tea > pu-erh tea > black tea > green tea. Pu-erh tea and oolong tea could lower the levels of triacylglycerol more significantly than green tea and black tea, but pu-erh tea and green tea were more efficient

than oolong tea and black tea in lowering the level of total cholesterol. It seemed that the fermented teas, including oolong, black, and pu-erh teas, are more effective than unfermented green tea in suppressing the body weight and lipogenesis in rats. It has been suggested that the inhibition of growth and suppression of lipogenesis may occur through downregulation of fatty acid synthase gene expression in the nucleus and stimulation of cell energy expenditure in the mitochondria. The experimental data indicate that the molecular mechanisms of fatty acid synthase gene suppression by tea polyphenols (EGCG, theaflavins) may be rendered through downregulating EGFR/PI3K/Akt/Sp-1 signal transduction pathways. The possible association of hypolipidemic and antiobesity effects of tea polyphenols with the expression of the uncoupling factor UCP-1, UCP-2, and UCP-3 genes in adipose tissues deserves further studies.

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15 Trapping of Methylglyoxal by Tea Polyphenols

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CONTENTS

15.1 INTRODUCTION

Diabetes is a heterogeneous disorder and generally accompanied with multiple complications. It involves resistance of glucose and lipid metabolism in peripheral tissues to the biological activity of insulin and inadequate insulin secretion by pancreatic β cells. Epidemiological and large prospective clinical studies have confirmed that hyperglycemia is the most important factor in the onset and progress of diabetic complications, both in type 1 (insulin-dependent) and type 2 diabetes mellitus.[1,2] Increasing evidence identifies the formation of advanced glycation end products (AGEs) as the major pathogenic link between hyperglycemia and diabetes-related complications.[3]

Nonenzymatic glycation is a complex series of reactions between reducing sugars and amino groups of amino acids, peptides and proteins, lipids, and DNA. As the first step of AGE formation, the free amino groups of proteins in the tissues react with a carbonyl group of reducing sugars such as glucose to form fructosamines via a Schiff base by Amadori rearrangement. Both Schiff base and Amadori product further undergo a series of reactions through dicarbonyl intermediates (e.g., glyoxal [GO], methylglyoxal [MG], and 3-deoxyglucosone) to form AGEs.[3] GO and MG, the two major α-dicarbonyl compounds found in the human body, are extremely reactive and readily modify lysine, arginine, and cysteine residues of proteins.[4] Reactive carbonyl compounds such as GO and MG have recently attracted much attention because of their possible clinical significance in chronic and age-related diseases. They are considered to accumulate in the body fluids and tissues mainly by accelerated oxidative stress, and modify proteins, DNA, and phospholipids to form biologically active adducts such as AGEs.[5–7] It has been shown that dicarbonyl compounds are more reactive than reducing sugars and are more important for cross-linking proteins in the glycation process.[5–7]

More and more evidence indicates the increase in reactive carbonyl intermediates is a consequence of hyperglycemia in diabetes. Carbonyl stress leads to increased modification of proteins, followed by oxidant stress and tissue damage.[5–7] Several studies have shown that higher levels of GO and MG are present in diabetic patients' plasma than those in healthy individuals' plasma.[8–10] In a most recent report,[10] the amount of MG from diabetic patients was found to be 16–27 μg/dl, compared with that of normal subjects of 3.0–7.0 μg/dl. Several dicarbonyl-derived products in proteins from diabetic individuals have been identified; these include imidazolium cross-links, imidazolysine,[11,12] carboxymethyllysine,[13] glyoxal-lysine dimer (GOLD),[14] carboxyethyllysine,[15] and argpyrimidine.[16,17] Thus, decreasing the levels of GO and MG may provide a useful approach for preventing the formation of AGEs. Some therapeutic agents such as aminoguanidine, L-arginine, OPB-9195, tenilsetam, pyridoxamine, and metformin have been reported to trap reactive carbonyl compounds, thereby preventing the formation of AGEs and protein cross-links.[18]

15.2 METHYLGLYOXAL

Methylglyoxal (MG), also called pyruvaldehyde, is one of the reactive carbonyl species. It is present in three forms in aqueous solution in rapid equilibrium, as shown below.[19,20] Among these, monohydrate is the most abundant (71%), followed by dihydrate (28%), and the unhydrated form is present at only about 1%.

$$CH_3COCHO \underset{-H_2O}{\overset{+H_2O}{\rightleftharpoons}} CH_3COCH(OH)_2 \underset{-H_2O}{\overset{+H_2O}{\rightleftharpoons}} CH_3C(OH)_2CH(OH)_2$$

Unhydrated Monohydrate Dihydrate

MG in food and beverages may come from sugars, the intermediates of Maillard reactions, and lipids. In addition, microorganisms growing during the processing

or storage might generate MG as well.[21] MG is generated from carbohydrates by auto-oxidation, or fragmentation and retro-aldol reaction through Maillard reaction intermediates.[21]

15.2.1 Analytical Methods for Quantification of Glyoxal and Methylglyoxal

To quantify MG, the derivatization process is needed prior to chromatographic analysis. Several derivatization agents listed in table 15.1 and figure 15.1, including diamino derivatives of benzene and naphthalene, react with MG to form quinoxalines.[21] Quinoxalines have been analyzed and quantified with high-performance liquid chromatography (HPLC) and can be monitored by UV detector at 300–360 nm, by fluorescent spectrometry at 300–360 nm with excitation wavelengths and 380–450 nm with emission wavelengths, or mass detector (MS). Other agents, like 6-hydroxy-2,4,5-triaminopyrimidine forming pteridin derivative, and cysteamine forming 2-acetylthiazolidine, have also been analyzed by HPLC. The reverse phase HPLC column is often applied.[21]

For the GC method, *O*-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine can be detected by flame ionization detector, MS/SIM detector, electron-capture detector, or flame photometric detector. 1,2-Diaminobenzene derivatives of MG can be analyzed using a flame ionization detector, MS/SIM, or a specific nitrogen/phosphorus detector.[21]

For biological samples, extra procedures might be needed to separate MG from proteins. More than 90% of MG was demonstrated to be bound to proteins, and perchloric acid was needed as a deproteinization agent.[21] Another benefit of the use of perchloric acid is to keep the samples in low pH, which can prevent degradation of dihydroxyacetone phosphate and glyceraldehyde 3-phosphate to MG.

TABLE 15.1
Examples of derivatization methods for MG analysis

	Derivatization reagent	Derivative products	Detector
HPLC	6-Hydroxy-2,4,5-triaminopyrimidine	Pteridin	UV
	Cysteamine	2-Acetylthiazolidine	UV
	Mesostilbenediamine	2,3-Diphenyl-5-methyl-2, 3-dihydropyrazine	UV
GC	1,2-Diaminobenzene	Quinoxaline	MS/SIM, NPD
	O-(2,3,4,5,6-pentafluorobenzyl) hydroxylamine hydrochloride (PFBHA)	Oxime	MS/SIM, EDC, NPD, FPD

Source: Nemet, I., Defterdarovic, L., and Turk, Z. 2006. Methylglyoxal in food and living organisms. *Mol. Nutr. Food Res*. 50:1105–17. With permission.

Note: EDC, electron-capture detector; NPD, nitrogen phosphorus detector; FPD, flame photometric detector.

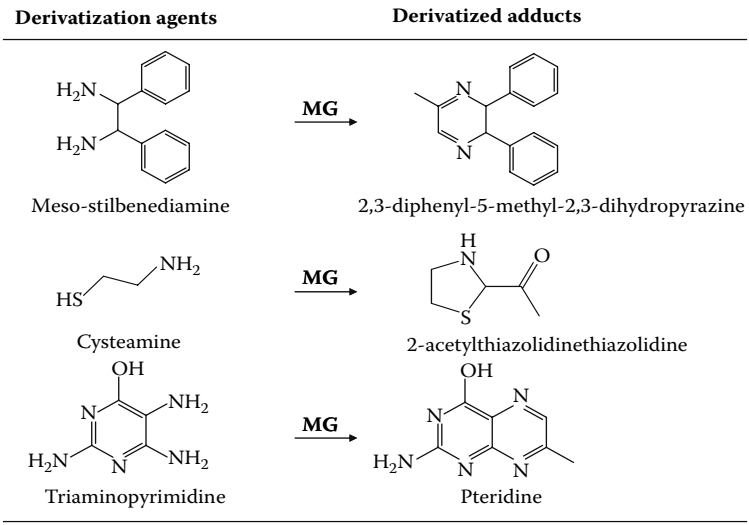

FIGURE 15.1 Examples of derivatization reagents for MG analysis and their adduct products.

15.3 EFFECTS OF TEA AND ITS POLYPHENOLS ON DIABETES

Tea is the most widely consumed beverage in the world and may be effective in the prevention and treatment of diabetes. Tea polyphenols, also know as catechins, are considered to be the active components responsible for prevention of diabetes. The major polyphenols in green tea include (–)-epigallocatechin gallate (EGCG), (–)-epigallocatechin (EGC), (–)-epicatechin-3-gallate (ECG), and (–)-epicatechin (EC).

15.3.1 Human Studies

The effects of tea on diabetes have received increasing attention as human studies' data accumulate. A recent study suggested that green tea promotes glucose metabolism in healthy humans in oral glucose tolerance tests.[22] The antihyperglycemic effect of oolong tea has been tested in a human study compared with a water control group; plasma glucose and fructosamine levels of type 2 diabetes patients decreased significantly after daily consumption of oolong tea.[23] Black tea is a fermented tea containing more catechin condensation products. Epidemiological evidence showed that increased black tea consumption significantly decreased levels of serum glucose.[24]

15.3.2 Animal Study

Several animal studies have shown that tea and tea polyphenols have potential antidiabetes effect. For instance, significant reductions of serum glucose were observed in EGCG-treated Sprague-Dawley rats, and male lean and obese Zucker rats.[24] Moreover, daily treatment with green tea of rats with streptozotocin (STZ)-induced diabetes showed inhibition of diabetic cataracts by lowering plasma and lens glucose levels. The same phenomenon was noted in a black tea treatment group.[25] Finally,

green tea lowered blood glucose concentrations in the genetically diabetic db/db mice 2–6 h after administration at 300 mg/kg body weight (bw), whereas no effect was observed in the control mice.[22]

15.3.3 *In Vivo* Study

The reduction of carbohydrate absorption from the intestine of rats with a saccharide supplemented by EGCG or green tea is based upon suppression of the activity of intestinal α-amylase, sucrase, or α-glucosidase.[25] Also, green tea enhances insulin sensitivity of normal and fructose-fed rats as demonstrated by increased glucose uptake by muscle cells.[26]

15.3.4 Effects of Tea and Its Polyphenols on Diabetes-Related Complications and AGE Formation

The ability of preventing diabetic-related complications by using tea and its ploy-phenols has been tested in several studies. For instance, oral administration of tea catechins retarded the progression of functional and morphological changes in the kidney of STZ-induced diabetic rats.[27] Also, the effects of ameliorating glucose toxicity, renal injury, and thus alleviating renal damage by EGCG have been pointed out in diabetic nephropathy model rats.[28]

The detailed mechanisms for the prevention of diabetic complications by tea and its polyphenols require further studies. Many studies have shown that those effects could partially be due to the inhibition of AGE formation. Rutter et al.[29] reported that green tea extract was able to delay collagen aging in C57Bl/6 mice by blocking AGE formation and collagen cross-linking. In addition, EGCG reduced renal AGE accumulation and its related protein expression in the kidney cortex based upon STZ-induced diabetic nephropathy model rats.[28] In an *in vitro* study, Ping et al.[30] demonstrated that green tea polyphenols dose-dependently inhibited AGE-stimulated proliferation and p44/42 mitogen-activated protein kinase (MAPK) expression of rat vascular smooth muscle cells (VSMCs).

Meanwhile, tea polyphenols may inhibit the formation of AGEs by trapping reactive dicarbonyl compounds. A recent study explored the inhibitory effect of tea polyphenols, including catechins, EC, ECG, EGC, and EGCG, on different stages of protein glycation, including MG-mediated protein glycation.[31] EGCG exhibited a significant inhibitory effect of 69.1% on MG-mediated protein glycation.

15.4 REACTION OF MG WITH TEA POLYPHENOLS

15.4.1 Detection and Quantification of Methylglyoxal with GC System

Methylglyoxal (MG) is a highly reactive α-dicarbonyl formed endogenously in numerous enzymatic and nonenzymatic reactions. MG is very volatile and often coelutes with the solvent peak when the mixture is prepared for GC analysis. Hence, many papers focusing on environmental, clinical, or food studies have been published on its detection and quantification using chemical derivatization.[21] After derivatization, the specificity of analyte, which carries a unique functional group,

allows detection with conventional detectors such as FID. In the present study, *O*-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine hydrochloride (PFBHA) was used as a carbonyl derivatization reagent. The chemical reaction between PFBHA and MG is illustrated in figure 15.2. The *O*-PFB-oximes are the derivative compounds utilized in MG quantification.

15.4.2 Trapping of Methylglyoxal by Tea Polyphenolic Compounds

In the tea polyphenol adducts investigation, the mole ratio of MG to each specific polyphenol was three, and MG decrease (%) was compared with that of the control sample at 0°C on an ice/salt bath for 1 h. After 1 h at 37°C incubation, MG was very stable, only decreasing by 5.8%, as shown in figure 15.3. All tea polyphenolic compounds scavenged. Nevertheless, the partial catechin moiety, gallic acid (GA) and pyrogallol (PY), showed 17.1 and 27.8%, respectively. Among four catechins tested, the MG decrease was relatively close for ECG and PY. ECG exhibited the lowest decrease among catechins. Most tea catechins decreased MG by about 33%, which indicates that one catechin molecule reacts with one MG molecule, since the initial mole ratio of MG and tea polyphenolic compounds is 3 to 1. On the other hand, EGC showed the highest decrease of MG. From the result (45.7% decrease), which is more than one-third, it is obvious that the active sites on EGC were more than one.

Theaflavins, the main black tea components, were more reactive toward MG than other polyphenols tested here (figure 15.3). Theaflavins showed high levels of MG reduction with respect to the control samples, which indicated that theaflavins will be excellent candidates in the treatment of MG scavenging in future *in vivo* studies. The decreased amounts of MG in TF1 (theaflavin), TF2 (theaflavin-3 and -3'-gallate), and TF3 (theaflavin-3,3'-digallate) were 63.1, 60.1, and 66.7%, respectively. All tested theaflavins decreased MG by about 66%, which implies that one theaflavin molecule can trap two MG molecules, as the observed decrease was greater than 33%.

The major adduct between EGCG and MG has been identified.[32] It is concluded that the reaction between EGCG and MG dominantly occurs at the C8-position in the A ring of EGCG. Figure 15.4 shows the structures of two peracetylated derivatives of adducts between EGCG and MG. The proposed mechanism of reaction between MG and EGCG is illustrated in figure 15.5. Whether theaflavin reaction with MG follows a similar mechanism to that of catechins needs to be further clarified.

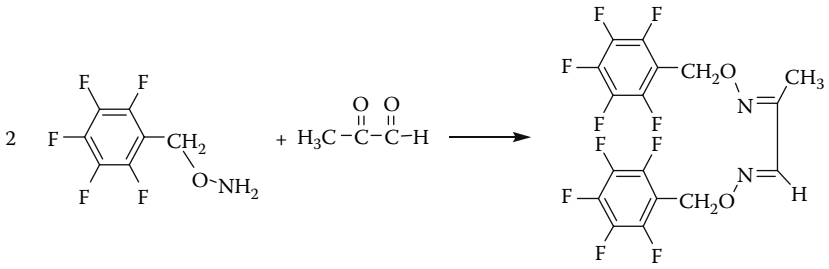

FIGURE 15.2 Reaction between PFBHA and MG forming PFBHA-MG oximes.

