

# Caffeinated Beverages





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# Caffeinated Beverages

## Health Benefits, Physiological Effects, and Chemistry

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# Foreword

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Before agreeing to publish a book, the proposed table of contents is reviewed for appropriate and comprehensive coverage and for interest to the audience. Some papers may be excluded in order to better focus the book; others may be added to provide comprehensiveness. When appropriate, overview or introductory chapters are added. Drafts of chapters are peer-reviewed prior to final acceptance or rejection, and manuscripts are prepared in camera-ready format.

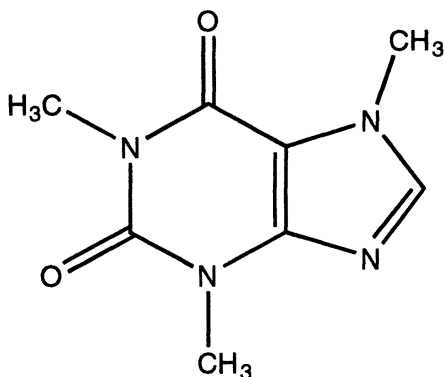
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## Preface

Caffeine-containing products have been consumed for hundreds of years for the delightful flavor and stimulating effects they possess. This group of products includes coffee, tea, and cocoa, as well as some less familiar products such as guaranà and kola. The two most commonly consumed beverages in the world today are coffee and tea, which are produced by hot water extraction of the roasted bean and leaf respectively.

The one common constituent they possess is caffeine, 1,3,7-trimethylxanthine, a white crystalline solid mp 238° C. Caffeine is a stable compound that is not destroyed by the roasting of coffee, the fermentation or roasting of cocoa, or the fermenting or firing of tea.



Caffeine

According to the Merck Index, caffeine is a CNS stimulant, and acts as a diuretic. As will become evident from this volume, caffeinated products possess additional positive health benefits for humans such as antioxidant properties, as well as possible anticarcinogenic activity. The ubiquitous consumption of caffeinated products throughout the world attests to the power these products hold on humans.

The symposium upon which this book is based was developed to explore recent advances in the flavor chemistry, physiological effects, and health benefits

of caffeinated products. It is based on the four day symposium: *Chemistry and Health Benefits of Caffeinated Beverages* that was part of the 217<sup>th</sup> National American Chemical Society (ACS) meeting in Anaheim, California, March 1999.

It was the goal of the organizers to bring together world experts in the field of caffeinated products and to provide a forum for the discussion of modern advances in the field. This book represents the editors' efforts to produce a work detailing the current state of research covering caffeinated beverages.

This book has been arranged into sections that cover the following topics. An introductory section introduces the topic of caffeinated beverages. The next section covers the physiological effects of caffeine and concludes that caffeine lacks addictive potential. The following three sections review the current state of the knowledge of the health benefits of these beverages and concludes that anticancer, antioxidative, and cardiovascular benefits may result from consumption. The book concludes with four sections on the chemistry of coffee, tea, and cocoa as well as methods of analysis of such products.

### **Acknowledgments**

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## Chapter 1

# Chemistry and Health Benefits of Caffeinated Beverages: Symposium Overview

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This symposium provided a unique opportunity for food scientists, analytical chemists, and biological researchers to engage in an interdisciplinary conversation exploring the potential health benefits inherent in the world's most popular beverages. This continued a long-standing tradition of the Division of Agricultural and Food Chemistry wherein advances in food chemistry knowledge have been described in relation to the food itself as well as to the physiological outcome in an end-user organism.

### Introduction

Caffeine, a 1,3,7-trimethylxanthine, is naturally present in over one hundred plant species. However, over the centuries, humans have selected only a few plant varieties as common sources for consumption: coffee beans, tea leaves, cocoa beans, kola nuts, guarana seeds, and mate. These natural sources of caffeine are generally consumed as beverages; cocoa and chocolate are enjoyed as beverages or solid confections; and fresh kola nuts are sometimes chewed.

If the common sources of naturally occurring caffeine listed above are considered as one "commodity," the global economic impact of this one substance is enormous. Indeed, green (raw) coffee beans alone are the second most widely traded commodity in the world (oil being first). Thus, understanding the basic chemistry that contributes to the desirable organoleptic qualities of these source plants is an important scientific and economic endeavor.

Although caffeine is a unifying theme of this workshop, it is by no means the total focus of the papers that follow. The occurrence of caffeine and other methylxanthines such as theobromine and theophylline in the tropical and subtropical plants of interest here may represent a common evolutionary strategy to

deter predators. However, caffeine and a host of other chemical compounds also account for the complex flavor and aroma profiles that make the food and beverage products of these plant species so pleasing to humans. Many of these plant-based chemicals, or phytochemicals, are currently under intense study by medical and health researchers for their putative beneficial health effects. For example, part of the well-recognized health benefit of fruits and vegetables appears to be a result of polyphenolic compounds acting as antioxidants in the body. Several papers in this book suggest that the food and beverage products made from caffeine-containing plants can deliver substantial levels of antioxidant polyphenols to the human diet.

This overview continues with a brief review of the various chapters in a slightly different grouping than as published. The purpose is to separate papers dealing with a food chemistry topic (*e.g.*, new analytical method, effect of processing step) from those dealing with a physiological topic (*e.g.*, tumor formation, blood lipoprotein oxidation, behavior). Due to the nature of the research database, the majority of the papers discuss caffeine, coffee, or tea; several address cocoa, but only a few cover guarana and kola.

## Chemistry

Knowledge of the mechanism and chemistry of compositional changes during growth and storage of caffeine-containing plants, and their processing to final products, may permit enhancement of compounds with beneficial organoleptic or health properties or reduction of less desirable compounds. Many papers concentrate on the aroma and flavor compounds that are important to consumer acceptance and enjoyment of these products. However, a few papers go beyond the traditional hedonic endpoints to consider these same compounds as potential contributors to human health based on their antioxidant capabilities.

## Analysis

Modern analytical methods increase knowledge of the chemical profile of complex foods and can identify specific compounds useful for quality control purposes. Stadler extends work on C-8 hydroxylated methylxanthines as markers of oxidation in raw coffee, tea and cocoa materials and finished products. Ames demonstrates the use of capillary electrophoresis to examine the color fraction of roasted coffees. Hammerstone presents a method (HPLC-MS with API-ES chamber) to identify a variety of flavonoids, especially procyanidins, in tea, cocoa and chocolate products. Speer notes that the unique coffee diterpenes kawheol and cafestol may be used to distinguish among Arabica blends, while 16-O-methylcafestol may distinguish Robusta coffee in Arabica mixtures and that the distribution of these compounds in roasted coffee tends to remain similar to that found in the respective green bean type.

Flavor and aroma play special roles in consumer acceptance of caffeinated beverages. Using HPLC/UV PDA and HPLC/DC Voltammetry, Cohen determines specific green coffee phenols and phenolic acids which may impact the flavor profile. Likewise, Glazier describes statistical methods to correlate traditional laboratory evaluations of cocoa bean quality with sensory panel data to develop a predictive model of cocoa bean flavor based on instrumental measures of cocoa bean volatiles generated during roasting.

### General Considerations—Coffee and Tea

Waller describes recent work on caffeine metabolism in coffee and tea plants. Caffeine biosynthesis uses a common pathway from xanthosine through theobromine to caffeine, but theophylline does not appear to be methylated to caffeine. Caffeine accumulation generally results from a slow degradation of caffeine to theophylline; the caffeine degradation sequence then proceeds by conversion of theophylline to xanthine via 3-methylxanthine and entry into the purine catabolic pathway. Caffeine metabolism is related to the stage of plant development, time of year, and species.

Segall compares and contrasts coffee and tea from farm to cup. The similarity of preparation of the final beverages (hot water infusion) belies the botanical, agronomic, processing, and compositional differences between coffee and tea.

### Coffee

Several papers examine the chemistry of coffee flavor development. Parliment outlines the major chemical changes occurring during the roasting of coffee beans that lead to desirable flavor compounds and discusses the most important flavor compounds in roasted coffee. Grosch finds that a quantitative mixture of 27 key odorants of roasted Arabica coffee produced an odor profile close to a real sample, but only 8 of these odorants were essential for the coffee flavor. A key thiol flavor component, 2-furfurylthiol, is present in coffee brews at about one-third the amount found in roasted coffee. Blank suggests that most of this loss may result from oxidative processes. The same sulfur-containing compound exhibits a distinct coffee-like aroma characteristic of brewed coffee. Rizzi examines model systems that suggest the key precursors in formation of this thiol may be pentose sugars and coffee bean proteins, the latter being responsible for the unique aroma developed on roasting. In a somewhat different approach, Steinhart attempts to model the effect on volatile aroma compounds of various dairy-based additives in typical coffee beverages.

Many processing conditions affect final beverage characteristics. Ko concludes that roasted bean quality improved with high temperature/short time roasting based on aromatic impact, cup strength, taste intensity, and extraction parameters.



Cammenga describes a filter bypass system that allows full caffeine extraction at a lower total solubles level, giving a “lighter” but fully stimulating brew.

## Tea

In his review of tea chemistry, Ho considers changes in the major chemical compounds, catechins and methylxanthines, during fermentation from green to black tea relative to the distinctive flavor and color of various teas. High levels of catechins in green tea decrease with fermentation, such that black tea contains predominantly undefined polyphenols. Catechins contribute to the astringency of the final brew, while bitterness may be a function of caffeine complexation with tea polyphenols. Tea aroma derives from critical changes in several compounds, but the predominant chemicals in green tea aroma and black tea aroma are different. Masuda describes flavor changes in canned green and black teas after retorting. Although significant variations in the volatile fraction contribute to off-flavors, some may be generated from nonvolatile precursors. An elegant report by Sakata suggests that the action of a specific enzyme,  $\beta$ -primeverosidase, during fermentation produces most of the alcoholic tea aroma (floral tea note) from stored disaccharide glycosides. Green tea catechins are important to the taste of green tea. Yoshida reports that lower pH improves extraction of major catechins and that pH decreases with increasing tea concentration; however, at higher tea concentrations, gallate catechins are less well extracted than non-ester catechins.

## Cocoa, Guarana and Kola Nuts

As with coffee, cocoa beans undergo a lengthy preparation process to produce the usable fractions, cocoa butter and cocoa powder. Schieberle follows the important chemistry of cocoa from growing conditions through manufacturing in relationship to the bitter taste, melting behavior and, importantly, characteristic aroma of cocoa products. Hashim argues that the complex flavor of cocoa develops mainly through the Maillard reaction during the roasting process. Chen further elucidates cocoa flavor development as reactions of sugars, amino acids, lipids and other precursors and identifies two compounds imparting strong chocolate-like notes to the overall flavor profile.

Much less is known about the chemical changes in guarana and kola nuts during growth and processing. Walker notes that guarana, mainly grown in Amazonian Brazil, is likely the richest known vegetable source of caffeine. Compositional changes on processing appear minimal, and little is known about the flavor profile. Most guarana is used commercially as a concentrate for beverages. Ringleib speculates that the characteristic flavor notes of the kola nut, a flavor ingredient in cola beverages, are likely due to borneol, geosmin and selected terpenoid compounds; however, data on processing chemistry are scarce.

## Antioxidant Properties

Several papers consider the antioxidant properties of the polyphenolic compounds in coffee, tea, and cocoa. Detailed studies provide information not only on the types and concentrations of these components in raw materials and final products, but also on mechanisms of action which are likely important to human health after ingestion. Engelhardt presents data showing that tea, coffee and cocoa drinks can contribute substantial amounts of total polyphenols to the diet, but only tea appears to be a significant source of flavonoids. Similarly, Lee notes that the total antioxidative activity of aroma chemicals in brewed coffee may be comparable to that of recognized antioxidant vitamins. Using a DPPH radical system, Bungert examines the relationship between phenolic compound structure and radical scavenging activity, while Suzuki begins to unravel the catechol type catechin radical scavenging mechanism and stoichiometry.

## Health

As with all organisms, the unique biochemistry and physiology of the caffeine-containing plants are the result of evolutionary strategies which ensure their survival—the chemistry is for the benefit of the plant. Although humans have long enjoyed the products of these plants for their taste and mildly stimulating effect, only during the latter half of this century have there been intense investigations of these plant components in relation to human health. Because of its widespread consumption in beverages, caffeine is one of the most studied chemicals in the world. Today, most of the concerns about the adverse effect of caffeine on major health problems (*e.g.*, cardiovascular disease, osteoporosis, cancer) have been refuted by carefully controlled studies. Now, many investigators are looking at potential beneficial impacts on human health that may derive from caffeine itself or from other compounds, particularly the polyphenols, in these plants. Perhaps the foods and beverages from caffeine-containing plants are just an early example of what in contemporary parlance are called “functional foods”—in this case, a common food whose unique phytochemicals may impart potential health benefits.

## Behavior

Controversy remains among researchers about the long-term effects of caffeine use on behavioral endpoints. Smith reviews a decade of work on the effects of caffeine on mood and cognitive performance. He suggests that caffeine at relatively low levels, comparable to those achieved with average consumption patterns, rapidly improves mood and sustained performance tasks, especially in low alertness situations. In a different interpretation of similar data, Rogers contends that consumers' liking of the taste, aroma, and flavor of caffeinated beverages is

reinforced by the stimulating effects of caffeine. Over time, consumers lose the expected beneficial stimulant effect and continue to drink these beverages to avoid unwanted consequences of caffeine deprivation and maintain a normal level of alertness. While the opposing conclusions of Smith and Rogers derive from similar psychological testing paradigms, both agree that there are conditions under which caffeine consumption is beneficial.

Another contentious area of behavioral psychology is whether or not caffeine should be considered a drug of dependence. In one approach to this question, Nehlig uses a sensitive animal model of cerebral energy metabolism to understand the behavioral effects of caffeine. Her data indicate that areas of the brain controlling locomotor activity and the sleep-wake cycle are very sensitive to low concentrations of caffeine which are in the general range of human consumption. However, brain structures involved with addiction, reward and motivation respond only to levels of caffeine that induce global brain stimulation and general adverse effects. Importantly, these results are being replicated in human studies. Thus, the data do not support a potential dependence on caffeine at normal levels of intake.