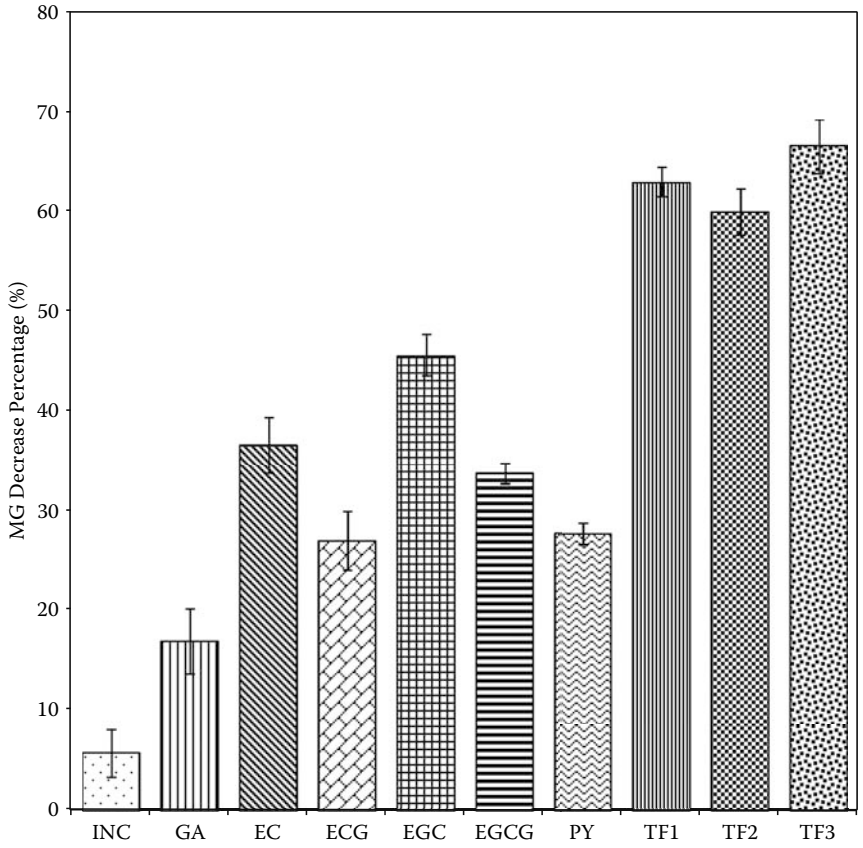

FIGURE 15.3 The comparison of polyphenol compounds on trapping efficiency of MG. Decreased percentages of MG were detected among MG only or added with each different polyphenol. Reactions were carried out under physiological conditions at 37°C, 40 rpm shaking speed water bath for 1 h. Methylglyoxal control sample was in salt added to ice bath. The decreased percentage values were expressed in mean ± SD (n = 3). INC, methylglyoxal only incubation sample; GA, gallic acid; EC, (–)-epicatechin; ECG, (–)-epicatechin gallate; EGC, (–)-epigallocatechin; EGCG, (–)-epigallocatechin gallate; PY, pyrogallol; TF1, theaflavin; TF2, theaflavin-3 or -3'-gallate; TF3, theaflavin-3,3'-digallate.

15.5 SUMMARY AND CONCLUSIONS

Methylglyoxal (MG) can be generated both *in vitro* and *in vivo*. The rate of MG formation is approximately 120 μM/day, which is about 0.1% of the flux of glucose under normal conditions measured in *in vitro* red blood cells.[33,34] Even with such a small fraction, MG is of importance and of threat because of its high reactivity. The regulation of MG levels and its close link to many chronic diseases is important. Among diseases, diabetes—and its complications—are of most concern; thus, hyperglycemia is the most important factor in the onset and progress of diabetic complications from epidemiological and large prospective clinical viewpoints.

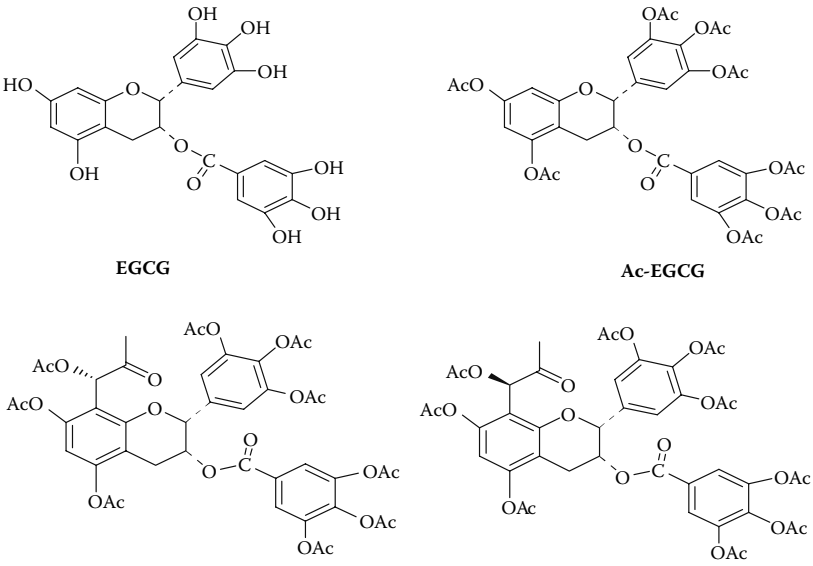

FIGURE 15.4 Structures of EGCG, acetylated EGCG (Ac-EGCG), and adducts C and D.

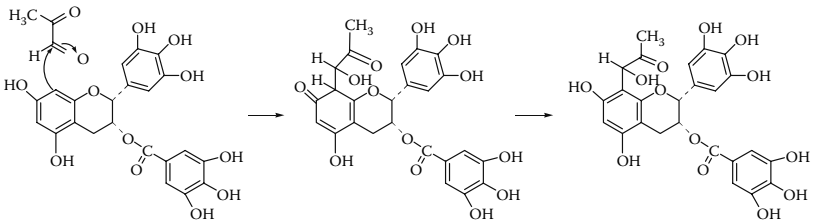

FIGURE 15.5 Proposed mechanism of formation of EGCG-MG adducts.

Many synthetic organic compounds have been demonstrated to have excellent AGE inhibitory activity. Thus, finding dietary compounds to minimize safety concerns is of interest. It is well known that tea is rich in polyphenolic compounds and has potential health benefits, including prevention of cancer, heart disease, and diabetes. We have shown in this study that all tea polyphenols, including pyrogallol, gallic acid, EC, ECG, EGC, EGCG, TF1, TF2, and TF3, have very good MG-trapping abilities. Catechins seemed to trap MG at a 1 to 1 ratio. The major adduct between EGCG and MG has been identified.[32] The reaction between EGCG and MG dominantly occurred at the C8-position of the A ring of EGCG. Our study indicated that tea catechins are able to effectively trap MG, the most important reactive carbonyl species, *in vitro*. Whether tea catechins can trap reactive carbonyl species *in vivo* and thus reduce the formation of AGEs and prevent the development of diabetic complications needs to be further studied.

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16 Chemistry and Biological Properties of Theanine

Xiaochun Wan, Zhengzhu Zhang, and Daxiang Li

CONTENTS

16.1 INTRODUCTION

Theanine, γ-glutamylethylamide (5-*N*-ethylglutamine) (figure 16.1), was first found in tea leaves (*Camellia sinensis* and others of the *Camellia* genus) in the early 1950s.[1] The only other reported natural source of theanine is in the mushroom *Xerocomus basius*,[2] but it is otherwise rare in nature. Theanine is a nonprotein amino acid. It

accounts for more than 50% of total free amino acids in tea. The content of theanine in tea is between 1.5 and 3% of the dry weight, which significantly contributes to the gracefully sweet taste of tea.[3,4] Natural theanine in tea is mostly in the L-form. Additionally, small amounts of the D-isomer were also found in tea leaves (table 16.1); each of the pure enantiomers and the racemic mixture of theanine have a similar, sweet taste, with no bitter aftertaste.[5]

Theanine crystallizes in the form of a colorless needle, molecular formula $C_7H_{14}O_3N_2$, melting point 217–218°C (decomposed), and $\alpha = +7.1$. It gives a strong ninhydrin reaction and is very soluble in water. One gram of theanine can be dissolved in 2.6 mL water at 0°C and 1.8 mL water at 100°C, but is insoluble in ethyl alcohol and ether. By hydrolysis of theanine, it gives L-glutamic acid and ethylamine.[1] L-Theanine is a weakly acidic amino acid, which is stable under acidic conditions

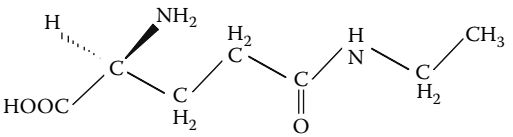


FIGURE 16.1 Chemical structure of L-theanine.

TABLE 16.1
Enantiomeric composition and total amount of theanine in various tea samples

Tea name	Tea type	Country where produced	D-Theanine (%)	SD	Total amount (g of theanine/ 100 g of tea)
African Flower	Black	Kenya	0.54	0.05	1.30
Assam FOP	Black	India	0.49	0.03	1.05
Ceylon Broken	Black	Sri Lanka	2.30	0.09	1.32
Ceylon Pekoe	Black	Sri Lanka	0.34	0.04	2.20
Cherry Blend FOP	Black	India/China	0.21	0.04	2.04
Darjeeling FOP	Black	India	0.45	0.03	1.45
Earl Grey	Black	China	0.42	0.03	1.07
Formosa Oolong	Half-green	Taiwan	12.7	0.4	0.60
Georgian FOP	Black	Georgia	0.46	0.02	1.16
Gunpowder	Green	China	2.20	0.20	1.78
Jasmine FOP	Half-green	China	0.45	0.04	1.72
Keemun FOP	Black	China	0.65	0.06	1.12
Lapsang Souchong	Black	China	1.04	0.12	0.82
Lemon Blend	Black	India/Sri Lanka	2.70	0.23	1.26
Rosen	Black	China	2.46	0.24	1.03
Sencha	Green	Japan	2.20	0.09	1.05
Yunnan	Black	China	1.79	0.16	2.38

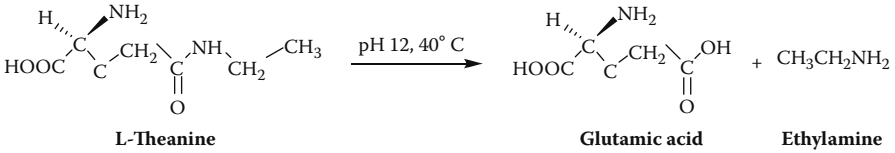


FIGURE 16.2 Decomposition of L-theanine under alkaline conditions.

of pH 3 for 1 year. There was little difference in hydrolysis of theanine between pH 3 and 7. However, it has been demonstrated that theanine has low stability under alkaline conditions. Theanine showed a far greater amount of hydrolysis to produce glutamic acid and ethylamine at pH 11 (figure 16.2).[5]

Theanine is one of the important chemical components that contribute to the taste of green tea infusion. Green tea flavor has four characteristic taste elements: bitterness, astringence, sweetness, and umami (a brothy or savory taste). The brothy, sweet, umami taste is due to amino acids, especially theanine. It is thought that theanine makes a significant contribution to the flavor of the tea beverage, which is dominated by the bitter taste of the polyphenols and caffeine, but is mellowed and broadened by the umami taste. Theanine and glutamic acid have been found to be the most important umami substances of green tea. Theanine is the predominant amino acid in tea, accounting for about 50% of the total free amino acids (constitutes between 1 and 2% of the dry weight of tea leaves). The threshold of theanine was about 0.06%, while the other amino acids were about 0.15%, which may be the reasons why theanine was used as one of the most important quality indices in world tea markets.[5]

High-grade tea product shows a high level of theanine content. Especially Anji Baicha (name of a Chinese green tea) and Gyokuro (name of a Japanese green tea), higher-grade green tea, normally plucked from shaded tea plants, contain about 2% of theanine on a dry weight basis. In shaded tea leaves, large quantities of theanine accumulated, but by contrast, the catechin content was lower than that in unshaded leaves.

16.2 BIOSYNTHESIS OF THEANINE IN TEA PLANT

Biosynthesis of theanine occurs in the root of the tea plant with the aid of the enzyme theanine synthetase (also called L-glutamate ethylamine ligase or L-glutamate:ethylamine ligase, EC 6.3.1.6) (figure 16.3).

Synthesis of theanine from ^{14}C-labeled glutamic acid and ethylamine in the presence of ATP and Mg^{2+} was first observed by the use of a homogenate of tea seedlings. The L-theanine synthesizing enzyme in tea seedlings might be a synthetase called L-glutamate:ethylamine ligase, which had a high affinity for ethylamine, and little affinity for ammonia, D-glutamate, and L-aspartate. ATP and ATP generating systems were necessary for theanine formation, but theanine synthesis was independent of glutamine synthesis.[6–8]

Theanine, synthesized from glutamic acid and ethylamine in the root of the tea plant, was found to exist in all parts of the tea plant.[9] The ethylamine moiety of theanine was formed from L-alanine via free ethylamine, which is formed by alanine

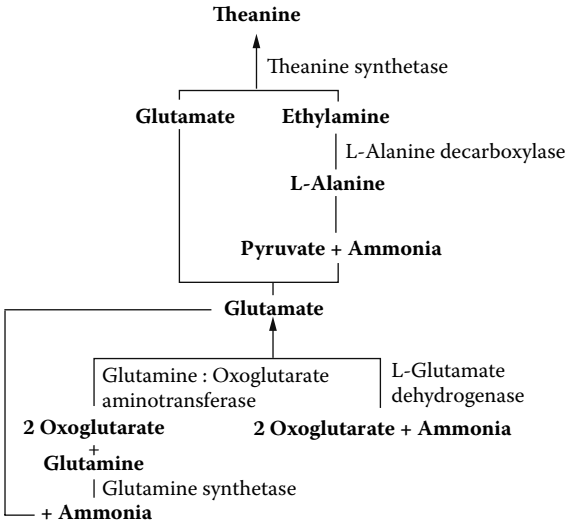


FIGURE 16.3 The biosynthesis route of theanine in tea root.

decarboxylase in the root of tea.[10,11] It accumulated more in young and active tissues and also in younger plants, which emphasizes its metabolic role in the tea plant.[9]

Since L-theanine synthetase is quite labile, its further purification may be hampered. There is no report on the purification and property on this enzyme. The biosynthesis of theanine from L-glutamate and ethylamine is shown in the following equation of the L-theanine synthetase catalytic reaction:

$$\text{ATP} + \text{L-glutamate} + \text{ethylamine} = \text{ADP} + \text{phosphate} + N^5\text{-ethyl-L-glutamine}$$

The biosynthesis route of theanine in tea roots was proposed as shown in figure 16.3.[10,11]

This synthetic reaction of theanine takes place in tea roots and acts as a means of storage or transport of nitrogen in a nontoxic form. After being synthesized in tea roots, the theanine is transported to plant tops, where it serves as the major source of soluble nitrogen for other carbon skeletal compounds, such as flavanols. The investigation of the physiological function of theanine in the tea plant showed that during germination the theanine level reached a maximum after 45 days. This indicates the growth of the synthesis of the carbon skeletal compounds during this period. It was observed that the *N*-ethyl carbon of theanine may be converted to the phloroglucinol nucleus of catechins.[12] The distribution of theanine in the shoots indicated that the first leaf was the principal site for the synthesis of polyphenolic compounds from L-theanine.[9]

16.3 PRODUCTION OF THEANINE

16.3.1 Biosynthesis of Theanine

Theanine has been considered a therapeutic/medicinal agent and additive in consumer products for its cardiovascular, neurological, and oncological effects. The

claimed pharmacological benefits of theanine are numerous, ranging from prevention of neuronal death[13] to reduction of tumor growth,[14] enhancement of antitumor activity of chemotherapeutic agents,[15] and reduction of hypertension and blood pressures.[16] Moreover, theanine relieves excitation induced by caffeine ingestion[17] and affects the brain levels of norepinephrine, serotonin, 5-hydroxyindole acetic acid, and dopamine.[18–20] In addition, ethylamine, a major metabolite of theanine, has been found to boost immune response.[21]

Because of its good taste and favorable physiological effects on mammals, theanine could be a new food additive, and several investigations on its effective production have been performed.

Theanine is not readily available and is expensive; its isolation from tea or other sources is complicated, and the cost of isolation is high. Many attempts have been made to produce theanine by biochemical and chemical routes, but up to now most of these attempts have remained in the developing stage due to the low yields, low purity, and complicated process.

Tachiki et al.[22] reported the production of theanine from glutamate and ethylamine through the coupling of baker's yeast preparations and bacterial glutamine synthetase. Because of the low reactivity of glutamine synthetase with ethylamine and the difficulty in pH control of the reaction mixture, the yield was not satisfatory. The production of theanine with cultured cells of *Camellia sinensis* has been performed, but it took a 4-week cultivation period.[23,24]

The biosynthesis of theanine from glutamate and ethylamine is catalyzed by theanine synthetase (L-glutamate:ethylamine ligase, EC 6.3.1.6), which is an ATP-dependent enzyme and quite unstable. All attempts to biosynthesize theanine using this enzyme should overcome the problems of the ATP generating system.

Enzymatic production of theanine with glutaminase from *Pseudomonas nitroreducens* has been reported.[25] In order to prepare adequate quantities of theanine efficiently, Abelian et al.[26,27] constructed a reactor by employing immobilized *Pseudomonas nitroreducens* cells as the catalyst to synthesize theanine continuously from glutamine and ethylamine. Cells of *Pseudomonas nitroreducens* were immobilized with *k*-carrageenan and filled in columns to construct a bioreactor (figure 16.4). After immobilization, the enzymes in cells became stable and the reactor maintained the synthetic activity for at least several weeks. Based on this industrial method, pure products of theanine have been produced in Japan.

An enzymatic method for the synthesis of theanine involving bacterial γ-glutamyltranspeptidase (GGT) was developed; the conversion from glutamine was 60%.[28]

16.3.2 Chemical Synthesis of Theanine

Theanine was first chemically synthesized with a 9% yield from pyrrolidonecarboxylic acid (PCA) treated with 33% (v/v) aqueous solution of ethylamine at 37°C for 20 days (figure 16.5).[29] L-Glutamic acid could be dehydrated, leading to 2-pyrrolidone-5-carboxylic acid. Abderhalden and Kautzsch[30] and Beecham[31] reported a yield of 70–90% for conversion of glutamic acid to PCA.

To chemically synthesize L-theanine from PCA, several procedures may be followed. A classical synthesis of γ-glutamyl peptides has been developed from carbo-

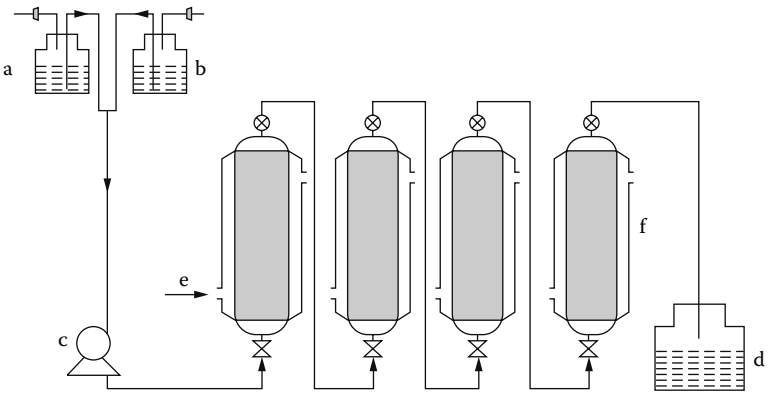
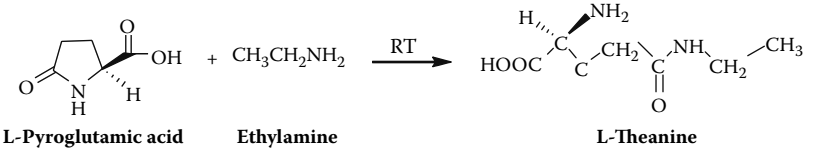


FIGURE 16.4 Schematic presentation of the bioreactor for synthesis of theanine from glutamine and ethylamine. (a) Glutamine solution vessel. (b) Ethylamine solution vessel. (c) Pump. (d) Production solution reservoir. (e) Temperature controlled water. (f) Immobilized cell columns (1.7 × 40 cm packed with 200 ml immobilized cells).



L-Pyroglutamic acid **Ethylamine** **L-Theanine**

FIGURE 16.5 Reaction of L-pyroglutamic acid with ethylamine to form L-theanine.

benzoxy L-glutamic anhydride that reacts with amino acid esters, after saponification and hydrogenation, to yield α-glutamyl peptides.[32]

A procedure for large-scale synthesis of theanine from γ-benzyl glutamate, as white crystals (m.p. = 214–216°C) with a yield of 33.9% and the specific rotation $[\alpha]_D = +8.6°$ (1% in water), was presented by Kawagishi and Sugiyama in 1992.[33] Theanine was synthesized in a similar manner using *N*-benzylcarbonatoglutamic anhydride with ethylamine hydrochloride to afford *N*-benzylcarbonato-L-theanine, which was catalytically reduced to L-theanine.[34] The latter chemical synthetic route was considered unsuitable because it required the protection and deblocking procedures of reactive groups.[35]

Recently, a Chinese patent reported a chemical procedure to synthesize L-theanine on an industrial scale. With L-glutamic acid, phthalic anhydride, and ethylamine as materials, theanine was synthesized with a yield of over 50% (figure 16.6).[36] The method includes four steps: (1) L-glutamic acid reacts with phthalic anhydride to produce *N*-phthaloyl-L L-glutamic acid and L-pyroglutamic acid. (2) *N*-phthaloyl-L-glutamic acid is boiled together with acetic anhydride to produce *N*-phthaloyl-L-glutamic anhydride. (3) *N*-phthaloyl-L-glutamic anhydride reacts with an ethylamine solution to produce *N*-phthaloyl-L-theanine. (4) *N*-phthaloyl-L-theanine reacts with hydrazine hydrate and L-theanine as given below.

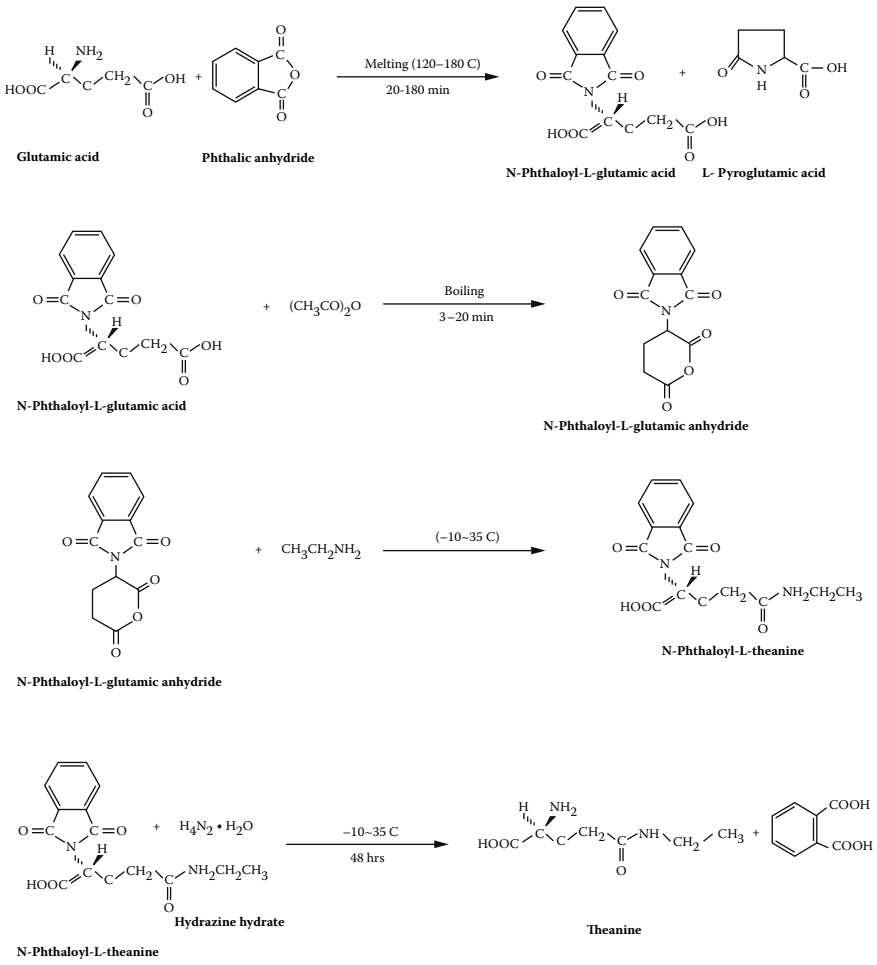


FIGURE 16.6 Chemical synthesis of theanine.

16.4 BIOLOGICAL PROPERTIES OF THEANINE

Regarding the pharmacological effects of theanine, previous reports have indicated a reduction in blood pressure in spontaneously hypertensive rats,[37] a relief from convulsions induced by caffeine,[38–41] and an influence on the brain levels of norepinephrine, serotonin, 5-hydroxyindoleacetic acid, and dopamine.[19,42,43] Moreover, oral intake of theanine results in the generation of α-electric waves in the occipital and parietal regions of the human brain.[44] Theanine-induced inhibition of glutamate transporter enhances the activity of an antitumor agent. It increased doxorubicin (DOX)-induced antitumor activity, and confirmed that this action contributed to the increase in the DOX concentration in a tumor with inhibition of the DOX efflux from tumor cells.[45,46]

16.4.1 Fate of Theanine after Oral Ingestion

After orally ingested, L-theanine is absorbed into the blood circulation through the small intestinal tract's brush-border membrane and then distributed to tissues.[47,48] It is easily transported into the brain through the blood–brain barrier's leucine-preferring amino acid transporter system.[49,50] L-Theanine does not appear to accumulate. The metabolic fate of theanine after its oral administration was verified to be enzymatically hydrolyzed to glutamic acid and ethylamine in the blood, kidney, liver, and brain; then most of the ethylamine generated was immediately excreted into urine, with only a part circulated in plasma. It is completely absent 24 h after administration.

Oral administration data indicated that gut absorption of D-theanine was far less than that of L-theanine. Data on urinary concentrations of D-theanine suggested that the D-isomer may be eliminated with minimal metabolism. L-Theanine appears to be preferentially reabsorbed and metabolized by the kidney, while D-theanine is preferentially excreted.[4]

Green tea has a long history of safe consumption. Until now, no toxic effects have been reported associated with theanine, even in large amounts.

16.4.2 Theanine Influences the Release and Concentration of Several Neurotransmitters

The administration of theanine has been reported to cause a significant increase in the dopamine concentration,[50] a decrease in the serotonin concentration,[19,37] and an increase in γ-aminobutyric acid levels in the brain.[38] Each of these neurotransmitters may play a role in mediating the effects of theanine to modulate mood (by creating a sense of relaxation and thereby promoting a sense of well-being), as well as its ability to perform a number of other actions demonstrated in animal studies, including enhancing memory and learning ability[51,52] and regulating blood pressure.[16,37,50,51]

By promoting a sense of relaxation and well-being without interfering with cognitive abilities, theanine allows for increased focus concentration, especially in those with high stress levels. This improves the ability of individuals to remember and learn.[51,52]

16.4.3 Relaxation Effect

It has been shown that theanine not only gives flavor and taste to green tea but also produces a noticeable relaxation effect in human beings.[51,52] Human brain-wave studies have confirmed that theanine produces a state of alert relaxation. In general, animals and humans always generate a very weak electric pulse on the surface of the brain, called brain waves. Brain waves are classified into four kinds, α-, β-, δ-, and θ-waves, according to frequency. Each brain wave is related to individual mental conditions (figure 16.7). Generation of α-waves is considered an index of relaxation. A volunteer test was performed to investigate the mental effect of theanine. Since it was expected that mental response to theanine could be varied with anxiety level, 50 female university students 18 to 22 years old were classified into two groups, a high-anxiety group and low-anxiety group, based on monitoring by manifest anxiety scale. Finally, the test was conducted with four high-anxiety female subjects and four low-anxiety students. These eight women took a theanine solution orally once

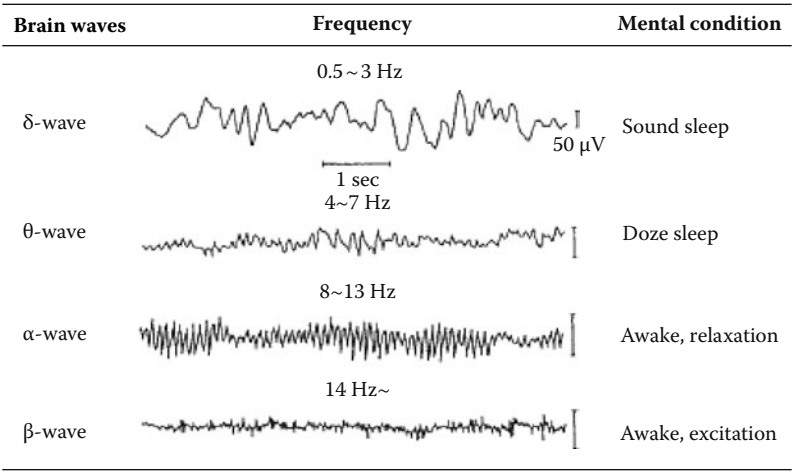


FIGURE 16.7 Classification and relation between length of brain waves and mental condition.

a week at a fixed time of day, and brain waves were measured and analyzed for 60 min after intake.

Results show that the most remarkable α-brain waves were observed from the back to the top area of the brain surface approximately 40 min after intake of theanine solution, indicating the subjects were in a relaxed state of mind. An oral dose of 200 mg of theanine dissolved in 100 ml of water resulted in the generation of α-brain waves in the occipital and parietal regions of the brains of the volunteers, while only a small amount of α-waves were observed with water intake. Accumulated intensity of α-brain waves showed clear tendency of dose-dependent generation of α-waves after 30 min during 1 h measurement, in that the 200 mg theanine solution produced more α-waves than the 50 mg solution, especially in the high-anxiety group. Moreover, the levels of θ-waves in both groups remained unchanged during the observation period for all the test solutions, indicating that theanine did not induce drowsiness. It is well known that α-brain waves are generated during the relaxed state, and it is therefore recognized that generation of α-waves is an index of relaxation. Theanine can promote the generation of α-brain waves and induce a relaxed state in humans. It should be noted that the study population in this research consisted only of females 18–22 years of age, and this effect has not yet been confirmed in other human populations.

16.4.4 Improvement of Learning Ability

It has been shown that administration of theanine has a significant effect on the release or reduction of neurotransmitters like dopamine and serotonin. It is also known that these neurotransmitters are closely related to memory and learning ability. Theanine does not change activity level or exploration behavior in normal animals. However, it does improve performance on a number of tests of memory and learning, including the active avoidance test and passive avoidance test.[51]

Theanine (180 mg/day) was administered to weanling male Wistar rats for four months to investigate its effect on memory and learning ability. The operant test was applied to test for memory and learning ability.[51] Two avoidance tests (passive avoidance and active avoidance tests) were applied to investigate memory.[51] The operant test was performed under the condition that feed comes out when a lever is pushed and a light turns on. The frequency of correct responses was greater in the theanine group than in the control group, especially with advancing sessions. Figure 16.8 shows the results of the avoidance tests. The same experiment was repeated at intervals of several hours to confirm memory. There is a general tendency that rats move to a dark place from a light place. In the passive avoidance test, an electric shock is applied soon after a rat moves from the light room to the dark. Animals that were administered theanine hesitated to move to the dark room and showed a tendency to remain in the light room longer than the control group (figure 16.8a). In the active avoidance test, the escaping behavior of rats from electric shock was examined. The avoidance reaction rate in the theanine group was higher and increased in proportion to the number of tests (figure 16.8b). Relatively good reaction frequency in a series of memory and learning ability tests was observed in the group administered theanine solution (1 g/100 ml water) for a long period (5 months). Those results showed the positive effects of theanine on memory and learning ability.

16.4.5 Regulation of Blood Pressure

Theanine is a derivative of glutamic acid, which is one of the major neurotransmitters in the brain. It is also known that the regulation of blood pressure involves catecholaminergic and serotonergic neurons in both the brain and peripheral nervous system.[53,54] Administration of theanine caused a significant increase in dopamine (DA) concentrations in the brain, especially in the striatum, hypothalamus, and hippocampus. Direct administration of theanine into brain striatum using the microdialysis technique caused a significant increase of DA release in a dose-dependent manner.[50] Oral intake of a high dose of theanine to spontaneously hypertensive rats (SHR) significantly decreased the blood pressure.[37]

16.4.6 Neuroprotection

Theanine is an analog of glutamic acid,[55] which is an excitatory neurotransmitter in the central nervous system and an integral component in the synthesis of GABA (an inhibitory neurotransmitter).[56] Because theanine is structurally similar to glutamate (a form of glutamic acid most abundant in bodily fluids), it can protect the brain from toxic levels of this neurochemical by serving as a competitive antagonist on glutamate receptors, thereby shielding brain cells from glutamate-induced toxicity.[55,57,58] Although glutamate is essential to brain chemistry, the high content of glutamate can destroy brain cells or cause degenerative brain disease. By binding to glutamate receptors, theanine helps guard against neuronal death and damage,[57,58] as well as vascular dementia.[58] These observations are based upon *in vitro* and animal tests.

The protective effect of theanine on ischemic delayed neuronal death in field CA1 of the gerbil hippocampus was examined (figure 16.9).[57] One microliter of theanine from each of the three concentrations (50, 125, and 500 mM) was admin-

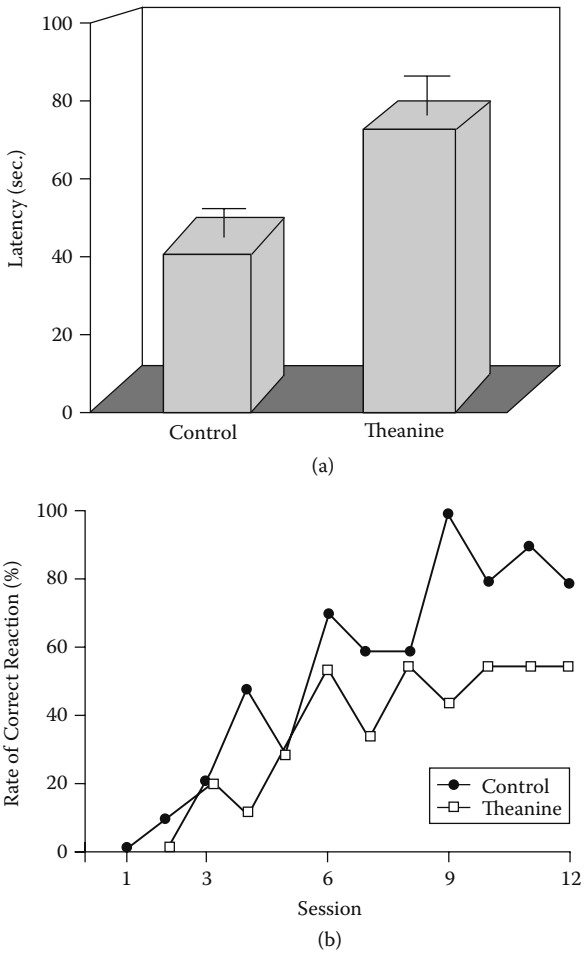


FIGURE 16.8 (a) Average time of latency (remaining time in a light room) in rats in passive avoidance test. An electric current is passed when a rat moves from a light room to a dark room. After 24 h, the same performance is repeated. (b) Learning performance ability of rats in active avoidance test. An electric current is passed 10 s after a rat moves from a light room to the dark room to make a rat learn the danger of the dark room.

istered through the lateral ventricle 30 min before ischemia. Transient forebrain ischemia was induced by bilateral occlusion of the common carotid arteries for 3 min under careful control of brain temperature at approximately 37°C. Seven days after ischemia, the number of intact CA1 neurons in the hippocampus was assessed. Ischemia-induced neuronal death in the hippocampal CA1 region was significantly prevented in a dose-dependent manner in the theanine-pretreated groups. These findings indicate that theanine might be useful clinically for preventing ischemic neuronal damage.