## Cancer

The influence of caffeine and coffee on carcinogenesis has been closely followed for many years. Although many reports suggest that some particular chemical found in coffee may produce a mutagenic or carcinogenic outcome in an isolated test system, Adamson reports that coffee is not mutagenic *in vivo* and that coffee or caffeine are not carcinogenic in long-term animal bioassays. Likewise, both animal and epidemiological studies suggest that caffeine and coffee may inhibit tumor induction and are not associated with various cancers. Ho continues these observations with detailed results on the reduction by caffeine of chemically-induced skin, stomach, mammary and lung tumorigenesis in animal models. Extending previous work on green coffee beans and fraction thereof, Miller demonstrates in a hamster model that consumption of roasted coffee beans or fractions inhibits the development of tumors. In a succinct summary of recent cancer research, an invited paper by Sivak concludes that coffee consumption does not affect the occurrence of cancer in humans; furthermore, coffee intake clearly reduces the risk of colon cancer, possibly due to the antioxidant-like activity of several compounds in coffee. Similarly, Lin attempts to elucidate mechanism for cancer prevention of tea based on studies with tea polyphenols.

## Antioxidants

A final series of papers may be grouped by a common focus on health-related outcomes based on antioxidant properties of coffee, tea or cocoa products. Elevated plasma cholesterol, particularly the low-density lipoprotein (LDL) fraction, is one of many recognized risk factors for cardiovascular disease; recent data suggest that

oxidized LDL may actually be the important fraction in development of arterial wall plaques. Richelle compares the ability of coffee, tea and cocoa beverages, under normal preparation conditions, to inhibit LDL oxidation in an *in vitro* test system. All beverages prolong the lag time before initiation of oxidation, based on polyphenol content. Other measures indicate that antioxidant polyphenols are likely absorbed at levels within an active physiological range. In similar work, Schmitz examines the structures of procyanidins in chocolate and cocoa in relationship to their activity in various *in vitro* tests related to potential cardiovascular health benefits. Chen concludes that the hypolipidemic activity of green tea catechins fed to hamsters is related to reduced absorption of cholesterol and triglycerides.

Osakabe presents results from a variety of *in vitro* tests and animal feeding experiments with a crude polyphenol extract from cocoa liquor. The data corroborate and extend the results from other investigators. The extract shows anti-mutagenic and anti-tumorigenic activity, reduction of oxidative stress and LDL oxidative susceptibility, and a slight level of anti-ulcer activity. Since catechins are only partially absorbed after ingestion, Hara offers a unique perspective on green tea catechins as antibacterial agents in the gastrointestinal tract. Studies suggest that isolated catechins, given at amounts present in normal tea consumption ranges, can improve fecal parameters associated with health benefits in a reversible fashion.

## Conclusions

Sophisticated analytical techniques continue to reveal in ever greater detail the complex chemistry of caffeine-containing plants and their products. Flavor and aroma chemistry remains a most important area of research. Effects of processes such as fermentation, drying, roasting and grinding on precursor compounds and mechanisms of action of flavor and aroma development are subjects of intense study. Coupled with sensory panels and statistical models, such research attempts to identify chemicals of importance to consumer enjoyment of tea, coffee and cocoa food and beverage products and to predict which raw materials may produce the best final products.

Research on potential health benefits of these products is in its early stages. Current data on polyphenols suggest that the protective antioxidative effects of these compounds observed in source plants may be transferred to humans. This exciting hypothesis must be confirmed by sound science. Human research needs to establish that these substances are absorbed in physiologically significant amounts and active forms. Meaningful *in vivo* endpoints of antioxidative activity need to be developed so that controlled human trials can corroborate experimental animal work and epidemiological observations. With more definitive human data, the foods and beverages from caffeinated plants may be prized for their health benefits in the future just as they are cherished for their organoleptic qualities today.

## Chapter 2

### Pathways Involved in the Metabolism of Caffeine by *Coffea* and *Camellia* Plants

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Extensive metabolic studies of purine alkaloids in leaves of tea and coffee have elucidated the caffeine biosynthetic pathway in great detail recently; the available data support the operation of a xanthosine→7-methylxanthosine→7-methylxanthine→theobromine→caffeine pathway as the major route to caffeine. Caffeine synthase (CS), the SAM-dependent *N*-methyltransferase involved in the last two steps of caffeine biosynthesis, was extracted from young expanding tea leaves (*Camellia sinensis*) which were soft and had few phenolic compounds or chlorophyll, purified 520-fold to apparent homogeneity with a final specific activity of 5.7 nkat mg<sup>-1</sup> protein. Purine alkaloid catabolic pathways based on recent evidence demonstrate salvage of 3-methylxanthine and xanthine for the resynthesis of not only theophylline but also caffeine via theobromine. These pathways operate in tea leaves but not coffee.

The widespread natural occurrence of purine alkaloids - caffeine and other methylxanthines such as theobromine and theophylline in a variety of plants has a major role in the long-standing popularity of caffeine-containing products, especially nonalcoholic beverages and foods (coffee, tea soft drinks, cocoa and chocolate products, etc.). More than 60 plant species throughout the world have been identified as containing caffeine; the more common are from the genera *Coffea*, *Camellia*, *Cola*, *Paullinia*, *Ilex*, and *Theobroma* (1). Caffeine also has been used as a drug to decrease sleeping and as a dietary aid since its original isolation from green coffee beans by Runge in 1820 (2).

Metabolism of purine nucleotides and purine alkaloids (caffeine, theobromine, and theophylline) occurs in coffee, tea, mate, and guarana plants; however, most of the research done is with coffee and tea. Purine metabolism is similar to that in other plants which do not contain caffeine. The young leaves of *Coffea arabica* contain ca 1-2 % caffeine on a dry weight basis while young tea (*Camellia sinensis*) leaves contain up to 2-4 % caffeine. Mature and aged leaves of both species contain lower levels of caffeine. Research on the biosynthesis of caffeine in coffee cherries (fruit) and in leaves of coffee and tea indicate that the main pathway is xanthosine → 7-methylxanthosine → 7-methylxanthine → theobromine → caffeine (3-6). In addition, a 7-methylxanthine → paraxanthine → caffeine pathway is one of a number of minor pathways operating in tea leaves (7). There is one report of an alternative entry in the caffeine biosynthetic pathway in coffee which involves conversion of xanthine monophosphate(XMP) → 7-methyl XMP → 7-methylxanthosine (8). These pathways are illustrated in Figure 1. Purification and characterization of caffeine synthase from tea leaves (6) represents a major step forward for the knowledge of the biosynthesis of caffeine.

### Caffeine Biosynthesis

The enzymes that are utilized in the caffeine biosynthetic pathway contain three SAM-dependent methylation steps, dependent upon *N*-methyltransferases. Activities of 7-methylxanthine *N*-methyltransferase and theobromine *N*-methyltransferase, which catalyze the second and the third methylation steps in the main pathway, were first demonstrated in crude extracts from tea leaves (9). This study shows that the two enzymes have identical pH optima and have similar metal ions and inhibitors. Since then, caffeine *N*-methyltransferase activities have been detected in cell-free extracts prepared from immature fruits (10), callus (11,12), and cell-suspension cultures (13) of *Coffea arabica*. The first methylation enzyme, xanthosine *N*-methyltransferase, which catalyzes the formation of 7-methylxanthosine from xanthosine, was demonstrated in tea leaf extracts (14,15). This confirmed the presence of the activities of the three *N*-methyltransferases in tea leaf extracts and found them to be quite high in very young developing leaves but absent in fully developed leaves.

The purification of *N*-methyltransferase(s) involved in caffeine biosynthesis has been attempted by several investigators. Mazzafera *et al.* (16) first reported the purification of a *N*-methyltransferase from fruits and leaves of *Coffea arabica* which possessed 7-methylxanthine and theobromine *N*-methyltransferase activity. However, the cell-free preparations were extremely labile and the specific activity of the enzyme diminished with each step in a sequential purification procedure. The specific activity of the final preparation was less than 1 fkat mg<sup>-1</sup> protein. Gilles *et al.* (17) purified *N*-methyltransferase from endosperm of *Coffea arabica* using Q-Sepharose in the presence of 20% glycerol. The final specific activity was 420 fkat mg<sup>-1</sup> protein. Kato *et al.* (7) partially purified *N*-methyltransferase from tea leaves by ion-exchange and gel-filtration chromatography. The partially purified enzyme preparation had three activities, suggesting that *N*-methyltransferases for caffeine biosynthesis are a single

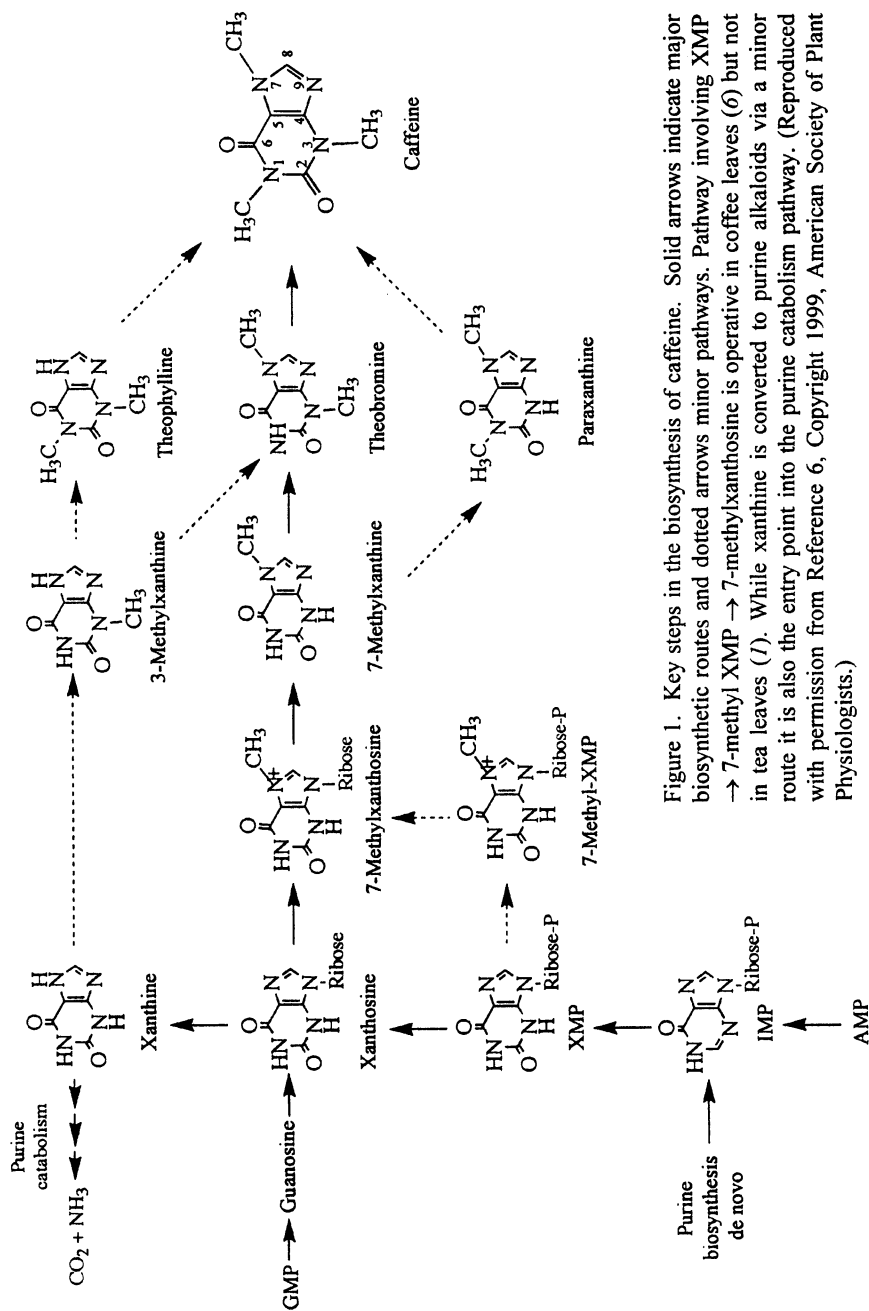


Figure 1. Key steps in the biosynthesis of caffeine. Solid arrows indicate major biosynthetic routes and dotted arrows minor pathways. Pathway involving XMP  $\rightarrow$  7-methyl XMP  $\rightarrow$  7-methylxanthosine is operative in coffee leaves (6) but not in tea leaves (7). While xanthine is converted to purine alkaloids via a minor route it is also the entry point into the purine catabolism pathway. (Reproduced with permission from Reference 6, Copyright 1999, American Society of Plant Physiologists.)

enzyme. Alternatively, two or more enzymes comprised of proteins with a similar molecular weight and comparable charge may participate in the three methylation steps. Waldhauser *et al.* (18) partially purified *N*-methyltransferases from coffee leaves up to 39-fold using ion-exchange chromatography and chromatofocusing and showed that XMP *N*-methyltransferase was a different protein from the other two *N*-methyltransferases. Moisyadi *et al.* (19) provided a preliminary report on the purification, partial amino acid sequencing and cloning of cDNA for xanthosine-7-methylxanthosine methyltransferase from coffee.

Kato *et al.* (6) reported the isolation of caffeine synthase (CS), the SAM-dependent *N*-methyltransferase involved in the last two steps of the caffeine biosynthesis, was extracted from young leaves of *Camellia sinensis*.

The *N*-methyltransferase was purified to apparent electrophoretic homogeneity, and is the first report of the isolation of the *N*-methyltransferase protein for caffeine biosynthesis with high specific activity. The protein exhibited broad substrate specificity and catalyzed the conversion of 7-methylxanthine to caffeine via theobromine. When dimethylxanthines were used as substrates, paraxanthine was the best methyl acceptor followed by theobromine which was 12% as active as paraxanthine which supported the findings of Roberts and Waller (10). 7-Methylxanthine was the most effective substrate of three monomethylxanthines and the product was theobromine. 3-Methylxanthine and 1-methylxanthine were not good substrates, yielding low levels of theophylline. This implies that CS catalyzed only 3*N*- and 1*N*-methyltransferase activity, with the former being generally more active than the latter. The purified CS did not catalyze the conversion of xanthosine to 7-methylxanthosine or XMP to 7-methyl-XMP.