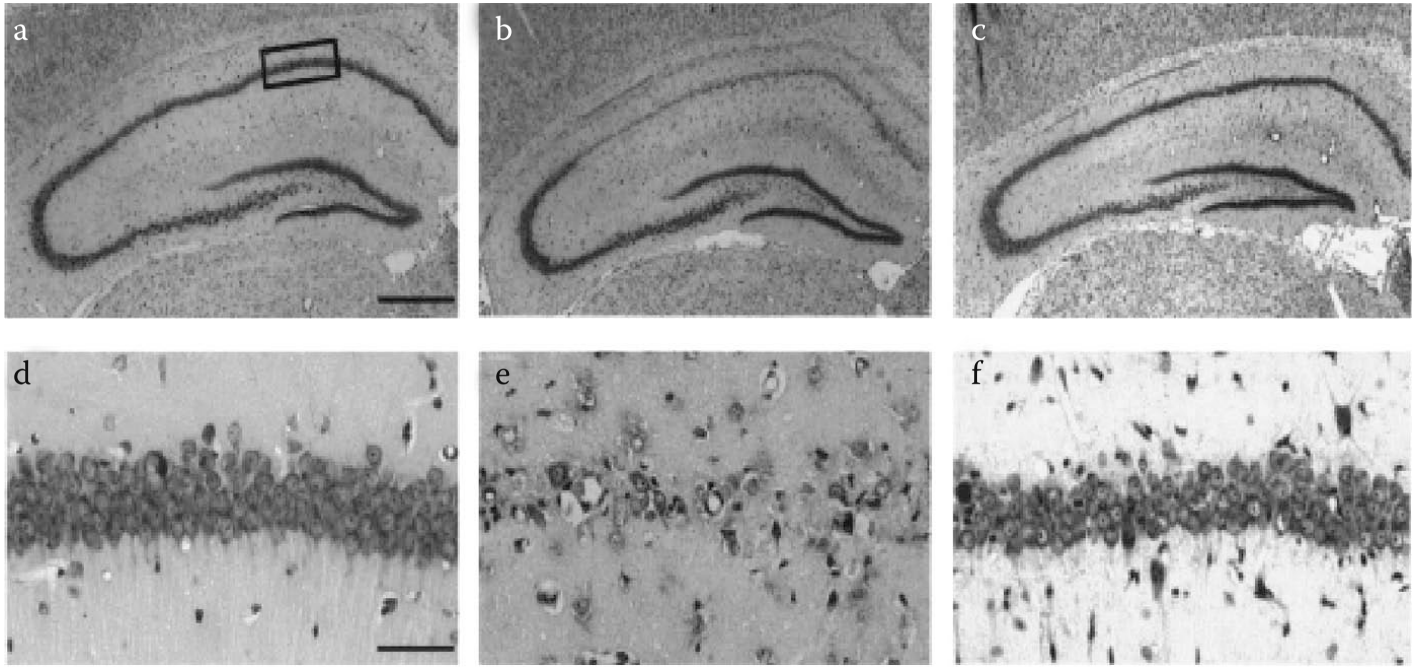


FIGURE 16.9 Photographs showing neuroprotective effect of theanine against ischemic delayed neuronal death in the gerbil hippocampus. Brain sections were obtained 7 days after 3 min transient ischemia. (a, d) Animals were administered theanine solution 30 min before sham operation (sham-operated group). (b, e) Animals were administered saline solution 30 min before ischemia (control group). (c, f) Animals were administered 1 ml of 500 mM theanine 30 min before ischemia (500 mM theanine-treated group). Lower photographs show the higher magnification of the field CA1 in each experimental group. Number of intact cells in the defined area was assessed (rectangle in (a)). Bars: 500 μm (a), 50 μm (d). Note that ischemic neuronal destruction in field CA1 was preserved by theanine administration.

16.4.7 Theanine Reduces the Adverse Reactions of Doxorubicin by Changing the Glutathione Level

16.4.7.1 Tumor Development Inhibitor

Theanine has been found to reduce tumor growth and proliferation of the AH109A hepatoma (liver cancer) cell line in animals.[14,59] It suppresses angiogenesis by intercepting the supply of nutrients and oxygen to tumors,[14] and inhibits the invasion and spread of cancer cells.[59]

16.4.7.2 Chemotherapeutic Drug Enhancement

Theanine may work by inhibiting the glutamate transporter, which increases the concentration of the drug in the tumor. It has been suggested that drinking green tea may improve the quality of life in cancer patients both by improving the outcome of therapy and through the positive psychological effects. In animal tests, theanine improves the antitumor action of cancer-fighting drugs like doxorubicin,[60–62] adriamycin,[45,63] idarubicin,[64] and pirarubicin.[65] When combined with these drugs, theanine increases their concentrations in cancer cells but inhibits their outflow from tumor cells to normal cells.[15] This reduces adverse side effects from chemotherapy and enhances its antitumor activity, especially in cases of drug-sensitive, drug-resistant, and metastatic tumors.[61,62,64]

In the case of tumor cell (figure 16.10), theanine decreases glutamate uptake through glutamate transporters (GLAST, GLT-1) as the first step, thereby decreasing intracellular glutathione (GSH) synthesis and GS-DOX conjugation, and subsequently suppresses the extracellular transport of GS-DOX by the multidrug resistance-associated protein (MRP)/glutathione S-conjugate export pump (GS-X pump).

In case of normal cells (figure 16.11), on the contrary, theanine is converted to glutamate, increasing the intracellular glutamate concentration, and leading to increased intracellular GSH, and then results in increased DOX efflux from normal cells. Therefore, theanine inhibits DOX-induced toxicity.

The glutamate transporter-mediated increase of antitumor activity caused by theanine is a novel mechanism, and it is hoped that this action will lead to the discovery of a useful cancer chemotherapy for drug-sensitive, drug-resistant, and metastatic tumors. It was expected that this study will greatly contribute to clinical cancer chemotherapy in the future.[15]

Glutathione (GSH) is a tripeptide composed of glutamate, cysteine, and glycine. The sulfhydryl side chains of the cysteine residues of two glutathione molecules form a disulfide bond (GSSG) during the course of being oxidized in reactions with various oxides and peroxides in cells.

It should be noted that L-BSO (buthionine sulfoximine) is a strong inhibitor against γ-glutamylcysteine synthetase,[66] the enzyme that catalyzes the first reaction of glutathione (GSH) biosynthesis. L-BSO has been accepted as an anticancer medicine.[67]

In fact, the stereochemical structures of L-theanine are more similar to L-BSO than glutamine, since both of these two compounds have a hydrophobic group in their structures (figure 16.12). Thus, L-theanine probably has the inhibitory activity

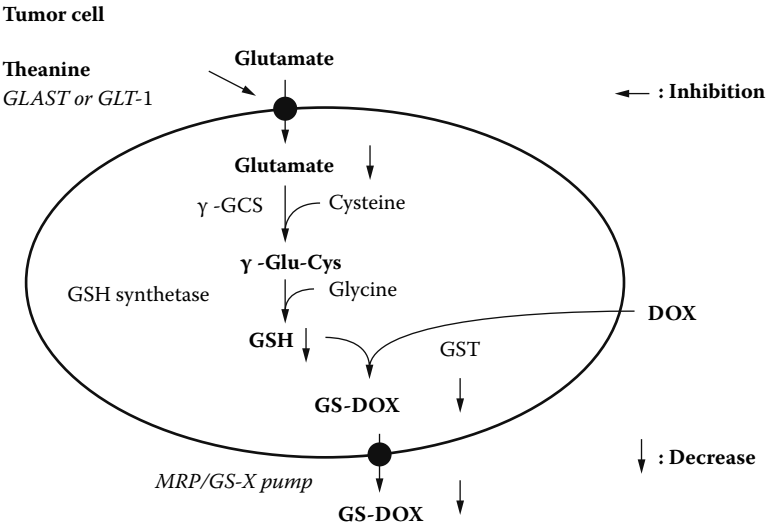


FIGURE 16.10 Clarified mechanism by which theanine inhibits DOX release from tumor cells.

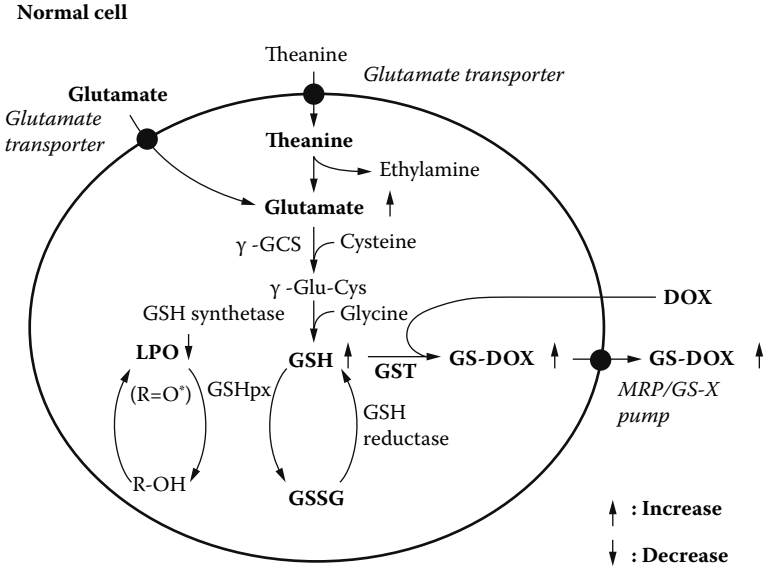


FIGURE 16.11 Proposed mechanism of theanine to protect normal tissue from DOX toxicity via the changing GSH level.

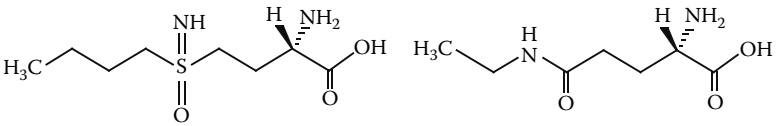


FIGURE 16.12 Chemical structure for L-buthionine sulfoximine (L-BSO) and L-theanine.

against γ-glutamylcysteine synthetase, and acts as a functional compound following the route of L-BSO.

16.5 ADDITIONAL BENEFITS OF THEANINE

16.5.1 Inhibition of the Stimulating Effects of Caffeine

By increasing dopamine and γ-aminobutyric acid, and influencing brain concentrations of serotonin, theanine can also help inhibit the stimulating effects of caffeine.[17,38,41]

Theanine creates a sense of relaxation approximately 30–40 min after ingestion via at least two different mechanisms. First, this amino acid directly stimulates the production of α-brain waves, creating a state of deep relaxation and mental alertness similar to that achieved through meditation. Second, theanine is involved in the formation of the inhibitory neurotransmitter, γ-aminobutyric acid, which influences the levels of two other neurotransmitters, dopamine and serotonin, producing the key relaxation effect.[52]

16.5.2 Promotion of Weight Loss

Theanine has been shown to work in conjunction with green tea catechins and caffeine to reduce body weight, fat accumulation, triacylglycerols, and free fatty acids in animals.[68] Theanine's antiobesity effects in reducing food intake[68] were most likely caused by the increase of dopamine release[50] and decrease of serotonin concentrations[19,37] in the brain. An animal test has suggested that these neurotransmitters may help regulate appetite and food intake.[69] A theanine and caffeine combination seemed to be responsible for suppressing body weight increase and fat accumulation. Caffeine also appears to work synergistically with green tea catechins in increasing thermogenesis and fat metabolism. Additionally, catechins and theanine were suggested to lower blood lipid levels.[68]

16.5.3 Enhancement of Immune System

The human immune system is complex and involves the digestive tract, the blood and lymph systems, hormones, and many other specialized cells that enable the human body to respond to exogenous substances that may come in contact with the body. This ranges from fighting off an *E. coli* infection to healing a small cut.

In most cases, the immune system is highly regulated internally and functions well. In some instances, the immune system attacks healthy cells, as in the case of autoimmune diseases, such as lupus, and some forms of diabetes and arthritis. In addition, some diseases and medications alter the body's immune system so that it is not as efficient. A healthy diet and lifestyle are known to help keep the immune system on track. Several nutrients, such as iron, zinc, and selenium, are essential for a healthy immune system, but emerging research suggests that compounds in tea may actually bolster the body's immune system too. Emerging data show that tea drinking, tea flavonoids, or theanine can positively affect the immune system to bolster the body's natural response.

Experiments show that 50% or more of the tea drinkers displayed strong gdT (gamma delta T) cell responses (increased γ-interferon production) within 2 weeks of tea consumption, which seemed to persist through the fourth week. No notable responses were seen among the coffee drinkers.[21]

Interestingly, the gdT cell function was stimulated by the addition of heat-killed bacteria or ethylamine and isobutylamine, two naturally occurring alkylamines found in wine, apples, and other plant products. Significantly, ethylamine occurs in tea and is found in the body after tea ingestion, most likely from the breakdown of theanine after it is absorbed.[47,70] Indeed, some *in vitro* work suggests that theanine does not itself elicit any gdT response, and only theanine that has been exposed to acid, thus yielding ethylamine, does.[70] Collectively, this work indicates that foods and beverages, especially tea brewed from *Camellia* species, may keep the gdT cells in a ready and primed state, and therefore more capable of mounting a swift and robust response to infectious invaders.[21]

16.6 CONCLUSIONS

Theanine, γ-glutamylethylamide, is a nonprotein amino acid, synthesized from glutamic acid and ethylamine in fresh tea leaves (*Camellia sinensis*). Theanine in tea is mostly in the L-form, which accounts for more than 50% of total free amino acids in tea. The content of theanine in tea is between 1.5 and 3% of the dry weight, which contributes to the gracefully sweet taste of tea.

Natural L-theanine is synthesized from glutamic acid and ethylamine in the root of the tea plant and transferred to young leaves. Theanine is absorbed by a common Na^+-coupled cotransporter in the intestinal brush-border membrane and incorporated into the brain via the leucine-preferring transport system of the blood–brain barrier. L-Theanine does not appear to accumulate; it is metabolized in the blood, liver, and brain, and then completely eliminated in the urine within 24 h.

It has been shown that theanine has numerous health effects, ranging from prevention of neuronal death to reduction of tumor growth, enhancement of antitumor activity of chemotherapeutic agents, and reduction of hypertension and blood pressure. Moreover, theanine relieves excitation induced by caffeine ingestion and influences the brain levels of norepinephrine, serotonin, 5-hydroxyindoleacetic acid, and dopamine. In addition, ethylamine, a major metabolite of theanine, has been found to boost immune response.

Theanine has been considered a therapeutic/medicinal agent and additive in consumer products for its cardiovascular, neurological, and oncological effects. The isolation of theanine from tea or other sources is complicated, and the cost of isolation is very high. Many attempts have been made to produce theanine by biochemical and chemical routes. Both biochemical and chemical routes for the synthesis of theanine have been established, and the theanine products are now readily available in the market but are expensive.

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17 Flavor Stability of Tea Drinks

Hideki Masuda

CONTENTS

17.1 INTRODUCTION

The tea drink market has been increasing every year. In Japan, the sale of green tea drinks has consistently grown compared to that of oolong and black tea drinks (figure 17.1).[1] The sales growth of green tea drinks has been due not only to the familiarity with green tea from the old days, but also the increase in health-oriented consumers in recent years. The sale of polyethylene terephthalate (PET) bottled drinks is increasing compared to that of canned drinks (figure 17.2),[1] because of its lightness, easier carrying after opening the container, and sense of security that is able to protect the contents from the outside.

In order to introduce tea drinks to the market, quality control is the most important criterion and most difficult problem for all drink makers. Among the many factors related to tea drink quality, flavor stability is the most important. The following conditions are responsible for flavor stability of tea drinks: (1) flavor stability of tea leaves during storage, (2) flavor stability during processing of tea drink, and (3) flavor stability during displaying of the tea drink in the hot vending machine or under light irradiation.[2] Unlike homemade tea drinks, there are many manufacturing processes that bring about a change in the flavor of commercially available tea drinks. Therefore, the manufacturing process of tea drinks is described first. Next, the stability of green and black tea drinks will be discussed in detail in connection with their sensory evaluation.

17.2 MANUFACTURING PROCESS OF TEA DRINKS

The manufacturing processes of green, oolong, and black tea drinks are shown in tables 17.1 to 17.3, respectively.[3] From the standpoint of flavor stability, one of the

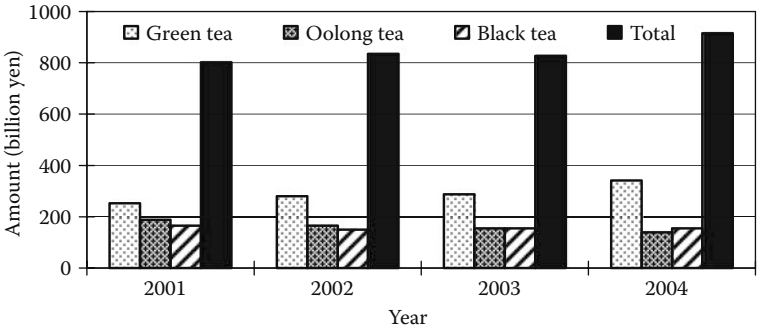


FIGURE 17.1 Sales of tea drinks in Japan.

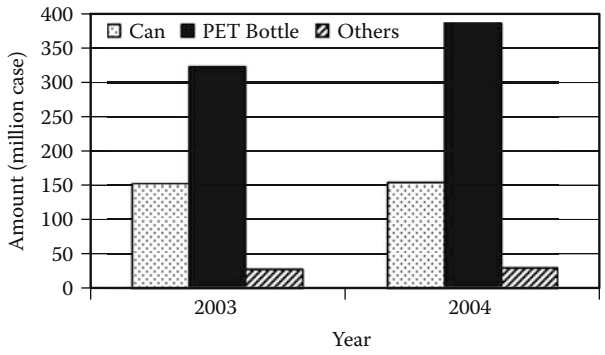


FIGURE 17.2 Sales of tea drinks in specific containers in Japan.

TABLE 17.1

Manufacturing process of green tea drinks

1	**Extraction**
	Extraction from Green Tea Leaves → Filtration → Cooling → Centrifugation → Extracts
2-1	**Mixing, Filling, Sterilization → Canned Drinks of Green Tea**
	Mixing (Extracts, Antioxidants, etc.) → Filtration → Heating → Filling of Can → Seal with Cap → Retorting Process → Cooling → Canned Drinks of Green Tea
2-2	**Mixing, Filling, Sterilization → PET Bottle Drinks of Green Tea**
	Mixing (Extracts, Antioxidants, etc.) → Filtration → Heating → UHT (Ultrahigh Temperature) Sterilization → Cooling → Pasteurization of PET Bottle → Filling of PET Bottle (Hot Filling or Aseptic Filling) → Seal with Cap → PET Bottle Drinks of Green Tea

TABLE 17.2
Manufacturing process of oolong tea drinks

1	**Extraction**
	Extraction from Oolong Tea Leaves → Filtration → Cooling → Centrifugation → Extracts
2-1	**Mixing, Filling, Sterilization → Canned Drinks of Oolong Tea**
	Mixing (Extracts, Antioxidants, etc.) → Filtration → Heating → Filling of Can → Seal with Cap → Retorting Process → Cooling → Canned Drinks of Oolong Tea
2-2	**Mixing, Filling, Sterilization → PET Bottle Drinks of Oolong Tea**
	Mixing (Extracts, Antioxidants, etc.) → Filtration → Heating → UHT (Ultrahigh Temperature) Sterilization → Cooling → Pasteurization of PET Bottle → Filling of PET Bottle (Hot Filling or Aseptic Filling) → Seal with Cap → PET Bottle Drinks of Oolong Tea

TABLE 17.3
Manufacturing process of black tea drinks

1	**Extraction**
	Extraction from Black Tea Leaves → Filtration → Cooling → Centrifugation → Extracts
2-1	**Mixing, Filling, Sterilization → Canned Drinks of Black Tea without Milk**
	Mixing (Extracts, Sugar, Flavor) → Filtration → Heating → Filling of Can → Seal with Cap → Retorting Process → Cooling → Canned Drinks of Black Tea without Milk
2-2	**Mixing, Filling, Sterilization → Canned Drinks of Black Tea with Milk**
	Mixing (Milk, Emulsifier) → Heating → Homogenization → Mixing (Extracts, Sugar, Flavor) → Filtration → Heating → Homogenization → Heating → Filling of Can → Seal with Cap → Retorting Process → Cooling → Canned Drinks of Black Tea with Milk
2-3	**Mixing, Filling, Sterilization → PET Bottle Drinks of Black Tea without Milk**
	Mixing (Extracts, Sugar, Flavor) → Filtration → Heating → UHT (Ultrahigh Temperature) Sterilization) → Cooling → Pasteurization of PET Bottle → Filling of PET Bottle (Hot Filling or Aseptic Filling) → Seal with Cap → PET Bottle Drinks of Black Tea without Milk
2-4	**Mixing, Filling, Sterilization → PET Bottle Drinks of Black Tea with Milk**
	Mixing (Milk, Emulsifier) → Heating → Homogenization → Mixing (Extracts, Sugar, Flavor) → Filtration → Heating → Homogenization → Heating → UHT Sterilization → Cooling → Pasteurization of PET Bottle → Filling of PET Bottle (Hot Filling or Aseptic Filling) → Seal with Cap → PET Bottle Drinks of Black Tea with Milk

important causes that produces a change in the flavor is considered to be the sterilization step. As for the canned drinks, the retorting process, which is used to sterilize the low-acidity drinks (pH > 4.6), such as tea drinks, requires heating at 120°C for more than 4 minutes. On the other hand, for PET-bottled drinks, at first, ultrahigh temperature (UHT) sterilization, at 135~140 °C for 30~60 seconds is used to sterilize the low-acidity drinks, followed by hot filling or aseptic filling of the pasteurized PET bottle. Needless to say, the flavor change of tea drinks is dependent not only on the type of manufacturing process, but also on the difference in the tea leaves

corresponding to their manufacturing processes. It is well known that the flavor characteristics of each tea are primarily dependent on the manufacturing process, as shown in table 17.4 for green, oolong, and black tea leaves. The characteristic flavor component of oolong and black tea leaves is due to the withering and fermentation process. However, as for green tea leaves, these processes are not necessary. Other than the flavor component, the remarkable difference in the nonvolatile component, that is, the flavor precursor, results from the difference in the manufacturing process.

It is well known that the aroma extract dilution analysis (AEDA) is a useful method for the recognition of the odor quality and odor intensity of each component.[4–6] Especially the AEDA is a useful method for the identification of trace amounts of the component that significantly affects the flavor of tea drinks. The odor intensity of the flavor component is expressed by the flavor dilution (FD) factor, that is, the ratio of the concentration of the flavor component in the initial extract to its concentration in the most dilute extract in which odor was detected by gas chromatography–olfactometry (GC-O). Therefore, hereafter, from the viewpoint of sensory evaluation, the change in the flavor of tea drink during heat processing by AEDA will be mainly discussed. Furthermore, in order to inhibit flavor deterioration of tea drink, the study of flavor precursor in a variety of foods, including tea drinks, will be proposed.

17.3 FLAVOR STABILITY OF GREEN TEA DRINKS

The effect of the retorting process and UHT sterilization on the flavor of green tea drink is shown in figure 17.3.[7] The intensity of off-flavor (potato-like, floral, sweet, heavy, spicy) increased during retorting and sterilization, and the characteristic green

TABLE 17.4

Manufacturing process of green, oolong, and black tea leaves

1	**Green Tea (Sen-cha) Leaves**
	Picking of Raw Leaves → Steam → Rolling (Coarsely → Finely)[1] → Drying[2] → Crude Green Tea Leaves → Refining (Heating)[3] → Green Tea (Sen-cha) Leaves
[1]	Water Content: 10–13%
[2]	Water Content: 5–6%
[3]	Water Content: less than 3%
2	**Oolong Tea Leaves**
	Picking of Raw Leaves → Withering[1] → Rolling → Drying → Oolong Tea Leaves
[1]	Water Content: 60%
3	**Black Tea Leaves**
	Picking of Raw Leaves → Withering[1] → Rolling → Fermentation → Drying[2] → Black Tea Leaves
[1]	Water Content: 60%
[2]	Water Content: 2–3%

odor decreased. As would be predicted, the retorting process brought about a greater flavor change than UHT sterilization due to the difference in each heat history. The off-flavor components present in green tea drinks are described in figures 17.3, 17.4, and 17.5. In order to ensure the flavor quality of tea drinks, tracings of the precursors of each off-flavor component shown in figure 17.4, considered to be important, are shown in figure 17.6. 2,3-Butanedione (diacetyl) is one of the characteristic components (threshold value: 10 ppb in water[8]) obtained from citrate via pyruvate in fermented dairy products.[9] On the other hand, under nonenzymatic conditions, the α-dicarbonyl components, such as 2,3-butanedione, glyoxal, or methylglyoxal, are reported to be formed from monosaccharides, such as D-glucose or D-fructose, or disaccharides, such as maltose or maltulose, during the heating process.[10,11] Addition of L-glycine under the above-mentinoned conditions enhances the yield of the α-dicarbonyl components. Methional (threshold value: 0.2 ppb in water[8]) and phenylacetaldehyde (threshold value: 4 ppb in water[8]) are reported to be formed from L-methionine and L-phenylalanine, respectively.[12,13] The oxidized tea flavanols, that is, the *ortho*-quinones, formed from tea flavanols by the action of catechol oxidase during tea fermentation produced aldehydes and other degradation products from α-amino acids by Strecker degradation. It is interesting that the yield of aldehydes is reported to depend on catechins present.[13] As for black tea leaves, fermentation is a necessary step for its manufacturing (table 17.4). However, on the other hand, the manufacturing process of green tea leaves does not contain the right fermentation reaction producing the *ortho*-quinones from the corresponding catechols. Therefore, in green tea drinks, the formation of aldehydes from α-amino acids is supposed to be formed via another pathway during elevated temperature.[14] The precursors of linalool and geraniol were isolated as their corresponding glycosides from tea leaves. As for linalool (threshold value: 6 ppb in water[8]), linalyl 6-*O*-β-D-xylopyranosyl-β-D-glucopyranoside (β-primeveroside) was isolated from oolong tea leaves.[15] On the other hand, for geraniol (threshold value: 7.5 ppb in water[8]), geranyl 6-*O*-β-D-xylopyranosyl-β-D-glucopyranoside (β-primeveroside) and geranyl 6-*O*-α-L-arabinopyranosyl-β-D-glucopyranoside (β-vicianoside) were isolated as precursors from oolong and green tea leaves, respectively.[16,17] Interestingly, these glycosides were identified for the first time as the alcohol precursors.

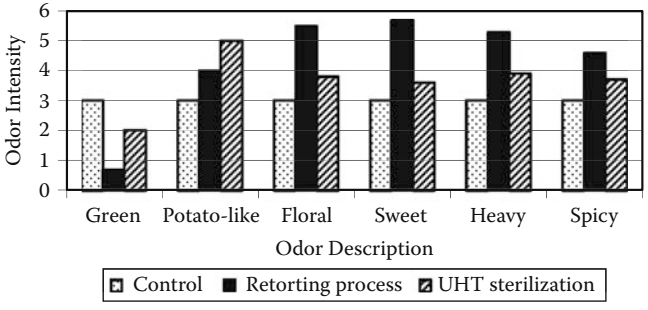


FIGURE 17.3 Odor intensity and odor description of green tea drinks before and after heating process. Retorting process: 121°C, 10 minutes. UHT sterilization: 134°C, 30 seconds. Odor intensity of nonheated green tea drink (control): 3. pH: 6.2 (control), 5.6 (after retorting process), 6.1 (after UHT sterilization).

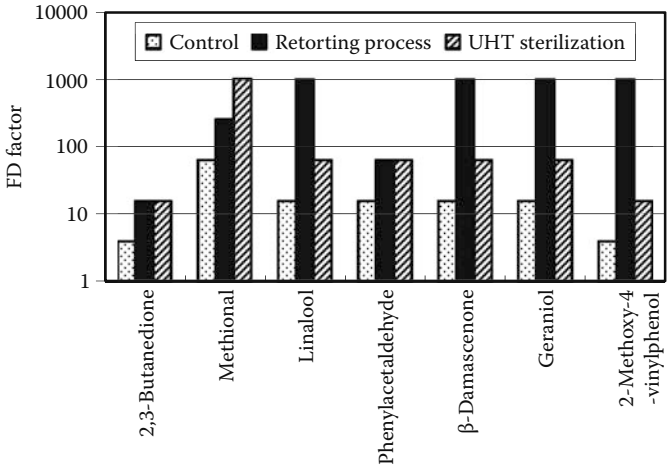


FIGURE 17.4 FD (flavor dilution) factors of flavor components of green tea drinks due to the heating process.

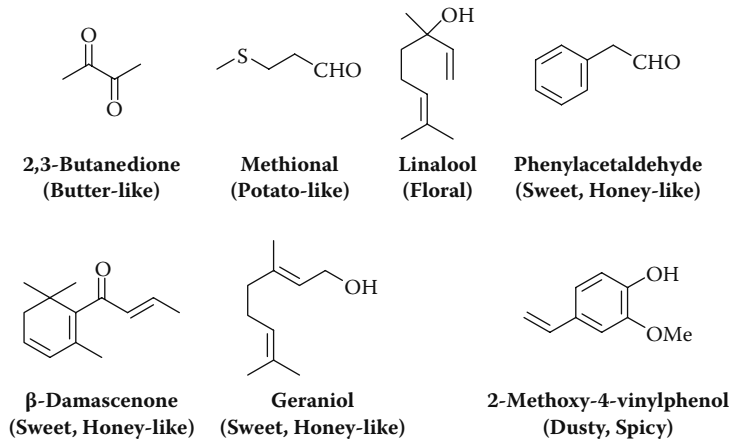


FIGURE 17.5 Structures of components shown in figure 17.4. Odor description is stated in parentheses.

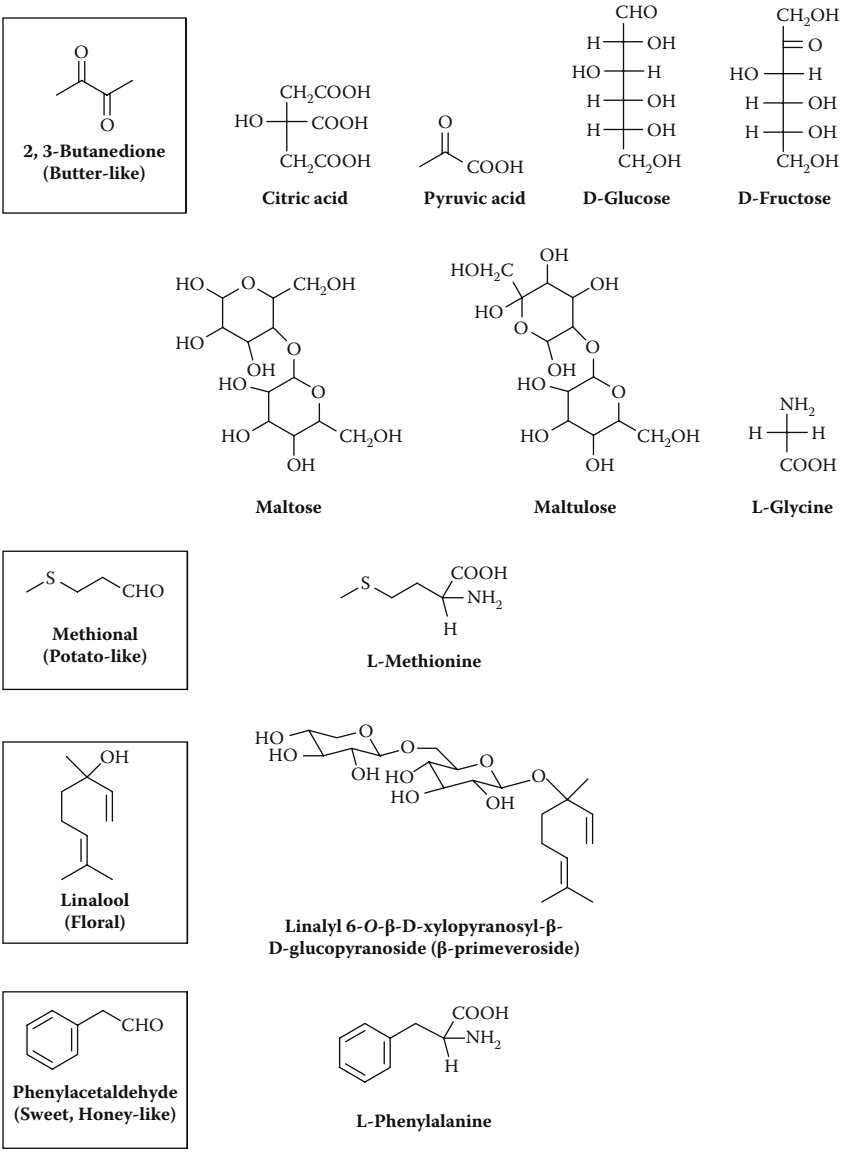


FIGURE 17.6A Proposed precursors of off-flavor components shown in figures 17.4 and 17.5.