The kinetic parameters of the enzyme-substrate affinity were determined from the Lineweaver-Burk plots which gave a  $K_m$  value of 21  $\mu\text{M}$  for SAM in the presence of a saturated concentration (200  $\mu\text{M}$ ) of paraxanthine. The  $K_m$  value for paraxanthine, 7-methylxanthine and theobromine in saturating 50  $\mu\text{M}$  SAM were 24, 186, and 344  $\mu\text{M}$ , respectively. The final specific activity of CS was 5.7, 2.7, and 0.72 nkat  $\text{mg}^{-1}$  protein respectively with paraxanthine, 7-methylxanthine, and theobromine as the substrate. These values were 1,000- to 1,000,000-fold higher than those reported for partially purified preparations from coffee (16,17,20) and 60-fold higher than those obtained in an earlier study with a partially purified preparation from young tea leaves (7). The apparent molecular mass of tea leaf CS estimated by gel filtration chromatography was 61 kD and 41 kD by SDS-PAGE. The value obtained from gel filtration is broadly comparable with the estimated molecular masses from partially purified enzymes from developing coffee endosperm (54 kD) (16) and coffee leaves (67 kD) (16,17).

The methyl-acceptor specificity of the *N*-methyltransferases in crude, partially purified, and highly purified preparations from tea, cocoa tea (*Camellia pitilophylla*), and coffee are summarized in Table 1. The broad substrate specificity of purified CS is quite similar to that of the crude tea leaf extracts (9). Although paraxanthine is the best methyl acceptor for both tea and coffee *N*-methyltransferases, the substrate specificity of tea CS is different from coffee and cocoa tea. Theobromine is a better methyl acceptor than 7-methylxanthine in coffee (16,21) but this is not the case with



the tea enzyme (see Table 1). The properties of the *N*-methyltransferase of cocoa tea, a theobromine-accumulating plant, are different from those of coffee and tea in that the cocoa tea enzyme can use 7-methylxanthine as a methyl acceptor, but not theobromine or paraxanthine (22). The data summarized in Table 1 indicate distinct variations in the properties of the *N*-methyltransferases which may be related to the different spectrum of purine alkaloids that accumulated in the three species.

**Table 1. Comparison of the Substrate Specificity of *S*-Adenosylmethionine Dependent *N*-methyltransferases from Various Plant Sources**

Substrate <sup>1</sup> Methylation position <sup>2</sup>	7-mX 3N <sup>2</sup>	3-mX 1N	1-mX 3N	Tb 1N	Tp 7N	Px 3N	X 3N	XR 7N	XMP 7N	Ref
<b>Tea (<i>Camellia sinensis</i>)</b>										
Leaves (Purified)	100	17.5	4.2	26.8	tr.	210	tr.	Nd <sup>3</sup>	nd	6
Leaves (Partially purified)	100	14.0	20.0	21.4	Nd	206	-	10.3	-	7
Leaves (Crude)	100	tr.	4.5	25	2.5	250	nd	nd	nd	9
Leaves (Crude)	100	-	-	20	-	-	16	56	nd	14
<b>Cocoa tea (<i>Camellia pitilophylla</i>)</b>										
Leaves (Crude)	100	-	-	nd	-	nd	-	-	-	22
<b>Coffee (<i>Coffea arabica</i>)</b>										
Endosperm (Partially purified)	100	-	-	185	-	-	-	-	-	16
Fruits (Crude)	100	-	5.7	127	4.6	175	nd	nd	-	10
Leaves Crude)	100	-	-	-	-	-	-	-	104	8

<sup>1</sup>7-mX, 7-methylxanthine; 3-mX, 3-methylxanthine; 1-mX, 1-methylxanthine; Tb, theobromine; Tp, theophylline; Px, paraxanthine; X, xanthine; XR, xanthosine; XMP, xanthosine 5'-monophosphate.

<sup>2</sup>See structure of caffeine with labeled nitrogen atoms in Fig. 1

<sup>3</sup>Nd, not detected; tr, trace; -, not determined.

Two routes for the synthesis of 7-methylxanthine have been proposed. Negishi *et al.* (14) demonstrated the presence of *N*-methyltransferase activity in tea leaf extracts where xanthosine, but not XMP, was an active methyl acceptor. In contrast, Schulthess *et al.* (8) reported that the *N*-methyltransferase from coffee leaves catalyzed the methylation of XMP as well as xanthosine, and proposed that XMP, rather than xanthosine, is the *in situ* acceptor for the first methyl group acceptor in the caffeine biosynthesis. Kato *et al.* (6) has shown that the purified tea CS does not methylate either xanthosine or XMP and therefore does not catalyze the first methylation step in the caffeine biosynthetic pathway.

The tea xanthosine *N*-methyltransferase appears to be either very labile or the amounts present in young tea leaves vary. The enzyme sometimes disappears in crude extracts during purification although it was found in semi-purified tea leaf extracts that we have prepared previously (7) (see Table 1). Although improbable, it is difficult to eliminate the possibility that *in situ* tea CS has an active site of 7-*N*-methylation which is lost in the *in vitro* preparations. It is, however, more likely that xanthosine *N*-methyltransferase protein is different from CS, and two different enzymes may participate in the three methylation steps of caffeine biosynthesis.

The  $K_m$  value for paraxanthine is low and  $V_{max}$  for this substrate is the larger, hence, paraxanthine is the best substrate for CS. However, as discussed in the previous paper (7), there is limited synthesis of paraxanthine from 7-methylxanthine so it is not an important methyl acceptor *in vivo*. The  $K_m$  value for theobromine is high (more than 0.3 mM), and this low affinity may explain the transient accumulation of theobromine in young tea leaves (23).

The effects of the concentration of SAM and several methyl-acceptors on the activity of CS show typical Michaelis-Menten type kinetics, and feedback inhibition by 200  $\mu$ M caffeine could not be detected. It is unlikely that allosteric control of the CS activity is operating in tea leaves.

The  $K_m$  of tea CS for SAM is 21  $\mu$ M in the presence of paraxanthine which was similar to the values for 7-methylxanthine and theobromine (25  $\mu$ M) obtained with crude tea enzyme preparations (9). These figures are lower than the  $K_m$  values of coffee leaf *N*-methyltransferase, 203  $\mu$ M with 7-methylxanthine and 67  $\mu$ M with theobromine (18).

One of the major factors affecting the activity of CS *in vitro* appears to be inhibition of SAH. As shown in the photo-affinity labeling with SAM, CS was completely inhibited by SAH. SAH binds to most methyltransferases with higher affinity than SAM (24). Therefore, control of the intracellular SAM/SAH ratio is one possible mechanism for regulating the activity of several methyltransferases, including CS. Nothing is known about such ratios in tea or coffee, but the SAM and SAH content of the leaves of 6-day old pea seedlings is 14.6 and 0.7 nmol g<sup>-1</sup> fresh weight (25).

Maximum CS activity was obtained at pH 8.5 which is the same as was obtained in an earlier study with *N*-methyltransferase activity in crude tea leaf extracts (9) and immature coffee cherry extracts (3). Similar alkaline pH optima have been reported for chloroplast stroma enzymes (26). CS is probably a stroma enzyme as a previous study with tea has shown that paraxanthine *N*-methyltransferase activity is located in chloroplasts (27). It is noteworthy that there is a marked decline in CS activity between pH 8.0 and 7.0. Stromal pH increases from about 7.0 to about 8.0 upon illumination (28), so it is feasible that the activity of CS is stimulated by light. Several stromal enzymes, including ribulose-1,5-bisphosphate carboxylase, fructose-1,6-bisphosphatase and sedoheptulose-1,7-bisphosphatase, have alkaline pH optima and the regulation of their activities by light is one of the important mechanisms involved in the control of the Calvin-Benson cycle (26).

The 20 amino acid N-terminal sequence obtained for CS does not show similarities with *N*-methyltransferase sequence from coffee (16). Many N-terminal sequences have been reported for plant *O*-methyltransferases but there were only a few relating to plant *N*-methyltransferases (29). Genes encoding plant *N*-methyltransferases, putrescine *N*-methyltransferase (30-32), large subunits of ribulose-1,5 bisphosphate carboxylase/oxygenase (33,34), have been cloned, but the amino acid sequences of these enzymes show little homology with the N-terminal sequence of tea CS.

As has been shown with many secondary metabolism pathways (24), the biosynthesis of caffeine is closely related to the stage of development and CS activity

can be detected only in young, expanding tea leaves. Detailed studies on the control of caffeine biosynthesis will be possible when CS antibodies and the cDNA encoding CS protein become available.

### Caffeine Catabolism

The degradation of caffeine to xanthine, which is further catabolised by the purine catabolism pathway via uric acid to  $\text{CO}_2$  and  $\text{NH}_3$ , was first demonstrated in *Coffea arabica* leaves (3,5,35,36). Recent reports on the metabolism of [ $8^{14}\text{C}$ ]caffeine, [ $2^{14}\text{C}$ ]theobromine, [ $8^{14}\text{C}$ ]theophylline and [ $2^{14}\text{C}$ ]xanthine by leaves of coffee and tea, in the presence and absence of allopurinol, which inhibits xanthine dehydrogenase activity, have provided detailed insights into the catabolic pathways that operate in the two species (5,37,38). In both species, [ $2^{14}\text{C}$ ]theobromine is converted almost exclusively to caffeine with little or no release of  $^{14}\text{CO}_2$  demonstrating that theobromine is a precursor and not a catabolite of caffeine. [ $8^{14}\text{C}$ ]Caffeine is degraded very slowly with only trace levels of  $^{14}\text{CO}_2$  being detected. In contrast, [ $8^{14}\text{C}$ ]theophylline was metabolized rapidly by both tea and coffee leaves, indicating that the accumulation of endogenous caffeine in these tissues is a consequence of a lack of adequate 7-demethylase activity to convert caffeine to theophylline.

Leaves of *Coffea salvatrix*, *C. eugenioides*, and *C. bengalensis* contain ca. 3-7-fold lower levels of caffeine than those of *C. arabica*. Degradation of [ $8^{14}\text{C}$ ]caffeine, is negligible in leaves of *C. arabica*, was also very slow in *C. salvatrix* and *C. bengalensis*. In contrast, [ $8^{14}\text{C}$ ]caffeine was catabolised rapidly by young leaves of *C. eugenioides* primarily by a caffeine  $\rightarrow$  theophylline  $\rightarrow$  3-methylxanthine  $\rightarrow$  xanthine  $\rightarrow$  uric acid  $\rightarrow$  allantoin  $\rightarrow$  allantoic acid  $\rightarrow$  urea  $\rightarrow$   $\text{CO}_2 + \text{NH}_3$  pathway. These results indicate that the low caffeine accumulation in *C. salvatrix*, *C. eugenioides*, and *C. bengalensis* is a consequence of a slow rate of caffeine biosynthesis while rapid degradation of caffeine also contributes to the low endogenous caffeine pool in *C. eugenioides* (39).

[ $8^{14}\text{C}$ ]Theophylline absorbed by young, aged and mature leaves of *Coffea arabica* is metabolized rapidly via 3-methylxanthine to xanthine which enters the purine catabolism pathway and is released as  $^{14}\text{CO}_2$ . Likewise, more than 80% of [ $2^{14}\text{C}$ ]xanthine taken up by coffee leaves is released as  $^{14}\text{CO}_2$  over a 42 h incubation period. The inclusion of 5 mM allopurinol in the incubation medium has a major effect on [ $8^{14}\text{C}$ ]theophylline metabolism. The production of  $^{14}\text{CO}_2$  declines dramatically, as a consequence of xanthine degradation being blocked and there is a concomitant increase in the incorporation of label into not only xanthine and 3-methylxanthine, but also 7-methylxanthine. It is especially noteworthy that both [ $8^{14}\text{C}$ ]theophylline and [ $2^{14}\text{C}$ ]xanthine metabolism by aged leaves in the presence of allopurinol results in greater than 70% of the recovered radioactivity being incorporated into 7-methylxanthine. The conversion of xanthine to 7-methylxanthine in such large amounts is unexpected as the established role of 7-methylxanthine is that of a precursor of theobromine in the caffeine biosynthesis pathway. Some form of strict compartmentation would appear to be operating as the allopurinol-induced

accumulation of 7-methylxanthine in coffee leaves was not associated with incorporation of label into either theobromine or caffeine shown in Figure 2 (4).

The main fate of [8<sup>14</sup>-C]theophylline incubated with mature and aged tea leaves, and to a lesser extent young leaves, is conversion to xanthine via 3-methylxanthine, and entry into the purine catabolism pathway (5,38). However, if [8<sup>14</sup>-C]theophylline is fed to young leaves, a significant amount of the label is used for the biosynthesis of caffeine via a 3-methylxanthine→theobromine→caffeine pathway. Trace amounts of [2<sup>14</sup>-C]xanthine are also salvaged for caffeine biosynthesis, and this is increased when purine catabolism is blocked by allopurinol. Salvage of xanthine occurs as a consequence of its conversion to 3-methylxanthine which is metabolized to caffeine via theobromine. Interestingly, feeding [2<sup>14</sup>-C]xanthine to young leaves show that 3-methylxanthine, as well as yielding theobromine, is also converted via 1-methylation to theophylline. Supporting evidence for the existence of these pathways, which are illustrated in Figure 2, comes from *in vitro* studies with *N*-methyltransferase activity from young tea leaves which have shown that (i) xanthine is metabolized to 3-methylxanthine (40,41), (ii) 3-methylxanthine is converted to theophylline, theobromine and caffeine (17), and (iii) theophylline does not act as a methyl acceptor and therefore does not undergo direct conversion to caffeine (7).

There are therefore distinct differences in the purine alkaloid catabolism pathways operating in leaves of *Coffea arabica* and *Camellia sinensis*. In both species caffeine accumulates, because its catabolism to theophylline is blocked in to the purine catabolism pathway which results in its breakdown to CO<sub>2</sub> and NH<sub>3</sub>. In young tea leaves there is detectable salvage of 3-methylxanthine and xanthine and resynthesis of (i) theophylline and (ii) caffeine via theobromine. There is no evidence for the operation of such pathways in coffee leaves. Instead, young, mature and aged *Coffea arabica* leaves, treated with allopurinol, convert xanthine to 7-methylxanthine which does not appear to be metabolized to any extent (Figure 2).