The amount of glycosides is reported to be highest in young leaves, but decreases as the leaves age.[18] Under acidic conditions, these β-D-glucopyranosides were hydrolyzed to form many terpene derivatives other than geraniol.[19] Therefore, during the heating process of tea manufacturing, a variety of acid-catalyzed hydrolysates are formed as off-flavor components. As for β-damascenone (threshold value: 0.002 ppb in water[8]),

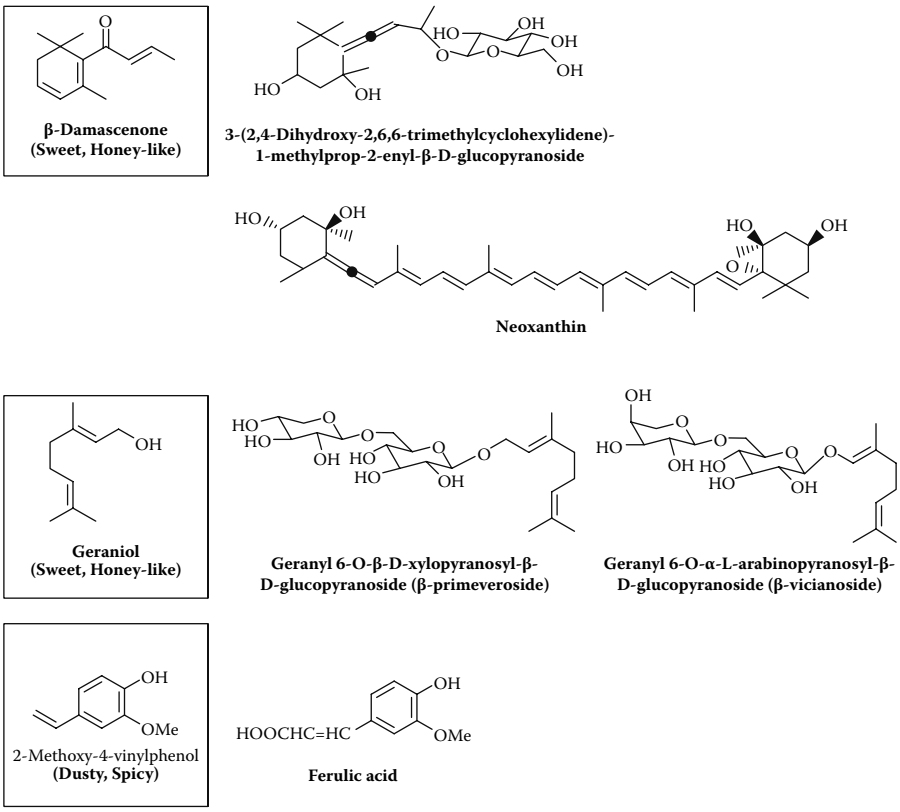


FIGURE 17.6B

3-(2,4-dihydroxy-2,6,6-trimethylcyclohexylidene)-1-methylprop-2-enyl-β-D-glucopyranoside, that is, the allenic triol glucoside, is reported to be its precursor. In addition, neoxanthin is thought to be the above-mentioned allenic triol glucoside.[20–23] Therefore, during the heating process under acidic conditions, β-damascenone is assumed to be formed from neoxanthin via 3-(2,4-dihydroxy-2,6,6-trimethylcyclohexylidene)-1-methylprop-2-enyl-β-D-glucopyranoside. 2-Methoxy-4-vinylphenol (threshold value: 5 ppb in water[8]) is reported to be formed from ferulic acid by thermal fragmentation in cooked corn and cooked asparagus.[24,25] This thermal fragmentation is considered to be popular during the heating process.

As for the potent odorants in green tea (sen-cha) drinks, the eleven components shown in figure 17.7 are found to be important contributors to its characteristics.[26] Using the AEDA method, the characteristic sulfur components, such as 4-methoxy-2-methyl-2-butanethiol, 4-mercapto-4-methyl-2-pentanone, and methional, were identified from the green tea for the first time. Among these eleven components, β-damascenone and methional were reported to increase after the heating process (figure 17.4), then theorized to have a remarkable effect on the tea drink quality.

Figure 17.8 shows the change in the flavor components of green tea drinks induced by the retorting process.[27] These data are obtained by the calculation of the GC area

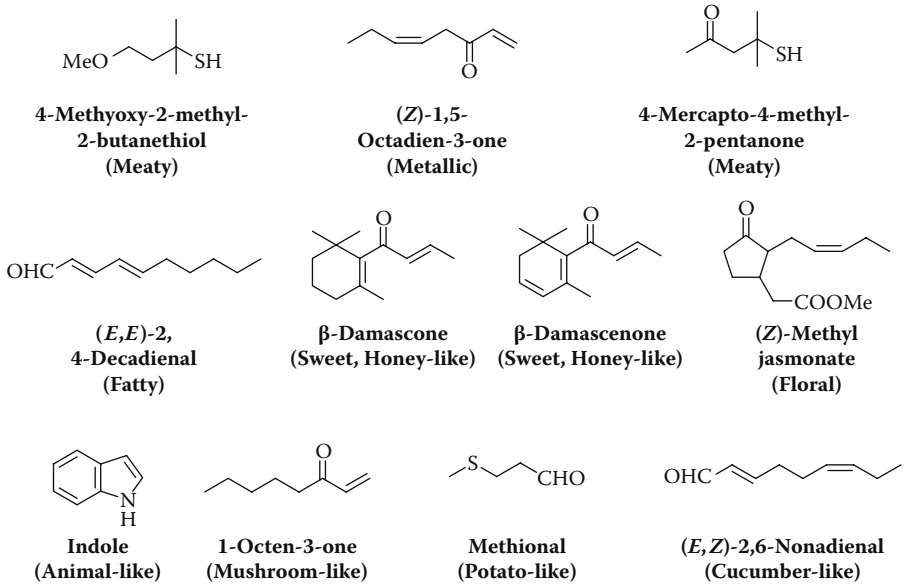


FIGURE 17.7 Potent flavor components of green tea drink identified by AEDA. Nonretorting process. FD factor of the flavor components: greater than 500.

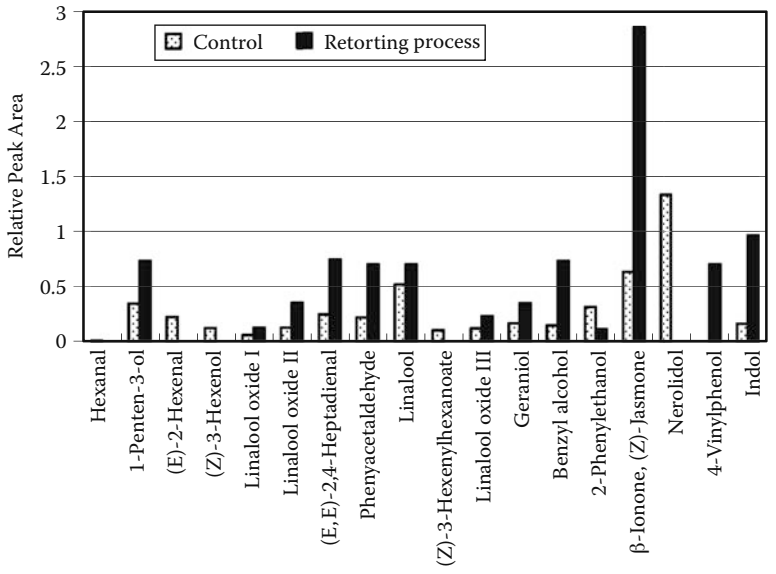


FIGURE 17.8 Relative peak area of flavor components in green tea drinks before and after retorting process. Retorting process: 120°C, 8 minutes. pH: 6.0 (control).

using an internal standard. Therefore, the effect of the level of these components on flavor quality of green tea drinks remains unclear. However, in order to study the precursor, it is important to compare green tea drinks before and after the retorting process. After the retorting process, the components that their levels increased and their proposed precursors are shown in figures 17.9 and 17.10, respectively. α-Linolenic acid is well known to produce many degradation products, including 1-penten-3-ol and (*E*,*E*)-2,4-heptadienal by auto-oxidation.[28] Furthermore, α-linolenic acid is also known to serve as a precursor for the formation of (*Z*)-3-hexenol, a leaf alcohol, and (*E*)-2-hexenal, a leaf aldehyde, during the biosynthetic pathway (figure 17.11).[29] These two compounds render a green odor in green tea leaves. As for 1-penten-3-ol, eicosapentaenoic acid, which is mainly present in marine animals and plants, is also reported as its precursor.[30,31] For the four species of the linalool oxides and benzyl alcohol, the corresponding glycosides are found from green tea leaves. Meanwhile, *trans*-linalool 3,6-oxide 6-*O*-β-D-xylopyranosyl-β-D-glucopyranoside (β-primeveroside), *cis*-linalool 3,6-oxide 6-*O*-β-D-xylopyranosyl-β-D-glucopyranoside (β-primeveroside), *trans*-linalool 3,7-oxide 6-*O*-β-D-apiofuranosyl-β-D-glucopyranoside, and *cis*-linalool 3,7-oxide 6-*O*-β-D-apiofuranosyl-β-D-glucopyranoside have been

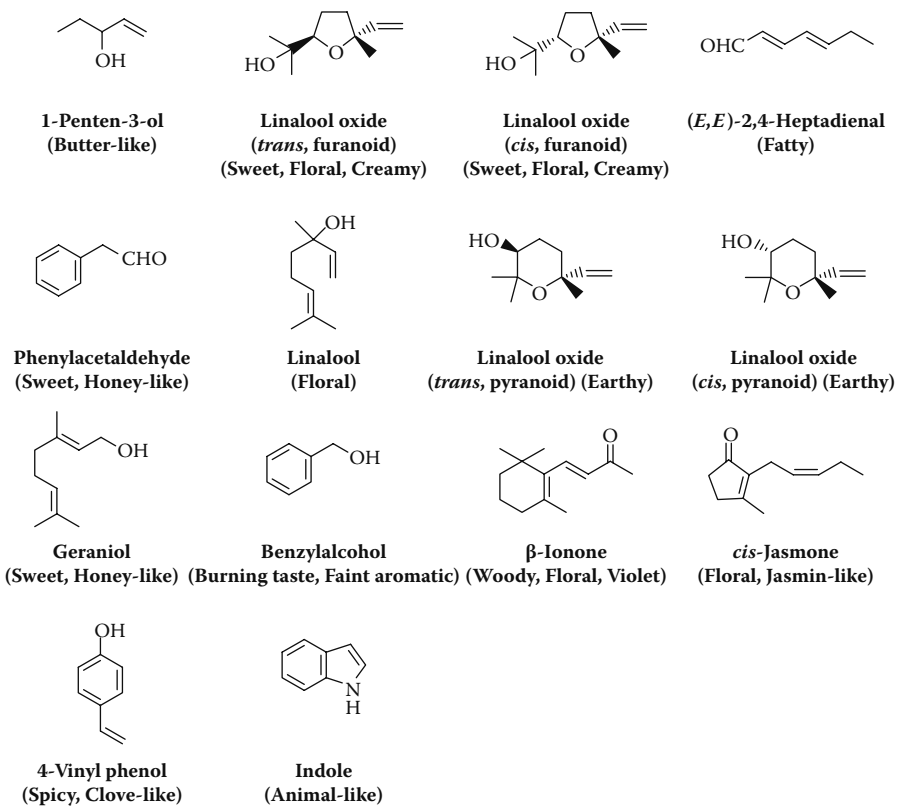


FIGURE 17.9 Structures of increased flavor components of green tea drink after the heating process among flavor components shown in figure 17.8.

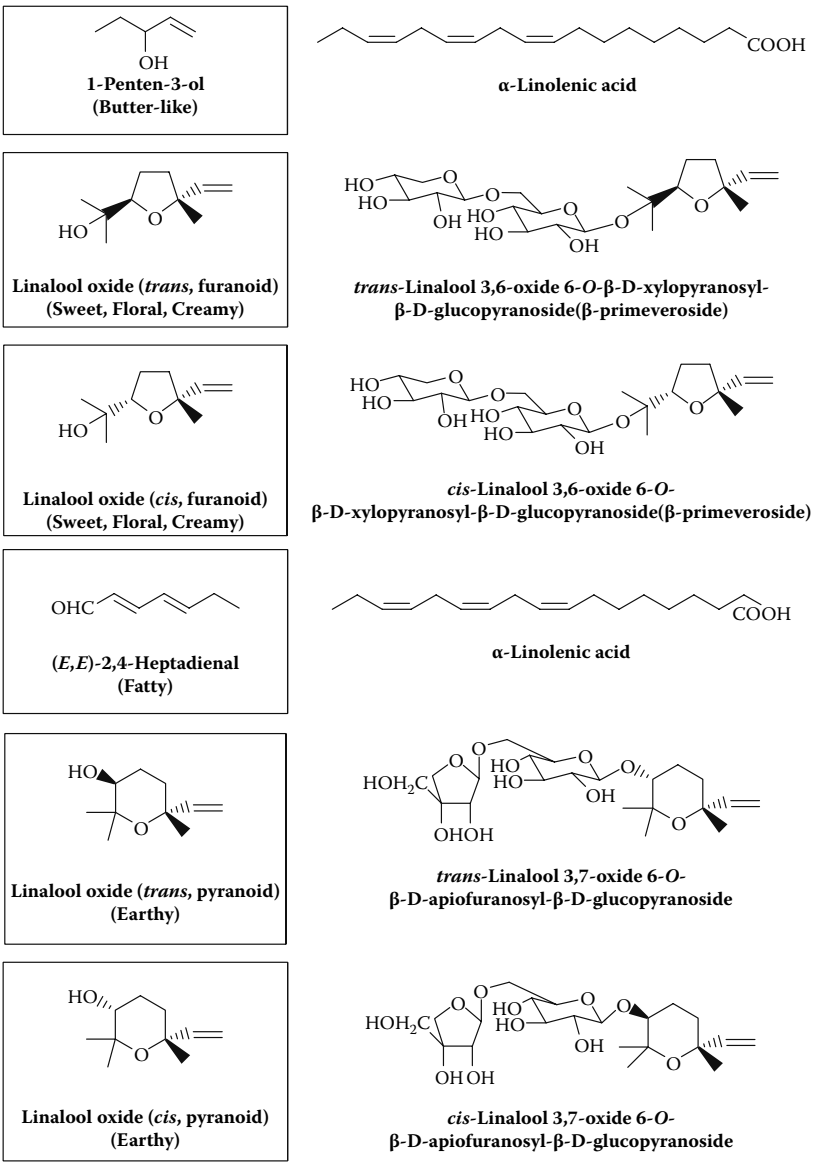


FIGURE 17.10A Proposed precursors of flavor components shown in figure 17.9. (Precursors of phenylacetaldehyde, linalool, and geraniol are shown in figure 17.6.)

isolated from oolong tea leaves.[32,33] Benzyl 6-*O*-β-D-xylopyranosyl-β-D-glucopyranoside (β-primeveroside) and benzyl β-D-glucopyranoside are isolated from oolong tea leaves and green tea leaves, respectively.[15,34] Other than above-mentioned glucosides, (*Z*)-3-hexenyl β-D-glucopyranoside has been isolated as the precursor of (*Z*)-3-hexenol from oolong and green tea leaves (figure 17.11).[33,35] The oxidized tea flavanols formed during tea fermentation are known as the oxidizing agents

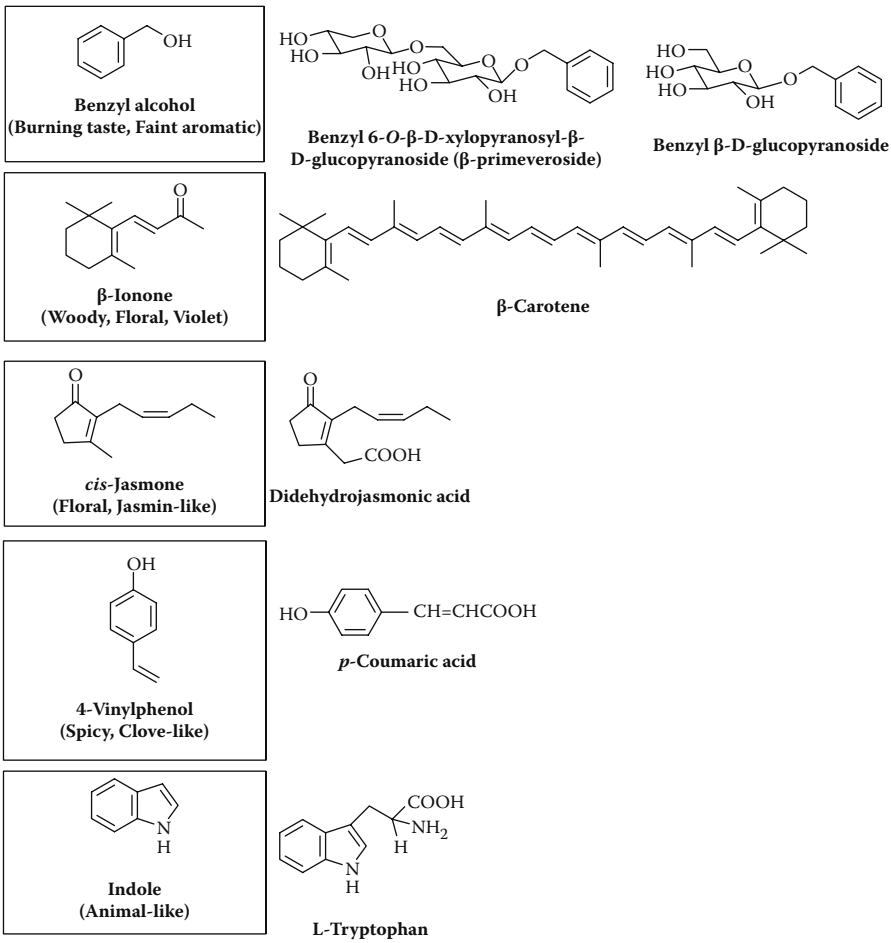


FIGURE 17.10B

responsible for oxidative degradation of β-carotene.[13] On the other hand, as for green tea which is nonfermented, β-ionone (threshold value: 0.007 ppb in water[8]) is produced from β-carotene by thermal degradation.[36] *cis*-Jasmone is reported to be formed from α-linolenic acid via didehydrojasmonic acid through a biosynthesis pathway.[37–39] The precursor of 4-vinylphenol (threshold value: 10 ppb in water[8]) is reported to be *p*-coumaric acid.[24,25] In addition to 2-methoxy-4-vinylphenol, 4-vinylphenol is estimated to be formed by thermal fragmentation during the heating process. Indole may be directly produced by the action of L-tryptophan and lyase in cheese.[40] However, upon heating, the activity of lyase is deactivated. Therefore, indole is formed from L-tryptophan Amadori compound under pyrolysis conditions when compared to the enzymatic reaction.[14]

The refining, that is, the heating process, is the last step in processing of green tea for producing the good-tasting and stable flavor of green tea leaves (table 17.4). Especially the refining step is very important for producing a roasted and sweet

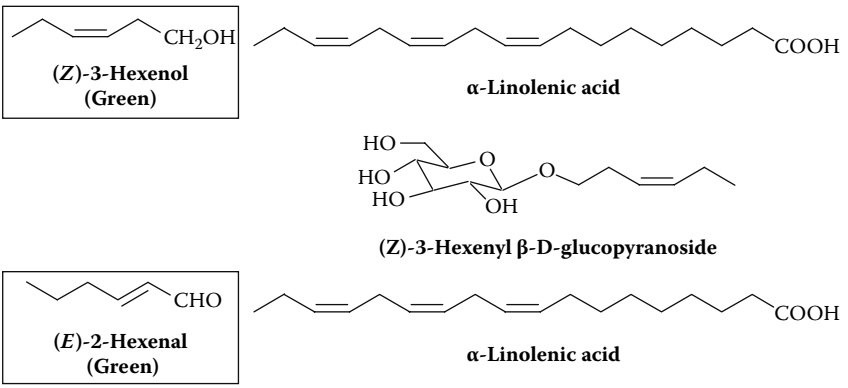


FIGURE 17.11 Proposed precursors of (Z)-3-hexenol and (E)-2-hexenal.