## Purine Alkaloids in Cultured Cells

Purine alkaloid formation in tea callus cultures was first reported by Ogotuga and Northcote (42,43) and by Tsushida and Doi (44). Using *in vitro* *Coffea arabica* cultures, Keller *et al.* (45) made the first observation of caffeine production. Caffeine is formed by *C. arabica* cultures in amounts as high as in the plant, but the productivity of cultured tea cells is significantly lower than that of *C. arabica* cells (11-14). In cultured *C. arabica* cells, caffeine is synthesized by the same biosynthetic pathway as in the plant; however, caffeine and theobromine are released more into the medium (11,12), but caffeine is also accumulated intracellularly to some extent and this is correlated with the 5-*O*-caffeol ester of quinic acid concentration in the cells of *C. arabica* (46,47). Little is known about cellular aspects of metabolism and storage of purine alkaloids in these intact plant tissues.

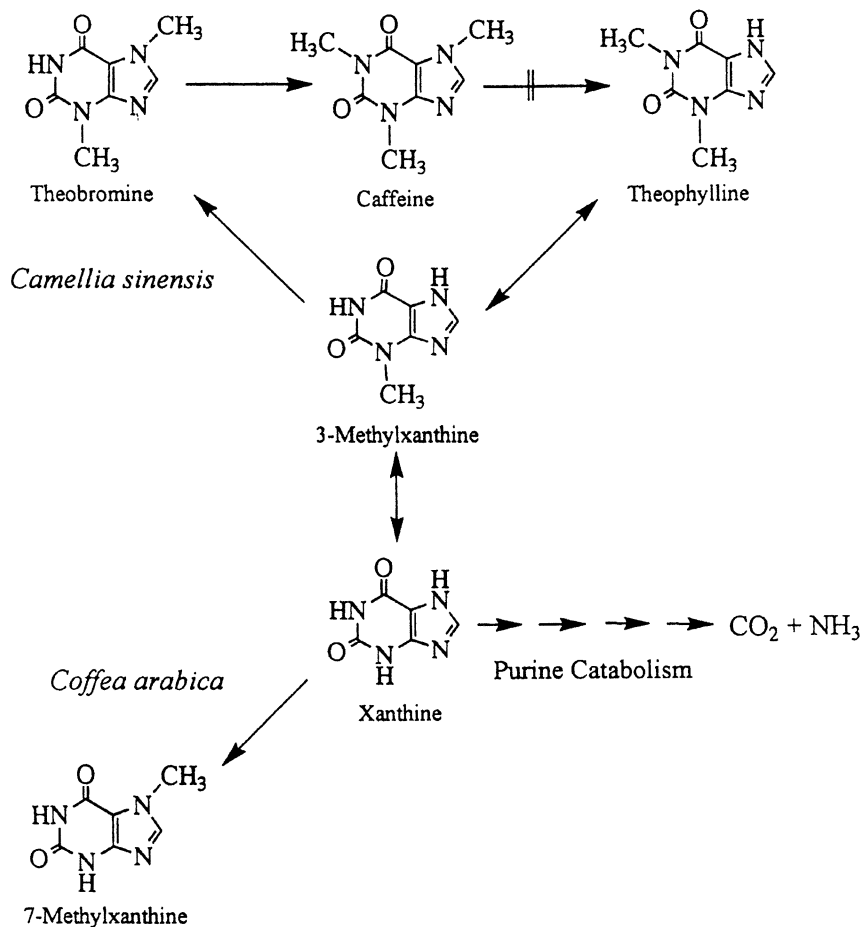


Figure 2. Purine alkaloid catabolic pathways operating in leaves of *Camellia sinensis* and *Coffea arabica*. Arrow with two vertical bars represents a blocked conversion by allopurinol. Double headed arrows indicate reversible conversions. (Reproduced with permission from Reference 34, Copyright 1997, Plant and Cell Physiology, Japanese Society of Plant Physiologists).

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## Abbreviations

CS, caffeine synthase; SAM, S-adenosylmethionine; SAH, S-adenosylhomocysteine; XMP, xanthosine 5'-monophosphate; AMP, adenosine 5'-monophosphate; IMP, inosine 5'-monophosphate; GMP, guanosine 5'-monophosphate.

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## Chapter 3

# Comparing Coffee and Tea

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Similarities in beverage preparation procedures, their twin roles as caffeinated stimulants, and wide familiarity with coffee and tea, encourage the common misconception that their technology and chemistry are similar. One (coffee) originates from a pyrolyzed seed, the other (tea) from a fermented leaf. Other differences include processing temperature, the nature of their oxidation, and the timing and control of enzymatic activity. Flavor development in coffee results from high temperature degradation of carbohydrate, protein, and lipid materials while in tea it stems mainly from oxidation of polyphenols. Differences in amounts of caffeine in the beverage result from differences in raw product processing and variations in beverage preparation practices.

For many years the major scientific interest in caffeine containing foods, such as coffee and tea, resulted mainly from curiosity about the sources of their unique organoleptic properties and in the need for manufacturers and processors to maximize and preserve these particularly pleasing and commercially important qualities. This benign attitude began to change with the rise of the environmental movement in the 1960's and the consequent extension of environmental interest and concern to the health effects of the air, water, and food on the well-being of the public. Caffeine containing foods and beverages, long treated as metabolically insignificant pleasure-providing oral rewards, are in the forefront of this new concern.

Interest in caffeine containing beverages comes as no surprise to those who have long recognized that caffeine is widely known to be a pharmacological active agent which has been listed for at least a century or more as a drug in standard pharmacopoeias and in drug references. Concern for the physiological and behavioral effects of caffeine led logically to questioning its deliberate inclusion in food and beverage formulations as well as the metabolic effect of the foods naturally containing



this active agent. Wide use of caffeine in medications makes a logical case for questioning its presence in a food, whether put there by nature or not.

The almost ubiquitous and long term use of coffee and tea as a common caffeine containing beverage by much of the world's population, coupled with the nearly universal practice of preparing both as beverages by means of simple hot water extraction, leads to an expectation that the two products might be very much alike in other ways. An examination of factors involved in the botanical nature, production, processing, preparation, and composition of these two foods, and their beverages, shows that in fact significant differences exist.

## Comparing the Botanical Nature of Coffee and Tea

Tea of commerce is derived from the young leaf and leaf buds of the tea plant genus *Camellia sinensis* in the *Theaceae* family (1). Coffee, on the other hand, is obtained from the fruit, or more properly from the seed contained within the fruit, of the coffee plant belonging to the genus *Coffea* in the *Rubiaceae* family. The two major coffee species used for commercial purposes are *Coffea arabica* and *Coffea canephora*, and of this latter species particularly the variety *Coffea robusta* (2).

Mature coffee trees generally range in size and can be up to fifty feet high. Tea plants come in a wide variety of shapes and size and can vary from a small bush to medium and tall trees ranging up to sixty feet in height. Both tea and coffee plants produce white fragrant flowers. The tea flowers are globular in shape, about one and one-quarter inches in diameter and are found singly or in pairs, although sometimes there are up to five flowers on a stalk. The coffee flower is star shaped, and usually is found coexisting with green and ripening fruit on the same branch. The coffee fruit resembles cherries and contains a pair of seeds which will eventually become the coffee beans of commerce.

Coffee is commercially cultivated in wide areas of sub-Saharan Africa, South and Central America, Asia, and Oceania. Tea is cultivated over an even wider latitude, ranging from as far north as Georgia in the Trans-Caucasus, through Asia Oceania, Japan, Latin America as far south as Argentina near Santos in Brazil, and throughout Africa down to the upper regions of South Africa. Interestingly enough, since colonial times tea has even been grown here in the continental United States, in South Carolina (3). Though not widely available at retail, it is possible to purchase U.S. grown tea produced at a commercial tea plantation and production facility near Charleston (The Charleston Tea Plantation, Wadmalaw, South Carolina). There is no analogous commercial coffee production in the continental U.S., albeit some commercial coffee (Kona) is grown and processed in Hawaii. This coffee is especially popular on the U.S. West coast.

Though the origin of both beverages is in the shoot section of flowering plants, they are plants of two distinctly different genera. In addition, a most striking difference in the botanical origin of both beverages is that each derives from a different functioning segment of its plant shoot: coffee from the reproductive or propagating structure of its plant genus, that is the seed, and tea from the energy and solar powered photosynthesizing food substance producing structure of its plant

genus, that is the leaf. Finally, although the plants share some similar growing areas they can also tolerate quite different growing regions.

## Comparing Processing Methods for Coffee and Tea

Considering that tea comes from a leaf and coffee from a seed, it is not unexpected that processing of the initial raw material and its eventual conversion into a form from which beverage can be prepared, should be markedly different.

In the case of tea, the leaf is picked and then treated in one of several ways to convert it relatively simply into the form required for marketing for beverage production. With coffee, the seed is initially encased inside a pulpy fruit body from which it has to be separated before it can be prepared by further processing to a form suitable for conversion into a final product suited for beverage production.

### Tea Processing

The bulk of tea leaf is processed by one of three distinctly different methods, depending upon the desired end-product. Most tea leaf is processed for manufacture of Green tea or Black tea. A smaller, but still significant, quantity of leaf is processed to yield Oolong tea (4).

Regardless of which of these methods is used, in the end each results in a product which, except for variations in the method of packaging, is suitable for beverage preparation without need for further processing.

#### *Black Teas*

There are a number of individual variations employed in the processing of the leaf into final product so what follows is a general description.

The leaf is picked or "plucked" from the plant. Plucking can be a hand or machine operation depending on practices prevalent in particular areas or on individual "estates" or farms. The initial processing step is termed "withering" and is a brief moisture reduction period. In simple operations this consists of a sun exposure up to two hours, usually with frequent turning of the leaves. The withering step can last up to sixteen to twenty-four hours. During withering, respiration continues in the leaf with considerable loss of water (25-30%), and the solids content falls slightly.

Withering is followed by a bruising step in which the leaves are gently broken, either by careful manipulation in the hand or by machine, a step termed "rolling". The object is to break open the vein structure of the leaves and allow leaking of internal juices out onto the surface of the leaf, thus permitting exposure of the leaf fluids to air. A typical rolling step lasts for about half an hour. After rolling, the leaf may be spread out and then rolled again. This process of spreading and re-rolling may be carried out two to as many as six times. This allows for a varying period of "fermentation", so-called though it is not a microbial action but an oxidative enzymatic process which is taking place. It is generally believed that for best color and organoleptic

characteristics, the total fermentation time should not exceed three hours. In general, temperatures around 60-70°F are best. Care must be taken that leaf temperature not exceed 80°F in order to avoid premature enzyme inactivation. The number of rollings and the time of fermentation varies depending upon the degree of flavor and color development desired. Fermentation is “killed” or stopped by a process of “firing” or heating the leaves to a temperature above 160°F, usually beginning with an initial temperature of 180-220°F, thus stopping the oxidative process by inactivating enzymatic activity. In addition, there is reduction in the microbial load and further removal of moisture from the leaf. Cooling may be followed by additional alternate rolling and continued drying until the leaves reach the desired moisture (usually below 3%), aroma, and color. By the end of this process the leaf has taken on the dry crisp characteristics associated with the tea product of retail commerce.

### *Green Teas*

In the case of Green tea, it is necessary to avoid the oxidative enzymatic activity so carefully encouraged for Black tea production.

The leaf must be heat treated as soon as possible after plucking in order to inactivate oxidative enzymes. In some cases, where there is a relatively long time lapse between plucking and a subsequent heating step, special effort is made to avoid sun exposure. The leaf may be deliberately cooled in order to minimize or prevent natural enzyme activity until the leaf can be carefully heat treated to inactivate the enzyme. This is accomplished by “roasting” or “firing”, or by steam treatment. After initial heat treatment, the leaf is rapidly cooled. The leaf is again alternately “rolled” and “roasted”, followed by cooling until the desired degree of processing is completed and the leaves have taken on the desired dark greenish to blue-olive color and the leaf has been dried to the extent where it can no longer be easily manipulated (around 15-17%). A final drying normally reduces the moisture level to under 4% although some Green teas are found with moisture levels as high as 10%.

### *Oolong Teas*

Oolong teas represent a middle ground between Black and Green teas. They are considered semi-fermented and therefore their processing closely resembles that of Black tea.

After plucking, the leaf is “withered”, traditionally by a short sun exposure. Withering is followed by cooling and a relatively gentle turning and leaf manipulation. Though not as vigorous as that involved in Black tea production, this still induces oxidative enzyme activity, or “fermentation”. Fermentation is allowed to continue until development of a reddish color and characteristic aroma, but the process is halted well short of the degree of development usually associated with Black tea. The enzymatic process is “killed” by “firing”, or dry heating, and then followed by “rolling” in the manner previously described for Black tea preparation. This is then followed by additional rolling and firing until the leaves are appropriately dry. There are a number of variations of Oolong produced depending upon the practices of individual factories. In some cases there may be a mixture of darker and lighter reddish, as well as yellow-green, leaf mixed into the tea.

## Coffee Processing

Once the snowy flowers have disappeared from the coffee tree, it takes about two months for the fruit, or green berries, to appear. After an additional seven months, these berries, now called cherries, are ripe for picking.

Cherries are picked one of two ways. In one case, all of the coffee berries, ripe, overripe, and underripe, are stripped from the branches. This can be done manually or by machine. The other way is to manually pick only the red ripe cherries and go back over the trees several times as the berries ripen. In either case, the problem of extracting the green coffee bean from the picked cherry remains.

Two different methods are used to accomplish this separation: the "dry" method or the "wet" method, so-called. Generally, the "dry" method is used where water is not plentiful and the amount of sun is abundant, for example in Southern Brazil. Conversely, the "wet" method is used primarily where water is abundant, for example, in Colombia (5). Which-ever method is used, its purpose is to free the bean from the fruit and remove the bean's outer hull, its parchment, and the gummy layer between them.

Whereas processing methods for tea leaf, result at the estate in a product ready for direct beverage preparation, coffee bean processing methods do not yield a beverage-preparation ready product at the facenda or plantation. The coffee product must still go through several additional down-stream processing operations (roasting, grinding, and also packaging) before being ready for beverage preparation.

### *The "Dry" Method*

In the "dry" method the picked cherries are spread on the ground in thin layers to dry in the sun. They are covered at night and during inclement weather. Artificial drying in hot air tunnels is also used. After drying, the cherries are sent through a hulling machine where the outer hull and parchment are removed by mechanical abrasion. Some screening by size or sorting by quality may take place. The resulting green beans are then bagged (60 Kilo/bag) for shipment to the green coffee market. Green coffee prepared by the "dry" method is known as "unwashed" or "natural" coffee.

### *The "Wet" Method*

In the "wet method the fruit is generally fully mature. The picked cherries are fed into large concrete tanks filled with water. The ripe cherries tend to sink while the unripe and defective berries float and are skimmed off. The beans are then run through a sluice into a pulping machine where they are gently separated from their pulpy covering under streams of water. Mucilage on the beans must then be removed.

This can be done in one of several ways. In the fermentation method, the depulped beans are placed into another concrete holding vat for 12 to 24 hours. During this time the mucilage material digests so that the parchment enveloping the beans can be washed free of this gummy matter. In some few cases, the mucilage may be removed by treatment with chemicals such as lime or alkaline carbonate. It is also possible to use mechanical pulping and mucilage removing methods. Generally, the

method used for *robusta* coffees is mechanical while fermentation is used for *arabica* coffees.

Regardless of the mucilage removal method used, the resulting beans are washed and then dried, either by spreading out on concrete aprons in the sun or by artificial means. The dried beans are then fed into a hulling machine to remove the parchment and silverskin, thus freeing the bean. There may be some size screening following which the beans are bagged (60 Kilo/bag) for shipment to the green coffee market. Green coffee prepared by the “wet” method is also known as “washed” coffee and is generally of higher grade than “naturals”.

#### *Flavor Development (Roasting)*

Green coffee beans leaving the *facendas*, unlike the fully flavor developed tea products leaving the estates, are not directly suitable for beverage preparation because the chemical mix responsible for the characteristic taste and aroma (flavor) have not yet been produced in the product. Green coffee beans must undergo a roasting process to develop the appropriate chemical make-up. Roasting is usually carried out in a manufacturing plant near the retail distribution system into which roasted coffee will move. Chemicals responsible for coffee flavor derive from a controlled high temperature conversion or partial pyrolysis of the initial chemicals composing the green bean

The roasting process itself usually involves a roaster, most often a gas-fired rotating oven. Bean temperatures usually range between 390<sup>0</sup>F to 430<sup>0</sup>F, although some specialty coffees can go well above this, to about 450<sup>0</sup>F, near the point of charring. The time of roasting is quite critical to flavor development and is inversely related to the temperatures employed.

Because time and temperature are critical to proper flavor chemical development in the bean, rapid cooling at the end-point of the roast is carefully controlled. Rapid cooling is frequently achieved by introduction of water into the roasting coffee at the end of the roast time, a process known as “quenching”. Because the green beans lose water due to vaporization during the roasting process, the addition of water at the end of roasting restores a small part of that loss and provides for rapid cooling below the pyrolysis point. Care must be taken not to add too much quench water since there is an inverse relationship between the amount of water added and the quality and stability of the flavor developed. Final cooling to room temperature is usually by means of forced air circulation through a bed of the beans.

During the roasting process there is great change in both the chemical make-up of the coffee and in its bulk density. Because the bean is plant material, as the pyrolysis progresses, large volumes of gases produced during the heat induced chemical reactions are trapped inside the bean structure. The cell walls are quite flexible. Thus the bean expands noticeably during the roasting process. Near the end of roasting there is a rapid, almost explosive, sudden expansion of the bean resulting from the large internal pressures developed by expanding pyrolysis gases trapped inside the bean. The volume of the roasted bean is approximately 50% greater than its original. Much of the gas released is carbon dioxide. However, large volumes of gas still remain trapped inside the intact internal cellular structure of the bean.

At this point, the coffee bean is capable of being used for beverage preparation in most applications.

### *Grinding*

Again in a process unnecessary for tea manufacture, the roasted coffee must be ground to a small particle size so that it can finally be used for beverage preparation. While grinding is easily performed by most beverage preparers, many commercial coffee marketers carry out this operation for consumers who wish it. Because of this commercial practice and the significance of particle size reduction to beverage quality, a few words about grinding are in order.

During the grinding process large volumes of carbon dioxide are evolved as the cellular structure of the bean is broken and the trapped pyrolysis gases are released. Before the bean is ground, these trapped gases help protect the labile flavor compounds inside the bean from oxidation by contact with air. The high outward internal pressures of the bean prevent oxygen from easy access to the delicate flavor chemicals. The degree of size reduction or grinding is carefully controlled and monitored because this process greatly affects the rate of extraction of substances responsible for coffee flavor.

## **Comparing Flavor Stability of Coffee and Tea**

At the point coffee or tea has been processed into its pre-beverage preparation form, it is accepted that it possesses optimum flavor. As time passes, product quality is subject to various potentially degrading natural effects, such as temperature, oxygen, and cross flavor contamination. The extent of these effects and the steps taken to minimize them differ for each product.

### **Tea Packaging and Shelf-life**

Finished tea leaf has a relatively long commercial shelf-life. It is usually packed in foil lined plywood "tea chests" where no special effort is made to exclude air. It is important that the product be protected from light, possible wetting, or cross-aroma contamination from other volatile flavored materials (6). Tea is repackaged for consumer use in a variety of materials and sizes depending on the market to which it is directed, but again there is no special effort needed to maintain the integrity of the product other than protection against the factors already noted.

Teas stored at  $-40^{\circ}\text{F}$  or  $0^{\circ}\text{F}$  were comparable in organoleptic quality over a 40 week term whereas samples stored at refrigerator temperatures ( $36^{\circ}\text{F}$ ) were less preferred, while samples stored at room temperatures ( $68-98^{\circ}\text{F}$ ) developed objectionable qualities. Extension of the storage period to 60 weeks accentuated these differences, with the teas stored above  $68^{\circ}\text{F}$  rated unacceptable. Comparable results were reported for changes in color (7).

## Coffee Packaging and Shelf-life

Roasted coffee has a relatively short commercial shelf-life. Coffee can be packaged for consumer use in either ground or whole roasted bean form. In either case, the commercial shelf-life is seriously impacted by the after-roast release of large volumes of entrapped gasses, including the rich aromatic flavor volatiles and carbon dioxide. Grinding accelerates the rate of release. After passage into the surrounding atmosphere, unless protected in some manner, the coffee is highly vulnerable to oxidative staling. For this reason roasted coffee, particularly if ground, must be packaged to limit oxygen exposure. Use of oxygen barrier films, vacuum packaging, packaging in an inert gas (nitrogen) or carbon dioxide atmosphere, and storage at low temperature are all employed in an attempt to minimize oxidative deterioration. Fortunately, coffee exudes large volumes of carbon dioxide for some time (weeks) which results in a net positive pressure inside flexible film packs, and thus limits the amount of ambient oxygen which can enter the package. This can result in a problem unique to coffee in that the volume of gas evolving from the coffee can be great enough to cause package distortion or actual rupture. This problem can be minimized by use of one-way valve packaging and/or allowing an initial "gassing off" period before sealing freshly roasted and ground coffee into non-rigid relatively gas-tight packaging.

## Beverage Preparation and Caffeine levels for Coffee and Tea

Both coffee and tea beverage preparation require a hot water extraction, commonly referred to as brewing. However, the preparation of tea beverage is also termed infusion or steeping, stressing the need for a degree of contact time during the extraction process. Both require a residual hot water contact time around 3-5 minutes, depending upon water temperature and particle size.

Water temperature requirements for both are similar but not the same. Tea, brewing water should be close to the boiling point, ie 205-212°F. For coffee, the most favorable temperatures range from 195-205°F. Taste panel studies with coffee have shown a reduction in preference when higher brewing temperatures are used (8) or when any reheating of beverage occurs (9).

Under these circumstances it is easy to understand why the common perception is to view the two products in a similar light. Of course, as already pointed out, a myriad of differences between the two products exist. Coffee beverage flavor originates from the controlled high temperature partial pyrolysis (roasting) of a seed. Variations in the time and temperature of roasting produces corresponding variations in the qualitative nature of the flavor and color of the eventual coffee beverage. Tea beverage flavor stems from a moderate temperature, physically induced, oxidative "fermentation" of the leaf constituent chemicals by naturally present tea leaf enzymes. Heat inactivation of the enzymes at any point during the process (e.g.: by steaming or "firing") inactivates the enzymes and halts the process. Variations in the extent of this process results in subsequent variations in the color and flavor of the eventual tea beverage.

Obviously, the different flavors of the two beverages stem from their different chemical makeup. Still, they do share the distinction of being caffeine containing products. Due to an essentially intact leaf structure, the rate of caffeine extraction from tea may be comparatively slower than from the high surface area particles of ground coffee. However, since caffeine is readily soluble, the usual brewing times for both beverages are more than long enough for very high extraction rates to occur in both. The caffeine derived from coffee and tea is chemically the same, therefore there is some common thought that the two beverages are similar as caffeine delivery vehicles. Though both beverages are rich sources of caffeine, the average caffeine content of tea beverage (in the U.S.) is about 30 .45 mg/cup while that of coffee beverage is about 60-120 mg/cup (10). Since the caffeine content of roasted coffee beans is about 1%, compared to 2-3% for tea leaf, the question is often raised as to why coffee beverage caffeine content is so much higher than tea beverage. Arithmetic provides the obvious answer. The usual cups per pound yield of beverage from coffee is about 60-90, while that of tea is 200-400. Since a cup of tea is prepared using less caffeine-containing material as a source, the result is less caffeine in the finished beverage.

## Conclusion

Though similar in beverage preparation method, consumer use, and caffeine delivery system, coffee and tea are obviously different in source, processing method, and chemical composition. Despite the temptation to focus on their similarity as consumer beverages, their differences should be considered in examining the physiological roles they play during normal metabolic processes.

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## Chapter 4

### Behavioral Effects of Caffeine

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The present paper will give a review of 10 years of our research on the effects of caffeine on cognition and mood. Research has demonstrated that caffeine has beneficial effects on performance of tasks requiring sustained attention and that these effects are readily observed in low alertness situations (after lunch; at night; when a person has a cold). Although many of the studies of caffeine have used very large single doses, recent studies have demonstrated that beneficial effects can be observed with more realistic doses and drinking regimes that equate to real-life intake. Furthermore, the improved performance can be demonstrated using simulations of real-life tasks and in the context of a normal working day.

#### An Overview of the Published Literature

Smith (*1*) has reviewed the published literature on caffeine and behavior. This review has been sub-divided into a number of different sections and the conclusions about each section are summarized below.

The aim of the article was to review the effects of caffeine on the behavior of adult humans. The main areas covered were effects on mood, the efficiency of mental performance and sleep. In all areas it is important to make a distinction between effects of amounts of caffeine that are normally consumed from food and drinks and the very different effects observed with excessive amounts or in very sensitive individuals. Unlike other areas of research (e.g. studies of health effects) most studies of the behavioral effects of caffeine have examined acute changes following a single dose. Less is known about effects of regular consumption although there is now enough data on this topic to draw tentative conclusions. In addition to studying effects of caffeine consumption the research has also considered possible changes in behavior as a function of caffeine withdrawal. This issue is usually considered in the context of whether caffeine leads to dependence and this

topic is not reviewed here. Rather a critical appraisal of claims that caffeine withdrawal influences performance and mood is provided.

Overall, the literature suggests that the following effects may occur when individuals consume moderate amounts of caffeine:

1. Caffeine increases alertness and reduces fatigue. This may be especially important in low arousal situations (e.g. working at night).
2. Caffeine improves performance on vigilance tasks and simple tasks which require sustained response. Again, these effects are often clearest when alertness is reduced although there is evidence that benefits may still occur when the person is unimpaired.
3. Effects on more complex tasks (e.g. memory tasks) are difficult to assess and probably involve interactions between the caffeine and other variables which increase alertness (e.g. personality and time of day).
4. In contrast to the effects of caffeine consumption, withdrawal of caffeine has few effects on performance. There is often an increase in negative mood following withdrawal of caffeine but such effects may largely reflect the expectancies of the volunteers and the failure to conduct "blind" studies.
5. Regular caffeine usage appears to be beneficial, with higher users having better mental functioning.
6. Most people are very good at controlling their caffeine consumption to maximise the above positive effects. For example, the pattern of consumption over the day shows that caffeine is often consumed to increase alertness. Indeed, many people do not consume much caffeine later in the day as it is important not to be alert when you want to go to sleep.

In contrast to effects found with normal caffeine intake, there are studies which have demonstrated negative effects when very large amounts are given or sensitive groups (e.g. patients with anxiety disorders) studied. In this context caffeine has been shown to increase anxiety and impair sleep. There is also some evidence that fine motor control may be impaired as a function of the increase in anxiety.

Overall, the global picture that emerges depends on whether one focuses on effects that are likely to be present when caffeine is consumed in moderation by the majority of the population or on the effects found in extreme conditions. The evidence clearly shows that levels of caffeine consumed by most people have largely positive effects on behavior. Like most things excessive consumption will lead to problems and there are also some individuals who are more sensitive than others.

### **Effects of Low Doses of Caffeine on Mood and Performance**

Many of the generalizations about the behavioral effects of caffeine are based on studies which have used very large doses of caffeine, used students as subjects and tested them in the early morning only. It is clearly desirable to have further

information on the effects of the lower doses of caffeine which are more typically consumed, to examine effects in a representative sample of the population at several times of day and to consider the possible modifying effects of habitual caffeine consumption and individual differences.

Smith et al. (2) carried out a study to examine the above issues and comparing 60 mg caffeine with placebo. The results showed that even this dose of caffeine led to improved mood and enhanced encoding of new information. Interactions between caffeine and other factors were observed although these were usually specific to particular tasks. In other words, the general effects of caffeine were not influenced by demographic factors, time of testing, personality or habitual caffeine usage.