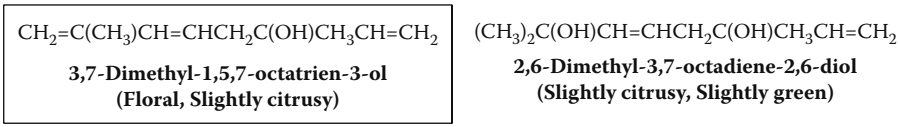


FIGURE 17.12 Structures of 3,7-dimethyl-1,5,7-octatriene-3-ol and 2,6-dimethyl-3,7-octadiene-2,6-diol.

flavor. 3,7-Dimethyl-1,5,7-octatriene-3-ol is reported to be effective in providing a roasted and sweet flavor in the high-grade oolong tea and black tea (Darjeeling tea),[41] and also in green tea, is formed from 2,6-dimethyl-3,7-octadiene-2,6-diol by dehydration during the refining (figure 17.12).[42] Figures 17.13 and 17.14 show the FD factors of ara-cha and sen-cha.[43] Sen-cha (refined green tea, usually called green tea) leaves are produced by the heating process, that is, the refining of ara-cha (crude green tea) (table 17.4). As already described, the difference in the ara-cha drink and sen-cha drink is important, because it enhances that content of the flavor components during heat processing, thus providing a more stable green tea drink. 2-Acetyl-1-pyrroline (threshold value: 0.1 ppb in water) and 2-acetyl-2-thiazoline (threshold value: 1.3 ppb in water) are characteristic impact components because of their lower odor threshold values compared to 2-acetylpyrrole (threshold value: 170 ppm in water) and 2-acetylthiazole (threshold value: 10 ppb in water), respectively (figures 17.14 and 17.15).[44] 1-Pyrroline and 2-oxopropanal, which are formed from L-proline and 1-deoxyglucosone during heating, are reported to be the important intermediates for generation of 2-acetyl-1-pyrroline (figure 17.16).[45] For 2-acetyl-1-thiazoline, 1-amino-2-mercaptoethane, also known as cystenamine, and 2-oxopropanal are considered to be its precursors (figure 17.16).[46] 2-Acetyl-1-thiazoline is reported[46] to be formed via 2-(1-hydroxyethyl)-4,5-dihydrothiazole as the key intermediate during the heating process. In general, the threshold values of tertiary thiols are extremely low (4-mercapto-4-methyl-2-pentanone, threshold value: 0.0001 ppb in water; 4-metoxy-2-methyl-2-butanethiol, threshold value: 0.00002

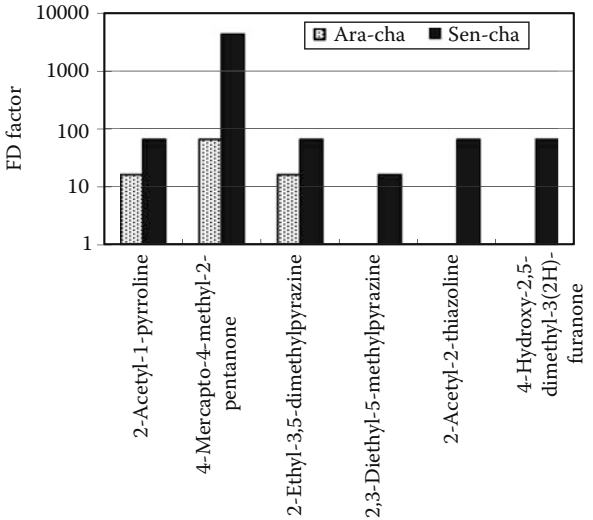
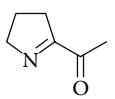
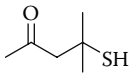


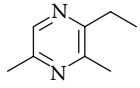
FIGURE 17.13 FD factors of flavor components of green tea drinks (ara-cha: crude green tea; sen-cha: refined green tea, usually called green tea). Refining (heating) conditions from ara-cha to sen-cha: 110°C, 15 minutes. Nonretorting process.



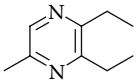
2-Acetyl-1-pyrroline (Popcorn-like)



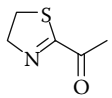
4-Mercapto-4-methyl-2-pentanone (Meaty)



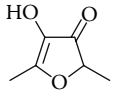
2-Ethyl-3,5-dimethylpyrazine (Nutty)



2,3-Diethyl-5-methylpyrazine (Nutty)



2-Acetyl-1-thiazoline (Popcorn-like)

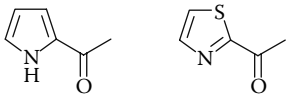


4-Hydroxy-2,5-dimethyl-3(2*H*)-furanone (Caramel-like)

FIGURE 17.14 Flavor components shown in figure 17.13.

ppb in water; 1-*p*-menthen-8-thiol, threshold value: 0.00002 ppb in water).[8] Therefore, a slight change in the amount of these hetero compounds can possibly produce a huge change in the flavor quality of green tea drinks. 4-Mercapto-4-methyl-2-pentanone is reported to be formed from *S*-4-(4-methylpentan-2-one)-L-cysteine by cysteine conjugate β-lyase in wine (figure 17.16).[47–49] It is interesting that β-lyase was reported to work at 75°C.[49] 2-Ethyl-3,5-dimethylpyrazine (threshold value: 0.04 ppb in water[8]), 2,3-diethyl-5-methylpyrazine (threshold value: 0.09 ppb in water[8]), and 4-hydroxy-2,5-dimethyl-3(2*H*)-furanone, that is, Furaneol™ (threshold value: 60 ppb in water[8]), are well known as Maillard reaction products.[50,51]

L-Rhamnose and L-proline are reported to be among important precursors that form 4-hydroxy-2,5-dimethyl-3(2*H*)-furanone (figure 17.16). In the study of strawberry flavor, its biosynthesis from 2,5-dimethyl-3(2*H*)-furanone 4-*O*-β-D-glucopyranoside is worthy of note as a storage in strawberries.[52]



2-Acetylpyrrole (Popcorn-like) **2-Acetylthiazole (Popcorn-like)**

FIGURE 17.15 Analogues of 2-acetyl-1-pyrroline and 2-acetyl-1-thiazoline.

For the drink manufacturer, it is most important to provide a stable drink after its production. Green tea leaves that have a more stable flavor quality under the required heating conditions have been examined in recent years. Kamairi-cha is one type of green tea, but unlike the manufacturing of sen-cha leaves, kamairi-cha leaves are produced by the rolling of raw leaves directly on a hot pan without the steaming process, as shown in table 17.4. Therefore, the difference of the heat history between sen-cha and kamairi-cha is postulated to cause the different FD factors for each flavor component (figures 17.17 and 17.18).[53] (*Z*)-1,5-Octadien-3-one is reported to be

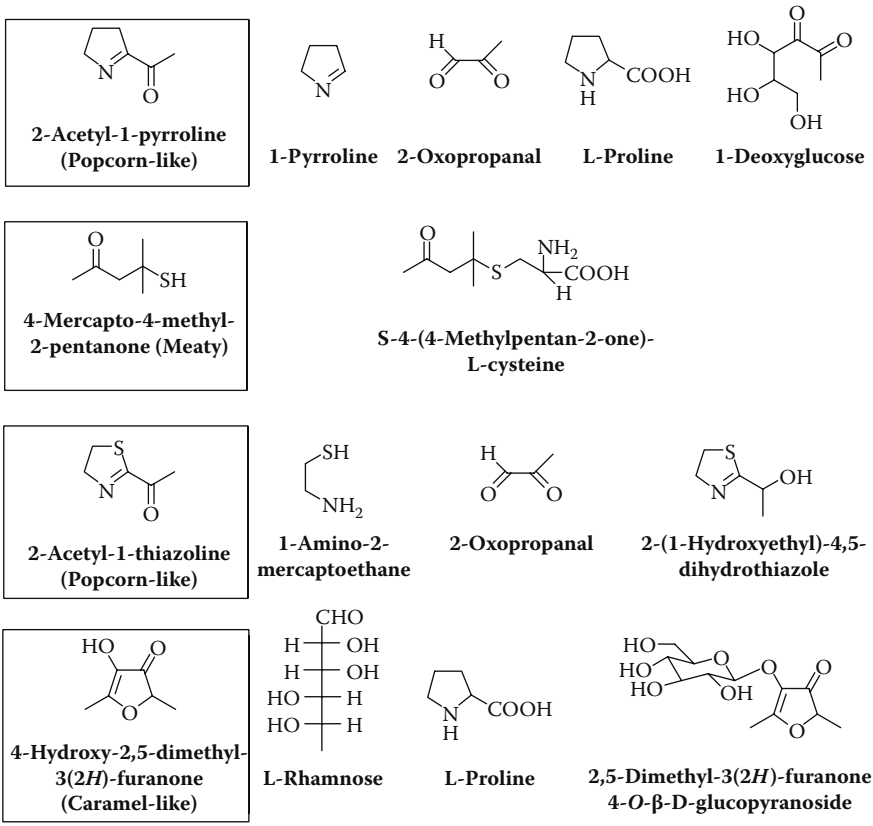


FIGURE 17.16 Proposed precursor flavor components of 2-acetyl-1-pyrroline, 4-mercapto-4-methyl-2-pentanone, 2-acetyl-1-thiazoline, and 4-hydroxy-2,5-dimethyl-3(2H)-furanone.

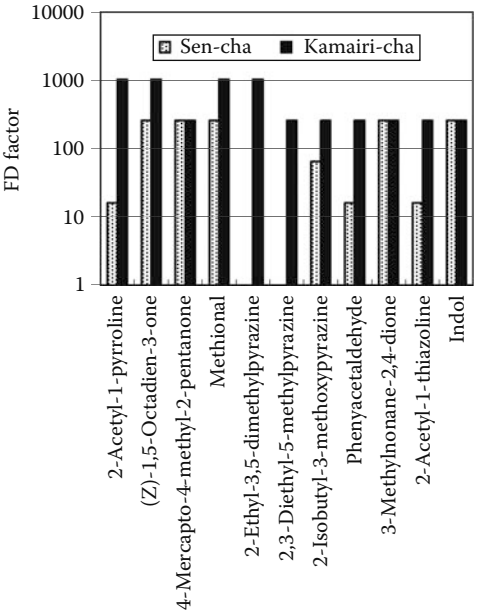


FIGURE 17.17 FD factors of flavor components of sen-cha and kamairi-cha drinks. Non-retorting process.

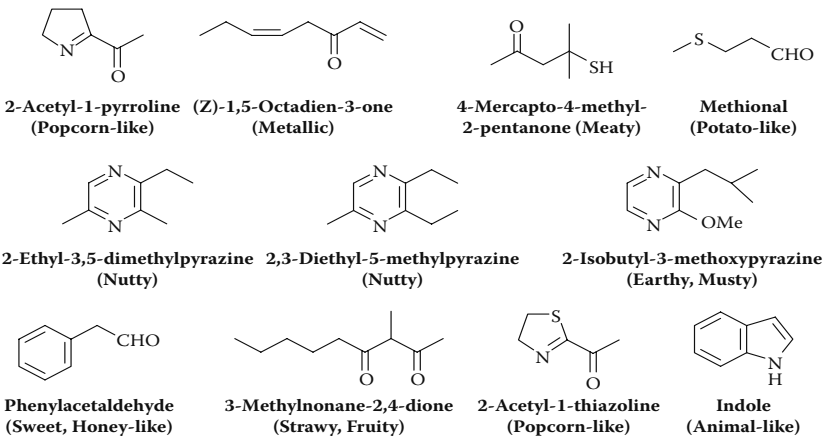


FIGURE 17.18 Structures of flavor components shown in figure 17.17.

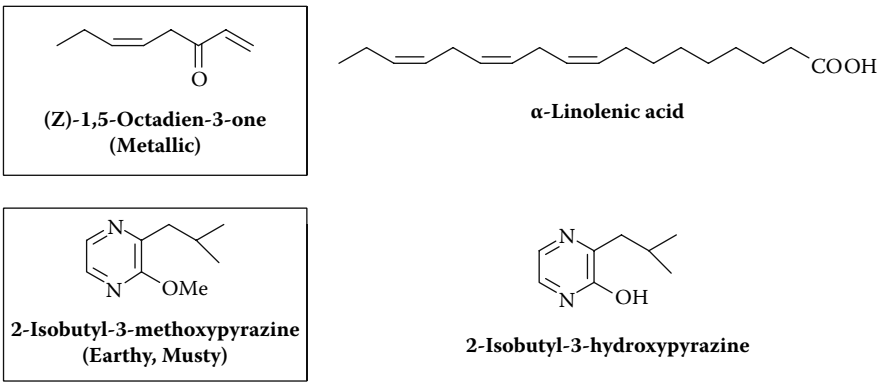


FIGURE 17.19 Proposed precursors of (Z)-1,5-octadien-3-one and 2-isobutyl-3-methoxypyrazine.

produced from α-linolenic acid as well as 1-penten-3-ol and (*E*,*E*)-2,4-heptadienal by auto-oxidation (figure 17.19).[28] In addition, eicosapentaenoic acid, present mainly in marine animals and plants, also serves as its precursor.[29,30] 2-Hydroxy-3-isobutylpyrazine is converted to 2-isobutyl-3-methoxypyrazine (threshold value: 0.002 ppb in water[8]) by the action of *S*-adenosyl-L-methionine-dependent *O*-methyltransferase in grapes[54] (figure 17.19). A variety of *O*-methylations have been reported[55]; however, whether a similar enzymatic *O*-methylation occurs in green tea drinks is not known.

17.4 FLAVOR STABILITY OF BLACK TEA DRINKS

The intensity of the off-flavor (sweet, clove-like, heavy, putrid) in black tea drinks increased after the retorting process (figure 17.20). Figures 17.21 and 17.22 show that the FD factors and the structures of the flavor components increased by the retorting process in black tea drinks.[56] As shown in figures 17.4 and 17.21, after the retorting process, the flavor component of black tea drinks was different from that of the green tea drinks. No alcohol formation was noted in black tea drinks; however, in green tea

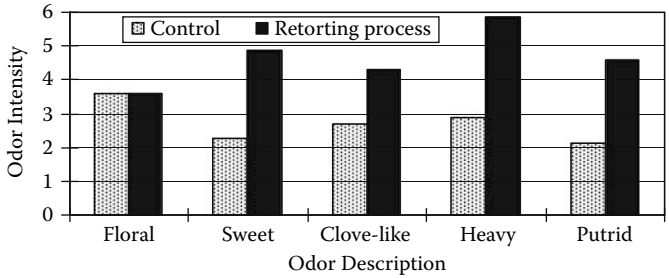


FIGURE 17.20 Odor intensity and odor description of black tea (Darjeeling tea) drinks before and after retorting process. Retorting process: 121°C, 10 minutes. pH: 5.4 (control), 5.1 (after retorting process).