### **Importance of the Mode of Administering the Caffeine**

It is important to consider whether effects of caffeine are modified by the vehicle in which it is administered. Smith, Sturges and Gallagher (3) examined this issue in a study with the following features.

An experiment was carried out to examine the effects of 40 mg of caffeine given in different drinks (coffee, water, tea, cola) on mood and performance. One hundred and forty four volunteers were randomly assigned to one of the groups formed by combining the caffeine/placebo and drinks conditions. Following a baseline session measuring mood and different aspects of performance the volunteers were given their drink and then carried out another test session one hour later. Administration of the caffeine/placebo was double blind. The results showed that those given caffeine reported greater alertness and anxiety at the end of the test session as well as improved performance on choice reaction time tasks involving focused attention and categoric search, a semantic memory task and a delayed recognition memory task. The effect of the caffeine was not modified by the nature of the drink in which it was given.

Overall, these results show that a dose of caffeine typical of the level found in commercial products can improve alertness and performance efficiency.

### **Effects of Regular Consumption Levels**

About 25% of the participants in the above study consumed virtually no caffeine on a day to day basis. These volunteers showed similar beneficial effects of caffeine to those who regularly consumed greater amounts. This finding has been replicated in another as yet unpublished study and the presence of beneficial effects of caffeine in very low/non-consumers argues against the beneficial effects of caffeine merely reflecting removal of the negative effects of caffeine withdrawal. These findings also support the view that regular levels of caffeine consumption have little effect on the caffeine-induced behavioral changes (4).

## **Caffeine and caffeine withdrawal**

In the majority of studies of caffeine the volunteers have caffeine withdrawn (usually over night) prior to testing. Positive effects of caffeine could, therefore, reflect removal of impairments produced by withdrawal. However, several studies (4, 5, 6) have demonstrated that one obtains similar effects of caffeine when volunteers have had caffeine withdrawn for at least 8 hours and when they have been free to consume caffeine prior to the experiment. Such results cause problems for an explanation based solely on the effects of caffeine withdrawal.

## **Consumption Regime**

Most studies of the effects of caffeine have administered a single large dose, often equivalent to the person's total daily consumption level. Caffeine is usually ingested in a number of smaller doses and it is unclear whether effects observed after a single large dose are the same as those produced by an identical level produced by consuming several caffeine containing drinks over a longer time period. Brice and Smith (unpublished) examined this issue and found that the improved mood and enhanced performance found after a single dose of 200 mg were also observed following 4 doses of 65 mg given at hourly intervals (which resulted in an identical final level to the single 200 mg dose).

## **Metabolism of Caffeine**

Most of the beneficial effects of caffeine show a linear dose-response relationship up to about 300 mg and this is then followed by either a flattening of the curve or, sometimes, impaired performance at higher doses. If one looks at the relationship between metabolism of a fixed dose of caffeine (as indicated by saliva levels) and mood and performance changes one finds that there is no strong association between the two. This is not too surprising in that it is not caffeine levels in the periphery per se which produce the behavioral changes but secondary CNS mechanisms. The individual differences in the metabolism of the caffeine may be very different from the individual differences in the CNS mechanisms which, plausibly, accounts for the lack of a strong association between plasma (or saliva levels) and behavioral changes.

## **Effects of Caffeine on Real-life Performance**

The previous sections have shown that doses of caffeine typically consumed in real-life, and presented in commercial products, can improve mood and aspects of

performance. The majority of studies have been laboratory experiments using artificial tasks. It is now important to ask whether similar effects are observed in simulations of real-life activities (e.g. driving).

### **Caffeine and Driving**

A number of studies (e.g. 8) show that caffeine can improve driving performance of fatigued drivers. In a recent as yet unpublished study caffeine was found to improve steering accuracy in drivers carrying out a one hour drive. Measures of mood and performance of artificial tasks were also taken during the last study and these also showed benefits of consuming caffeine. This suggests that changes in the laboratory may reflect a general benefit of caffeine that is also observed in real-life situations.

### **Changes Over the Working Day**

Another method of assessing the effects of caffeine involves monitoring changes over the course of the working day. Indeed, if performance is measured before starting work and then again at the end of the working day, then the difference between the two times reflects performance efficiency over the course of the day. Smith et al. (9) examined both the effects of controlled consumption and free choice of caffeinated drinks over the course of the day.

The results showed that caffeine consumption was greatest in the morning and that similar diurnal trends were seen in free living and free choice conditions. Consumption of decaffeinated drinks led to a reduction in alertness over the day and also slowed response times. Indeed, regular monitoring of alertness showed that decaffeinated drinks were associated with reduced alertness from 10.00 to 19.00. In the free living condition high caffeine consumption was associated with faster reaction times in the evening. Furthermore, there was evidence to suggest that it was the subjects with lower levels of alertness who subsequently drank more caffeine. Overall, these results demonstrate that caffeine consumption maximizes alertness over the working day and is often consumed to produce this effect.

## **Mechanisms Underlying the Effects of Caffeine on Mood and Performance**

It is highly likely that many different CNS mechanisms underlie the effects of caffeine on behavior. Caffeine's major effect is as an antagonist of the adenosine receptors which in turn affects the release of a variety of neurotransmitters (e.g. noradrenaline, acetylcholine, dopamine and the GABA/benzodiazepine system). It is important to link specific behavioral changes with CNS mechanisms and also to

develop profiles of the stages of information processing involved in and the energetics underlying the effects of caffeine.

Mechanisms can also be considered at the level of the cognitive processes and energetical mechanisms influenced by caffeine. Smith, Clark and Gallagher (10) conducted a study to elucidate the stages of processing underlying enhanced performance by caffeine of choice reaction time tasks. In addition, they attempted to identify the energetics of the mood and cardiovascular effects produced by caffeine. Ingestion of caffeine had no effect on initial mood but it did improve the encoding of new information and counteracted the fatigue that developed over the test session, resulting in greater subjective alertness at the end of the session in the caffeine condition.

Another approach to understanding the CNS mechanisms underlying the effects of caffeine has been to combine pharmacological challenges with administration of caffeine. It has been demonstrated that caffeine is especially beneficial when alertness is reduced. Alertness can be reduced by changing a number of the neurotransmitter systems. For example, by using clonidine, a drug which reduces the turnover of central noradrenaline, it is possible to mimic sleep deprivation in a period of a few hours. Smith et al. (unpublished) conducted a study combining caffeine/placebo and clonidine/placebo conditions. Caffeine was found to reverse the effects of clonidine but produce few effects when the volunteers were alert. However, some effects of caffeine (e.g. the beneficial effect on encoding of new information; the cardiovascular effects) were not related to changes in the noradrenergic system. Indeed, Rusted and Smith (unpublished) have shown that the encoding of new information reflects the cholinergic system and there is evidence from other studies that caffeine also influences this neurotransmitter.

## Conclusions

The published literature on the effects of caffeine on behavior shows that it increases alertness, reduces fatigue and leads to improved performance of vigilance tasks and simple tasks requiring sustained response. Our research shows that these effects are most apparent when alertness is reduced by other factors (e.g. sleep deprivation, working at night, prolonged work, consumption of lunch and minor illness). Our recent research suggests that these effects can be obtained with realistic doses of caffeine (and normal consumption regimes) and that the performance improvements can be observed using simulations of real-life activities (e.g. driving) and by assessing changes over the working day. The positive effects of caffeine can be observed in very low/non-consumers which suggests that withdrawal does not underlie the effects. This conclusion is supported by studies which have shown similar effects of caffeine in withdrawn and non-withdrawn volunteers. The mechanisms underlying these effects are now being identified. Caffeine improves the encoding of new stimuli and this may reflect cholinergic changes. In addition, it

improves performance when central noradrenaline is reduced which may be the mechanism underlying the large effects of caffeine in low alertness situations.

Effects on more complex tasks are less clear and probably depend on complex interactions between caffeine and other variables (e.g. personality and time of day). Indeed, the combined effects of caffeine and other variables is clearly an area which requires further study. Negative effects of caffeine have been found with extremely high levels of consumption and in certain sensitive individuals (e.g. patients with anxiety disorders). These effects must, however, be distinguished from the behavioral changes observed when caffeine is consumed in moderation by the majority of the population. Generally, consumption of caffeine is well controlled and the pattern of consumption suggests that individuals ingest caffeine to help restore levels of reduced alertness to a more optimum level. Similarly, consumption is reduced when high alertness is undesirable, such as when the person is trying to sleep.

### Acknowledgements

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## Chapter 5

# Why We Drink Caffeine-Containing Beverages, and the Equivocal Benefits of Regular Caffeine Intake for Mood and Cognitive Performance

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The consumption of caffeine-containing beverages may be motivated to a significant extent by anticipation of benefiting from the stimulant properties of caffeine, though almost certainly more important is the consumer's acquired liking for the beverage. The latter is reinforced, at least in part, by the psychopharmacological consequences of caffeine ingestion. Crucially, this reinforcement appears to occur most readily in the context of acute caffeine abstinence, an observation which in turn is consistent with recent findings on the impact of caffeine and caffeine withdrawal on mood and cognitive performance. These findings indicate that 'withdrawal relief' or 'reinstatement' is a major effect of caffeine consumption in everyday life, leading to the conclusion that the actual or net benefits of regular caffeine consumption are considerably smaller than the felt benefits.

### Why Caffeine is Important

Caffeine is the most widely and frequently consumed drug in the world. Coffee and tea account for the vast majority of this intake, which has been estimated at 70 mg of caffeine per person per day (1). This is about a cup-of-coffee equivalent of caffeine consumed by every person on earth every day. Therefore, even if caffeine has only a small effect on the health and functioning of individuals, its overall impact on human well-being is likely to be very significant. This impact will occur due to the effects of caffeine itself and through the effects of other constituents of caffeine-containing beverages. Some of these constituents may be beneficial to health, such as antioxidant compounds consumed in tea (2), whereas others may have adverse effects. For example, cafestol and kahweol, present in boiled and certain other styles of coffee, have been shown to markedly raise serum cholesterol concentrations (3). The evidence for beneficial effects of caffeine is also mixed. The generally accepted view that caffeine is a useful psychostimulant is challenged by findings showing negative



effects of short-term (e.g., overnight) caffeine abstinence in regular caffeine consumers, including fatigue, headache and impaired psychomotor performance (4, and see below). There is also concern that caffeine consumption has adverse effects on, for example, cardiovascular health. Experimental studies show that caffeine can increase blood pressure, but unfortunately the epidemiological data relevant to this issue are mostly flawed by inadequate measurement of caffeine exposure (5).

This variety of effects and their complexity clearly make it difficult to determine the net impact on health of the regular consumption of caffeine-containing beverages. Two aspects of this question are considered in the present paper. Both are about caffeine. The first concerns the role of caffeine in motivating the consumption of caffeine-containing beverages (and thereby indirectly promoting the intake of the other constituents of these beverages), and the second concerns recent evidence about the likely overall impact of caffeine intake on mood and cognitive performance.

### Why We Consume Caffeine-containing Beverages

One factor influencing the consumption of caffeine-containing beverages is a recognition of their potential psychostimulant properties. Thus the coffee drinker may choose to consume strong coffee at breakfast for its expected alerting effects, but may then avoid coffee late in the evening because of the expectation that consumption of caffeine can lead to difficulty in getting to sleep (6). However, if people are asked why they drink coffee or other caffeine-containing beverages, they are more likely to say that this is because they like the 'taste' of the beverage – people typically do not consume coffee as if it were a medicine, being prepared to tolerate its taste in the expectation of a benefit. At the same time, it is fairly certain that human beings are not born with a liking for the taste and flavour of either coffee or tea, at least partly, because these beverages contain bitter constituents, including caffeine, and bitterness is innately aversive (7). This then raises the question of how people come to acquire a liking for the sensory qualities of these beverages.

An important way in which preferences are modified is through the association of the orosensory and postingestive effects of eating and drinking (reviewed in 8). The most dramatic example of this is the strong and specific aversions which can develop when consumption of a food is followed by gastrointestinal illness. Similarly, there is now good evidence that association of a taste or flavour paired with positive postingestive consequences can result in increased preference for that specific taste or flavour. For example, a small number of studies on human participants have demonstrated conditioned increases in preference in children and adults for flavours associated with high carbohydrate content and high fat content.

A feature of these conditioned preferences and aversions is that they appear to involve changes in palatability or liking (i.e., alterations in the individual's affective response to the food's taste and flavour). Booth, for example, has said that "conditioned aversion is a nasty taste, not merely or at all the refusal to take something because it is perceived as dangerous" (9, page 566). Similarly, Sclafani proposes that pairing a flavour with intragastric nutrient infusion produces "changes in the rat's

evaluation of the flavour" (10, page 422). The most direct support for these conclusions comes from studies of the taste-elicited, fixed-action patterns displayed by rats (11). In these experiments solutions are infused into the animal's mouth through a fixed intraoral catheter, and its behaviour is recorded on video tape for subsequent detailed analysis. Aversive reactions, for example, to innately disliked bitter tastants, include gaping movements, chin rubs, head shakes and paw wipes, varying in intensity and frequency. Aversions established by flavour-illness pairings result in a shift away from acceptance reactions towards this aversive pattern even for the innately liked taste of sucrose, which was interpreted as showing not that this taste is now perceived as bitter, but simply as unpleasant. Furthermore, it has also been argued that the assignment of positive or negative values to conditioned stimuli is a basic feature of all Pavlovian conditioning (12).

Studies on conditioned preferences have demonstrated the existence of a capacity to adapt liking for a food according to the benefit or otherwise derived from consuming that food. Not surprisingly, the main focus of this research has been on the 'nutritional' effects (e.g., high versus low energy density) of eating and drinking. However, certain constituents of foods and drinks can also have potent pharmacological activity, caffeine and alcohol being the most obvious examples. Perhaps, therefore, liking for coffee, tea, beer, wine etc. are reinforced by the psychoactive effects of caffeine and alcohol (6, 13, 14).

Recently, this suggestion was tested directly in studies in which caffeine ingestion was paired with the consumption of novel-flavoured fruit juices. Caffeine was given either in the beverage or in a capsule swallowed with the beverage. A beverage of a different flavour was given without caffeine or with a placebo capsule. The design of these studies is, therefore, similar in principle to methods used in the work on flavour preferences conditioned by nutrient manipulations, and the straightforward prediction is that if caffeine has beneficial effects (e.g., on mood) then pairing consumption of a beverage with the consumption of caffeine should promote increased liking for that beverage. Initial results showed that under these circumstances caffeine was not a strong positive reinforcer, although it did have aversive effects (15) and, more importantly, it appeared to act as a negative reinforcer by removing or alleviating the negative effects of overnight caffeine abstinence (16). In this latter study caffeine was found to have no significant effects on beverage liking or mood in participants with habitually low intakes of caffeine, whereas moderate consumers of caffeine (mean caffeine intake of 205 mg/day) developed a relative dislike for the beverage lacking caffeine. These moderate consumers, moreover, showed lowered mood following overnight caffeine abstinence (e.g., less lively, clearheaded and cheerful), and this was significantly alleviated by caffeine.

An earlier study indicated similar effects in rats (17). Rats which had been previously given caffeine by injection for 12 consecutive days developed a relative aversion for a novel taste (saccharin) paired with the absence of caffeine. In addition, aversion for saccharin was also seen when this taste was paired with the injection of caffeine in caffeine-naïve rats. Again, these results point to a negative but not a positive reinforcing effect of caffeine. That is, caffeine acts most reliably as a reinforcer in the context of caffeine withdrawal, and perhaps when relieving other

negative states such as feelings of fatigue and tiredness experienced by some people soon after lunch (the 'post-lunch dip') (18).

This general conclusion is strengthened further by a replication and extension of the results reported by Rogers et al. (16). In this fourth conditioning study (19), participants were again moderate caffeine consumers deprived of caffeine overnight before each daily trial. Their liking for a herb tea beverage was significantly higher after four trials when it was paired with caffeine than when it was consumed without caffeine, demonstrating reliable and rapid conditioning. A very similar effect was observed for a caffeinated versus non-caffeinated fruit-juice beverage consumed 2 hours later on each trial, but only for participants who had not received caffeine in the herb tea. In other words, caffeine reinforced liking when participants were caffeine deprived but apparently not when they had recently received caffeine – although it remains possible that the non-deprived participants did learn about the caffeine content of the fruit juice, but failed to express this learning in their non-deprived state (if this were the case, this would be an example of irrelevant incentive learning).

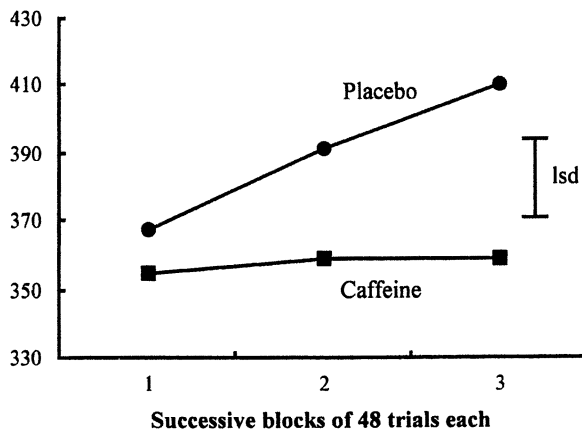
### **Equivocal Psychostimulant Benefits of Caffeine Consumption in Everyday Life**

As suggested above, caffeine appears to be widely regarded as a useful psychostimulant. It is, for example, available as an over-the-counter remedy to "relieve tiredness and help maintain mental alertness", and stopping for a cup of coffee is recommended as a cure for driver fatigue and sleepiness. However, recent reviews of the scientific literature on this subject have concluded that the evidence of a net psychostimulant benefit of regular caffeine consumption is equivocal, even suggesting that mood and performance are adversely affected overall (4, 5).

In many placebo-controlled studies acute administration of caffeine has been found to increase self-ratings of alertness, to improve mood, and to enhance psychomotor and cognitive performance. An example is presented in Figure 1, which shows significant differences in performance after caffeine versus placebo treatments during the last two thirds of a long-duration simple reaction time task. The most obvious conclusion from such findings is that caffeine improves performance by ameliorating the effects of fatigue. However, in this as in the vast majority of studies on the psychostimulant effects of caffeine the study participants had a history of regular caffeine consumption, and they were tested on caffeine and placebo after a period of (overnight) caffeine abstinence. What this experimental protocol leaves open is the question of whether the results obtained are due to beneficial effects of caffeine or to deleterious effects of caffeine deprivation. That is, the poorer performance on placebo might be due to the fatiguing effects of caffeine withdrawal, which are reversed by caffeine administration, thereby merely reinstating performance to a 'normal' level (i.e., the level displayed by individuals completely free of the effects of caffeine and caffeine deprivation).

The impact of caffeine withdrawal is confirmed by many results showing temporary adverse effects of short-term (e.g., from 10 hours to 3 days) caffeine

abstinence in regular consumers, including increased headache, drowsiness and fatigue (4, 5, 20). For instance, a study of the acute and chronic effects of caffeine withdrawal compared morning mood in caffeine consumers and non-consumers (21). The group of regular consumers, with a mean caffeine intake of 250 mg/day, was divided into three matched sub-groups who avoided all significant sources of caffeine for either 1.5 hours (given 70 mg caffeine after overnight abstinence from caffeine), 13 hours, or at least 7 days, before beginning an intensive morning test session. Two patterns were apparent in the results. First, the 13-hour deprived group showed markedly greater levels of tiredness and drowsiness, and were more angry and dejected compared with all of the other groups, including the non-consumers, who did not differ significantly on these moods. The second pattern was less definite, but tended to be characterised by poorer mood (e.g., lower clearheadedness and cheerfulness) and more headache in both the



*Figure 1. Performance of moderate to high caffeine consumers (mean daily caffeine intake 453 mg) on a long duration simple reaction time task. These participants were deprived of caffeine overnight and then given caffeine (1.2 mg/kg) or placebo 45 minutes before testing began at 11 am. Each trial consisted of a 'get ready' signal and, to prevent anticipatory responding, an interval of 1, 3, 7 or 16 seconds before stimulus onset. Participants were instructed to press the space bar on the computer keyboard as quickly as possible when they detected the stimulus (a star appearing in the centre of the computer screen). The vertical bar is the least significant difference at the 5% significance level. (M. Robelin and P. J. Rogers, unpublished results.)*

13-hour and 7-day groups. Other data collected during the 7-day period, when the participants were given regular or decaffeinated coffee and tea to consume, confirmed the different time-courses of these adverse effects of caffeine abstinence. That is,

increased headache and depressed mood appeared to persist for at least 7 days during withdrawal, whereas the effects on alertness were relatively short-lived. Moreover, these various adverse symptoms resulting from caffeine deprivation cannot be explained as 'expectancy' effects, because participants were blind to the administration of caffeine or placebo both during chronic deprivation and after overnight deprivation. Similar findings from a study comparing just overnight abstinent caffeine consumers with non-consumers were published over 30 years ago (22).

The finding that caffeine withdrawal is associated with fatigue leads, in turn, to the prediction that psychomotor and cognitive performance will also be disrupted by caffeine deprivation, although until very recently this had not been tested adequately. Comparing the effects of caffeine in caffeine consumers and non-consumers provides one approach, but these groups are self-selected and therefore pre-existing differences might account for any differences found in response to caffeine administration. The strongest test is to measure the psychostimulant effects of an acute caffeine versus placebo challenge in long-term caffeine-deprived consumers compared with overnight-abstinent consumers. The withdrawal-relief hypothesis predicts that in the placebo condition long-term deprived participants will perform better than overnight-abstinent participants, and that acute caffeine administration will affect performance only of the latter participants, bringing their level of performance up to but not exceeding that of the long-term deprived participants. More or less exactly this result was obtained in two independent studies, one using a letter recognition memory task (23) and the other using the long-duration simple reaction time task shown in Figure 1 (24). Moreover, the latter study indicated, if anything, a detrimental effect of caffeine on reaction time performance of caffeine consumers deprived of caffeine for 9 days, which might have arisen indirectly due to an adverse effect of caffeine such as 'jitteriness'. This is a frequently reported symptom occurring in normally caffeine-abstinent individuals given caffeine (e.g., 22).

An important feature of both of these studies on the effects of caffeine in relation to acute and chronic caffeine abstinence is that the acute caffeine challenge involved administration of several moderate doses of caffeine across a testing period lasting until early afternoon. This models the typical pattern of caffeine intake of regular caffeine consumers, and at the same time rules out the possibility that the failure of caffeine to affect performance in the context of long-term caffeine abstinence, or to raise performance above 'baseline' in overnight abstinent participants, was due to the administration of inadequate amounts of caffeine on the challenge day. Nor can these results be explained in terms of a 'ceiling effect', since performance was always below the maximum achievable level (see also 25).

Recent evidence therefore strongly suggests that although regular caffeine consumers feel a benefit from caffeine intake, especially first thing in the morning after overnight caffeine abstinence, the actual or real benefits gained for mood and performance are very small, or even negative overall (because of the repeated adverse effects of overnight caffeine abstinence). A small number of studies have claimed to show that caffeine administration can improve cognitive performance in non-caffeine-deprived individuals (e.g., 26, 27). However, with this approach it is always conceivable that there was some residual caffeine deprivation which was responsible

for the poorer performance in the placebo condition. The only unequivocal way to test for a net benefit of caffeine consumption is to carry out further studies of the effects of caffeine in long-term abstinent caffeine consumers. It may be that under certain conditions, for example when fatigued by sleep deprivation or after eating a large meal (e.g., 18), real benefits of caffeine consumption will be demonstrated. At present such evidence does not appear to have been published.

## Conclusions

The evidence reviewed above contradicts the widely held view that caffeine consumption benefits mood and cognitive performance. Recent studies on caffeine reinforcement and on the mood and performance effects of acute and chronic caffeine deprivation are consistent in showing that the major effect of caffeine for the regular caffeine consumer is withdrawal relief or reinstatement. These results also help to, but do not fully, explain the enormous popularity of caffeine-containing beverages. Thus negatively reinforced liking for the vehicles (coffee, tea, etc.) in which caffeine is present may be the single most important factor motivating consumption of these beverages, but other effects of regular caffeine intake are probably critical as well. For example, daily consumption presumably would be much reduced if there was no tolerance to potentially aversive effects of caffeine, such as the jitteriness reported by caffeine-naïve individuals (22). Apart from caffeine, thirst may motivate consumption of these beverages on at least some occasions, and liking could be reinforced by the consequent reduction of fluid need. Added milk, cream and/or sugar may also significantly affect liking by directly improving the sensory appeal of the beverage, through nutrient reinforcement, or through flavour-flavour conditioning (14). In the latter, the response to a neutral or even disliked flavour is modified as a result of being paired with an already liked flavour or sweet taste. Finally, situational influences will play a role, by cueing an appetite for the beverage (cf. 8), through social facilitation, and through indirect effects on, for example, mood. Caffeine-containing beverages are very often consumed in social contexts and during breaks from work (e.g., the 'tea break') or other activities. Positive shifts in mood occurring in these situations paired with drinking coffee, tea, cola, etc. may result in a conditioned increase in liking for the beverage.

Perhaps the most telling finding from this recent research is the marked negative impact of overnight caffeine abstinence. Fatigue and headache resulting from longer periods of caffeine abstinence, such as may occur at weekends, have been widely recognised (20), but the implication of these overnight effects is that tiredness and negative mood are experienced on a daily basis by caffeine consumers (who are the majority of the population). These symptoms are present on getting up in the morning and are removed by the consumption of caffeine. Caffeine intake does not, however, appear to improve mood or cognitive performance above this 'baseline'. In other words, while the functioning of the typical regular caffeine consumer might be restored soon after breakfast, the overall impact of caffeine consumption remains negative. Furthermore, on this basis, individuals who consume caffeine fairly

infrequently, including many children, could be even more adversely affected. At present there is uncertainty about the net benefit or harm accruing from caffeine consumption, but this is an improvement on the uncritical acceptance of research that apparently showed clear benefits for mood and performance but which failed to take into account the impact of caffeine withdrawal.

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## Chapter 6

# Caffeine Effects on the Brain and Behavior: A Metabolic Approach

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Caffeine is a worldwide consumed stimulant with known effects on behavior and sleep while its addictive potential is debated. With [<sup>14</sup>C]2-deoxyglucose autoradiography which allows to correlate cerebral functional activity to the behavioral effects of a drug, we studied the effects of increasing doses of caffeine on cerebral glucose utilization in adult rats. At 1 mg/kg, caffeine activated the caudate nucleus involved in the control of locomotion, the raphe nuclei and locus coeruleus mediating mood and sleep. After 2.5 and 5 mg/kg caffeine, metabolic activation spread mainly to the other brain regions mediating locomotion. The functional activation of the shell of the nucleus accumbens, involved in addiction and reward was only induced by 10 mg/kg caffeine. Thus, conversely to amphetamine and cocaine which quite specifically activate the latter structure at low doses, the caffeine-induced activation of the nucleus accumbens shell occurred together with that most other brain regions. These data confirm the high sensitivity of locomotion, mood and sleep to low doses of caffeine and provide functional evidence of the lack of addictive potential of caffeine at the usual level of human consumption.

Caffeine is the most widely used psychoactive substance in the world. Caffeine is consumed in a variety of forms, i.e., drinking coffee, tea, mate, soft drinks; chewing cola nuts; consuming cocoa products; taking over-the-counter pain or slimming medication. The mean daily caffeine consumption for all adult consumers and from all sources reaches 2.4-4.0 mg/kg for a 60-70 kg subject in the North America and the UK and 7.0 mg/kg in Scandinavia (3,4). Among children under 18, the mean daily intake of caffeine is about 1.0 mg/kg in the United States and less than 2.5 mg/kg in Denmark (3). Mild positive subjective effects occur at low

to moderate doses of caffeine (50-300 mg, i.e., 1-3 cups of coffee) and are described as feelings of well-being, alertness, energy and ability to concentrate. At high doses (300-800 mg), rather negative feelings such as anxiety, nervousness, insomnia are reported (for review, see refs. 1 and 2). Most of the effects of caffeine on the central nervous system reflect its antagonism at the level of adenosine receptors (1,5,6).