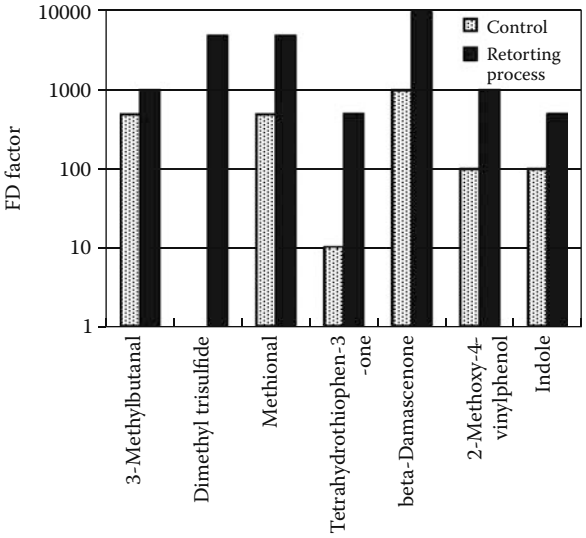


FIGURE 17.21 FD factor of flavor components increased by retorting process in black tea (Darjeeling tea) drink.

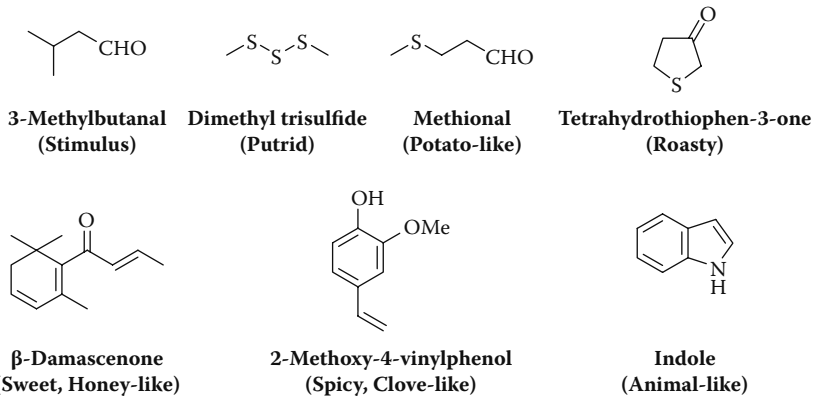


FIGURE 17.22 Structures of flavor components of black tea drink shown in figure 17.21.

drinks, linalool and geraniol were identified as important off-flavors after the retorting process. This difference is considered to depend on the decrease in glycosides during the manufacturing process, especially during the rolling and fermentation stages.[57] As for 3-methylbutanal (threshold value: 0.2 ppb in water[8]), as well as methional and phenylacetaldehyde, the corresponding α-amino acid, that is, L-leucine, is the precursor of the Strecker degradation (figure 17.23).[12,13] Trimethylsulfide is the characteristic component with a lower odor threshold value (0.01 ppb in water[8]) than dimethylsulfide (odor threshold value: 1 ppb[8]) and dimethyldisulfide (odor threshold value: 7.6 ppb[8]).[58] Dimethyldisulfide, which is formed via a dimerization of meth-

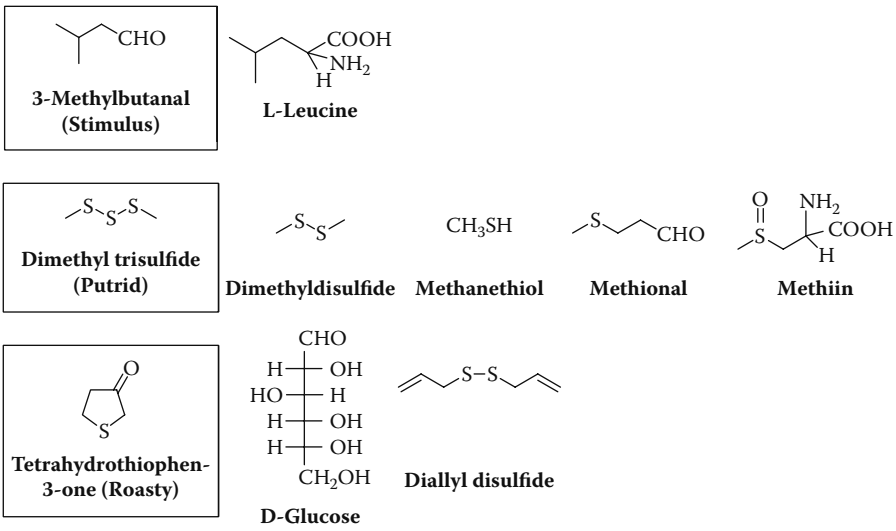


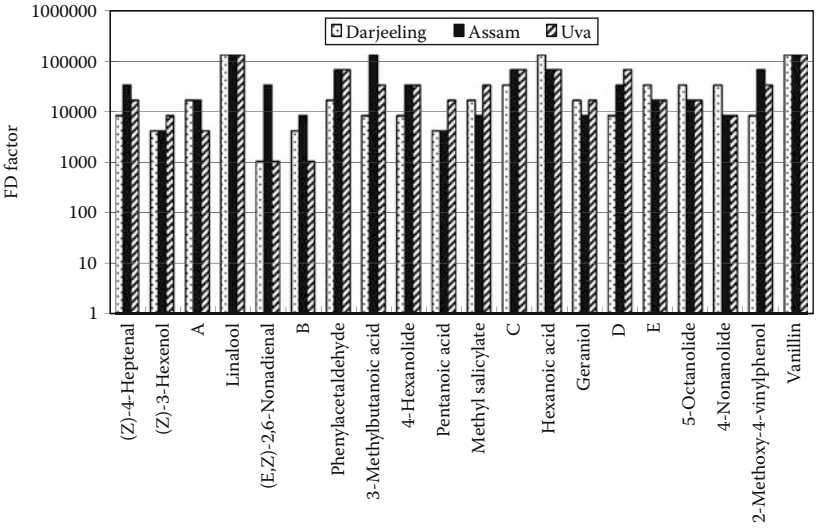
FIGURE 17.23 Proposed precursors of 3-methylbutanal, dimethyltrisulfide, and tetrahydrothiophen-3-one.

anethiol (threshold value: 0.02 ppb in water[8]) and produced by the degradation of methional, can disproportionate to trimethylsulfide and dimethylsulfide (threshold value: 1 ppb in water[8]) (figure 17.23).[58] Precursors of dimethyltrisulfide in green tea have not been reported. However, its formation mechanism has been studied in a variety of other foods. In the study of beer flavor, dimethyltrisulfide is reported to be formed from methional at low pH.[59,60] In addition, for fried potato flavor, sulfide compounds, such as dimethylsulfide, dimethyldisulfide, and dimethyltrisulfide, are reported to be formed from methional.[61] On the other hand, in *Brassica* vegetables, methiin, that is, *S*-methylcysteine sulfoxide, is reported to be the precursor of sulfide compounds such as dimethylsulfide, dimethyldisulfide, and dimethyltrisulfide.[62] As for tetrahydrothiophen-3-one, the sulfur compound serving as its precursor is unclear. However, it was reported to be formed from glucose and diallyl disulfide during the heating process in the model system (figure 17.23).[63]

The potent odorants in four kinds of black tea drinks, the characteristic impact components, and their structures are shown in figures 17.24 to 17.27.[64,65] The off-flavor components, β-damascenone and 2-methoxy-4-vinylphenol, shown in figures 17.21 and 17.22, are among components shown in figure 17.24 that, upon heating, render a remarkable effect on the quality of tea drinks. However, in order to provide a more stable drink to the consumer, it is necessary to study the flavor change due to these compounds in the future.

17.5 CONCLUSIONS

It is well known that flavor changes under various influences, such as light irradiation, heating, oxygen, pH change, microorganisms, and metals. Therefore,



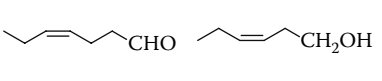
A	2-Ethyl-3,5-dimethylpyrazine, 5-Ethyl-2,3-dimethylpyrazine
B	Acetylpyrazine, 2-Acetyl-3-methylpyrazine
C	4-Heptanolide, (*E*,*E*)-2,4-Decadienal, beta-Damascenone, 2-Hydroxy-3-methyl-2-cyclopenten-1-one
D	Guaiacol, alfa-Ionone, Geranyl acetone
E	(*Z*)-3-Hexenoic acid, Heptanoic acid

FIGURE 17.24 Potent flavor components of black tea (Darjeeling, Assam, and Uva) drinks identified by AEDA. FD factor of the flavor components: greater than 210. Nonretorting process.

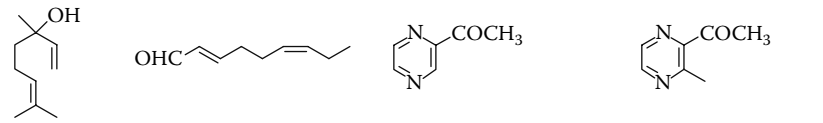
maintaining the flavor stability of tea drinks is important for manufacturers. The commercially available tea drinks produced by many manufacturing steps, which are different from homemade tea drinks, result in flavor deterioration, especially during the sterilization process, such as the retorting process and the ultrahigh temperature (UHT) sterilization. From the viewpoint of flavor quality, aroma extract dilution analysis (AEDA) is an effective method to determine the delicate change in flavor components before and after the heating process. Thus, the components having a higher flavor dilution (FD) factor by heating process compared to nonheating flavors have been the focus of studies. The nonvolatile flavor precursors, such as glycosides, carbohydrates, fatty acids, and amino acids, are considered to thermally generate off-flavor components. The residual amount of the flavor precursor is dependent on the manufacturing process of tea leaves. In green tea leaves, due to the deactivation of enzymes during the early steps of the manufacturing process, the precursor produces a variety of degradation products under heating compared to that of black tea leaves.

ACKNOWLEDGMENT

I appreciate Dr. Kenji Kumazawa at Ogawa & Co., Ltd., deeply for his helpful advice.

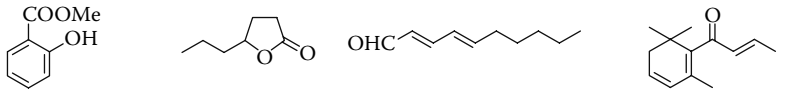


(*Z*)-4-Heptenal (Hay-like) **(*Z*)-3-Hexenol (Green)** **2-Ethyl-3,5-dimethylpyrazine (Nutty)** **5-Ethyl-2,3-dimethylpyrazine (Nutty)**

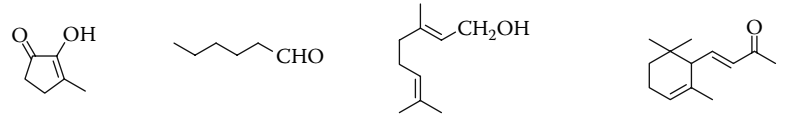


Linalool (Floral) **(*E,Z*)-2,6-Nonadienal (Green)** **2-Acetylpyrazine (Roasty)** **2-Acetyl-3-methylpyrazine (Roasty)**

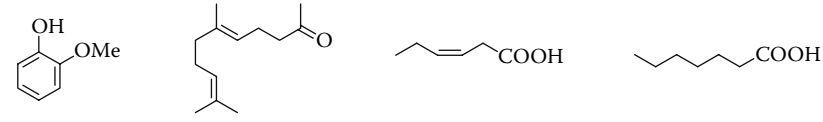
Phenylacetaldehyde (Sweet, Honey-like) **3-Methylbutanoic acid (Buttery, Rancid)** **4-Hexanolide (Nutty, Oily)** **Pentanoic acid (Buttery, Rancid)**



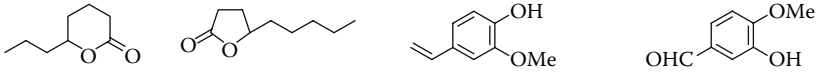
Methyl salicylate (Minty) **4-Heptanolide (Sweet)** **(*E,E*)-2,4-Decadienal (Fatty)** **β-Damascenone (Sweet, Honey-like)**



2-Hydroxy-3-methyl-2-cyclopenten-1-one (Caramel-like) **Hexanoic acid (Green, Acid)** **Geraniol (Floral)** **α-Ionone (Hay-like)**



Guaiacol (Smoky, Burning, Sweet) **Geranyl acetone (Hay-like)** **(*Z*)-3-Hexenoic acid (Green, Acid)** **Heptanoic acid (Green, Acid)**



5-Octanolide (Sweet) **4-Nonanolide (Sweet)** **2-Methoxy-4-vinylphenol (Dusty, Spicy)** **Vanillin (Vanilla-like)**

FIGURE 17.25 Flavor components of black tea drink shown in figure 17.24.

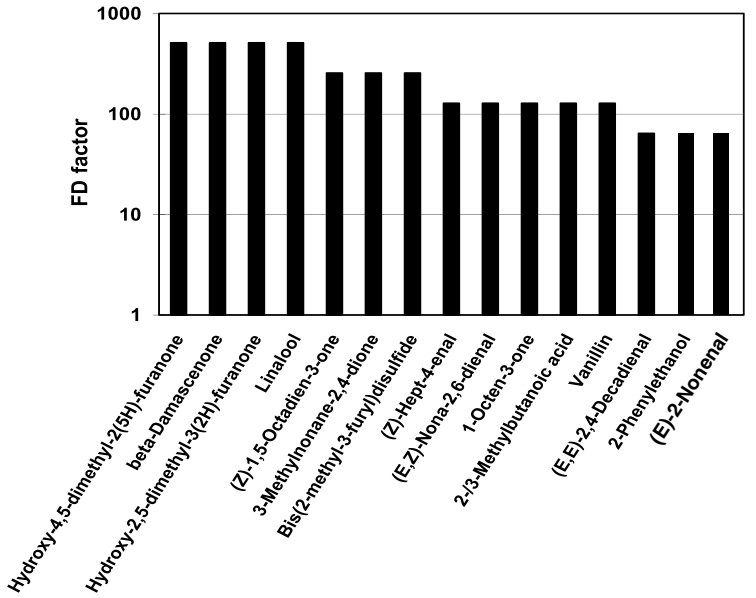


FIGURE 17.26 Potent flavor components of black tea (China Gold Yunnan, Flowery Orange Pekoe) drinks identified by AEDA. FD factor of the flavor components: greater than 64. Nonretorting process.

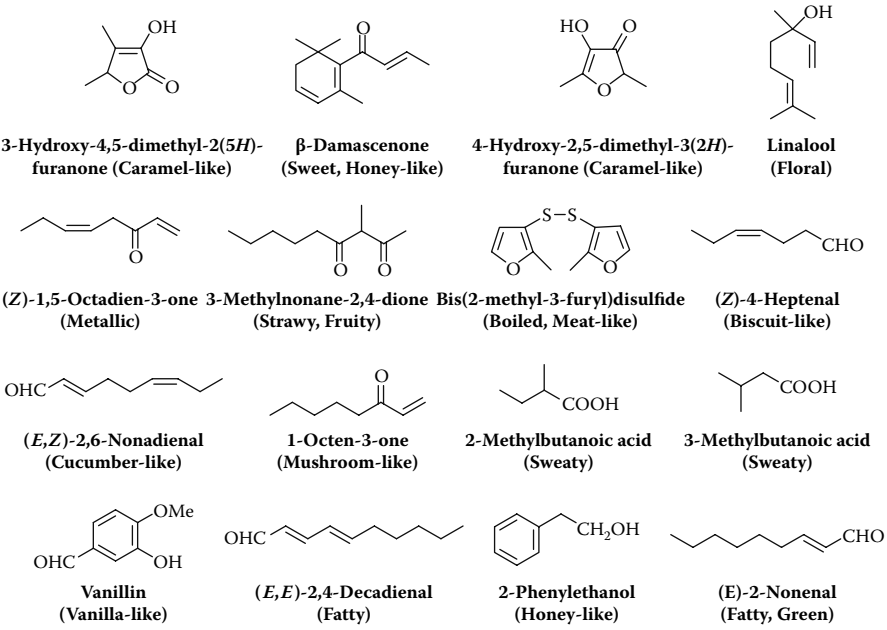


FIGURE 17.27 Flavor components of black tea drink shown in figure 17.26.

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Index

D

E

F

G

N

O

P

Q

R

S

T

U

V

W

X

Tea and Tea Products

Chemistry and Health-Promoting Properties

The past decade has seen considerable interest and progress in unraveling the beneficial health effects of tea, particularly its polyphenolic components and its antioxidant activity. Understanding the science behind the claims will help in the production and marketing of teas and tea products. Pulling together recent research and presenting it in an organized format, **Tea and Tea Products** discusses the manufacturing and chemistry of various teas including green, black, Pu-erh, white, and GABA teas.

Providing comprehensive and authoritative reviews and perspectives, this book includes:

- Comprehensive studies of tea chemistry, the bioavailability of polyphenols, and flavor chemistry
- Explorations of cellular and animal models in carcinogenesis research
- Discussions of the application of chromatographic techniques and analytical tools for the analysis of bioactive components
- More than 100 black and white illustrations, more than 40 tables, and 50 equations
- Coverage of the metabolism and bioavailability of EC, ECG, EGC, and EGCG in humans
- Examinations of the evidence that supports anticancer, anti-inflammatory, and antioxidant claims

Emphasizing black and green teas equally, the book presents comprehensive and up-to-date reviews and perspectives on the chemistry of tea components and the molecular biology of green tea catechins and black tea theaflavins. It covers the analysis, formation mechanisms, and bioavailability of tea polyphenols and discusses bioactivities of teas including anticancer, anti-inflammatory, anti-obesity, and anti-diabetes. The editors of this volume have more than 100 research publications in tea, and experience in editing more than 50 books among them. Under their expertise and editorial guidance, the contributors present chapters that explore the science behind the health claims of teas.

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