Here, I will review the effects of caffeine on cerebral glucose utilization in order to correlate the data with the well-known effects of caffeine on functions known to be sensitive to caffeine such as locomotor activity, mood and sleep and I will also consider the possible dependence to caffeine.

## Caffeine and Cerebral Energy Metabolism

The effects of caffeine on cerebral metabolic rates for glucose (LCMRgls) described in this review have been explored by the quantitative autoradiographic [ $^{14}\text{C}$ ]2-deoxyglucose method (7) which allows the simultaneous visualization of functional activity in discrete areas of the brain of conscious animals. This technique permits the identification of neuronal pathways affected by a pharmacological agent and is very useful for relating behavioral effects to the central action of a drug.

### Effects of Caffeine on the Nigrostriatal Dopaminergic System and on Locomotor Activity

The nigrostriatal dopaminergic system originates in neurons located in the substantia nigra, mainly in the pars compacta and to a lesser extent in the pars reticulata. They project via the median forebrain bundle and lateral hypothalamus to the globus pallidus and terminate in the caudate nucleus (8,9). This system is involved in the control of locomotion.

The caudate nucleus appears to be very sensitive to the effects of caffeine since LCMRglc is already significantly increased in this structure after the administration of the lowest dose of caffeine, 1 mg/kg to adult male rats (Figure 1). The functional activity of this nucleus is further increased at 2.5 mg/kg, about 40% over control levels and remains activated after 5 and 10 mg/kg caffeine. In the substantia nigra pars compacta and globus pallidus, LCMRglc increases after 2.5-10 mg/kg of caffeine while the sensorimotor cortex shows an increase in LCMRgls only after 5 mg/kg of caffeine. In accordance with the present data, it has been shown that a direct iontophoretic administration of caffeine modifies the spontaneous electrical activity of neurons in the caudate nucleus (10), and that caffeine is able to induce dopamine release (11,12) and the expression of immediate early genes in the caudate nucleus of the rat (13,14).

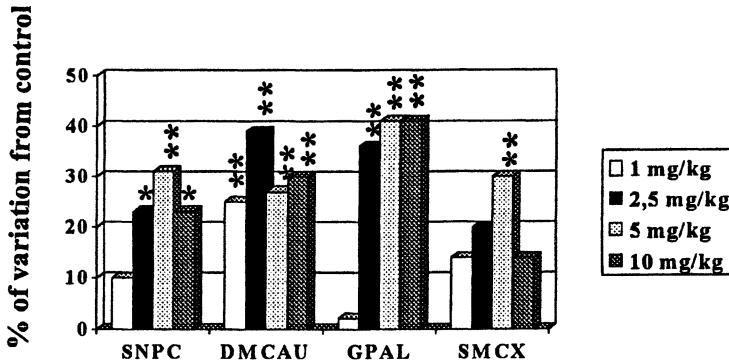
There is a good correlation between caffeine-induced functional activation of structures belonging to the nigrostriatal pathway and the well-known stimulant effects of caffeine on locomotor activity. This effect is dose-dependent; the minimal

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dose of caffeine necessary to affect locomotion is 1.5 mg/kg which correlates well with the increase in LCMRglc recorded in the caudate nucleus after 1 mg/kg caffeine. The stimulant effect of caffeine on locomotion increases with doses up to 20 mg/kg and decreases with doses higher than 40 mg/kg (1,2).

## Nigrostriatal dopaminergic system



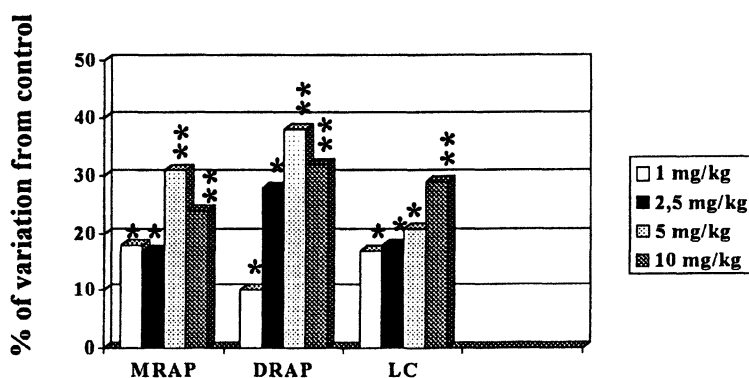
*Figure 1: Effects of increasing doses of caffeine on LCMRglcs. Data are presented as percent of variation from control values. Rats received caffeine i.v. at 15 min before the onset of the [ $^{14}\text{C}$ ]2-deoxyglucose procedure (13). Abbreviations: SNPC: substantia nigra pars compacta, DMCAU: dorsomedial caudate nucleus, GPAL: globus pallidus, SMCX: sensorimotor cortex. \*  $p < 0.05$ , \*\*  $p < 0.01$ , statistically significant differences from controls (Dunnett's  $t$ -test for multiple comparisons).*

### Effects of Caffeine on the Noradrenergic and Serotonergic Cell Groupings and on the Sleep-Wake Cycle

The serotonergic cell groupings, the medial and dorsal raphe nuclei as well as the noradrenergic cell grouping, the locus coeruleus are very sensitive to caffeine. These structures are involved in the control of sleep, mood and well-being (18). In the 3 structures, LCMRglcs are already activated after 1 mg/kg and remain increased at the higher doses of caffeine used, 2.5-10 mg/kg (Figure 2). These data

correlate well with the known sensitivity of sleep and mood to caffeine (18,19). Indeed, in the rat, caffeine stimulates spontaneous electrical activity in neurons of the reticular formation; this effect appears at low doses, 1-2.5 mg/kg i.v. and its extent and duration increase with the dose (20,21). Caffeine (0.1-0.5 mg/kg) lowers also electrical activity in the thalamus which appears as an important site for the arousal induced by caffeine (22,23). Moreover, caffeine reduces serotonin availability at postsynaptic receptor sites (24) which elicits a reduction in the sedative effect of the amine on activity and influences sleep mechanisms and motor function (25,26).

## Noradrenergic and serotonergic cell groupings



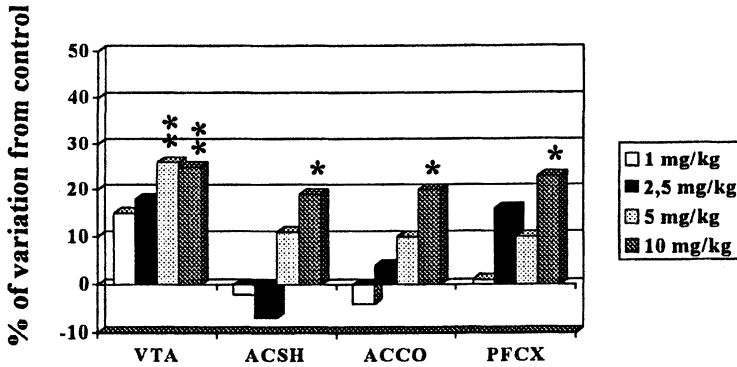
*Figure 2: Effects of increasing doses of caffeine on LCMRglcs. Data represent percent of variation from control values. Rats received caffeine i.v. at 15 min before the onset of the [ $^{14}$ C]2-deoxyglucose procedure (13). Abbreviations: MRAP: medial raphe, DRAP: dorsal raphe, LC: locus coeruleus. \*  $p < 0.05$ , \*\*  $p < 0.01$ , statistically significant differences from controls (Dunnnett's  $t$ -test for multiple comparisons).*

### Effects of Caffeine on the Mesolimbic Dopaminergic System and Addictive Effects

The possible dependence on caffeine has been questioned for over a decade (27-31). The mesolimbic dopaminergic system which plays a critical role in drug

dependence (32) originates in the ventral tegmental area, projects to the nucleus accumbens and terminates in the medial prefrontal cortex. The nucleus accumbens that plays a central role in the mechanism of drug dependence is divided into a core and a shell part. The medioventral shell is related to the limbic “extended amygdala” assumed to play a role in emotional, motivational and reward functions, whereas the laterodorsal core regulates somatomotor functions (33). The specificity of cocaine, amphetamine, morphine, alcohol and nicotine is to selectively activate dopamine release in the shell of the nucleus accumbens (34,35), a property that has been related to the strong addictive properties of these drugs (32,36). Conversely caffeine at doses ranging from 0.5-5 mg/kg does not trigger any release of dopamine in the shell of the nucleus accumbens (37).

### Mesolimbic dopaminergic system



*Figure 3: Effects of increasing doses of caffeine on LCMRglcs. Data are presented as percent of variation from control values. Rats received caffeine i.v. at 15 min before the onset of the [<sup>14</sup>C]2-deoxyglucose procedure (13). Abbreviations: VTA: ventral tegmental area, ACSH, nucleus accumbens, shell, ACCO: nucleus accumbens, core, PFCX: medial prefrontal cortex. \*  $p < 0.05$ , \*\*  $p < 0.01$ , statistically significant differences from controls (Dunnett's t-test for multiple comparisons).*

Our data show that the increase in LCMRglcs recorded in the structures of the mesolimbic dopaminergic system are of lower amplitude than those recorded in

the other brain regions studied (Figure 3). Moreover the significant activation of functional activity appears only at quite high doses, 5 mg/kg for the area of origin, the ventral tegmental area and 10 mg/kg for the two subdivisions of the nucleus accumbens and the medial prefrontal cortex. These data show that at the doses daily consumed by most people (2-2.5 mg/kg), caffeine does not activate the brain circuitry of dependence and reward. Moreover, the activation of functional activity in the shell of the nucleus accumbens occurs only at high doses of caffeine (10 mg/kg, i.e., about 4-5 times the average daily human consumption) at which the methylxanthine activates also the core of the nucleus and induces widespread non specific metabolic increases in a majority of brain regions (1,15-17). These widespread effects of high doses of caffeine on brain functional activity are likely to reflect the numerous adverse side effects of the ingestion of large amounts of caffeine. Conversely the effects of amphetamine, cocaine and nicotine on the neural substrates underlying addiction are rather specific and occur at doses that do not usually lead to the activation of many other brain regions (35,38,39).

The difference in the functional consequences of the psychostimulants, cocaine and amphetamine compared to caffeine could relate to their respective mechanism of action. Amphetamine and cocaine induce a release or inhibit the uptake of dopamine (40) which will bind to both D1 and D2 dopamine receptors in the striatum. At low doses caffeine acts preferentially at the level of adenosine A2a receptors (41) that are mainly found in the striatum where they colocalize with dopamine D2 receptors (42). When the circulating levels of caffeine increase, the methylxanthine binds also to adenosine A1 receptors (41) which are among other regions located in the striatum where they colocalize with D1 dopamine receptors (42). In the present study, it appears that caffeine mimics the effects of amphetamine and cocaine only at rather high doses (10 mg/kg) when the binding to A1 adenosine receptors is likely to occur.

## Conclusion

The areas controlling locomotor activity and the sleep-wake cycle appear to be highly sensitive to low concentrations of caffeine while the structures involved in addiction and reward are only activated after high doses of caffeine. These doses activate also numerous brain regions and are likely to induce also the adverse effects occurring after the ingestion of large doses of caffeine (4,43). Our data are rather in accordance with the reported observation that the effects of caffeine are used consciously or unconsciously to manage the mood state and to alleviate the adverse effects of caffeine deprivation (44,45). Moreover, human caffeine consumption is fractionated over the day while the doses given in the present study were injected as an i.v. bolus. Thus, the present data are rather in favor of caffeine acting as a positive reinforcer at doses reflecting the general human consumption and do not support the participation of the brain circuitry of addiction and reward in the dependence on caffeine reported even at very low doses (1 cup/day) in a recent human study (31).

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## Chapter 7

### The Anticancer Activity of Coffee Beans

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Earlier studies have shown that green coffee beans (GCB), defatted GCB, and GCB oil can inhibit the development of cancer in animals. The purpose of this study was to see what effect roasting might have on this activity. Sixty-eight hamsters were separated into 4 groups. The animals in group 1 were fed a normal diet, while the hamsters in groups 2, 3, and 4 were fed the same diet supplemented with 15% roasted coffee beans (RCB), 12.75% defatted RCB, or 2.25% RCB oil. The pouches of the hamsters were painted 36 times (3 x weekly) with a 0.5% solution of the carcinogen, 7,12-dimethylbenz[a]anthracene. The results showed that each of the special diets significantly reduced tumor burden. The mean values in mm<sup>3</sup> were 238 (1), 29 (2), 38 (3), and 115 (4).

#### General Introduction

In less than 400 years coffee has gone from a regional drink consumed primarily in Arab countries to an international beverage consumed throughout the world. Today more than five million tons are produced annually in approximately 50 coffee-growing countries. In international trade, green coffee beans are the second most important commodity in the world. In most of the industrialized countries of the world, coffee drinkers on average consume 3-5 cups per day. In 1991, the average was 3.4 cups per day for consumers in the United States (1).

A variety of factors contribute to the chemical mixture that is labeled a cup of coffee. Some of these factors include the soil where the coffee is grown, the variety of the coffee plant, the degree of roasting, and the way the coffee is prepared. With so many variables, it is not surprising that each cup of coffee contains several hundred different chemicals. Some chemicals have been identified, others await discovery. Interest in the chemical composition of coffee partially stems from the fact that a large number of these chemicals contribute to the unique aroma and

flavor of the beverage. A second reason is that a cup of coffee has very little nutritional value. Consumption is instead linked to a stimulant, caffeine. Since coffee contains at least one pharmacologically active agent, it has been postulated that coffee might contain other chemicals, which might affect the health and well being of the consumer.

### **Mammary Carcinogenesis**

Work on the cancer chemopreventive activity of green coffee beans and coffee bean chemicals was initiated in the early 1980's in Dr. Lee Wattenberg's laboratory at the University of Minnesota. In the first experiment, it was shown that the addition of 20% green coffee beans to the diets of the experimental animals inhibited by approximately 50-60% the development of carcinogen-induced mammary tumors (2). Further research led to the isolation of two antineoplastic agents (3,4). The chemicals, kahweol and cafestol, are both diterpenes that are structurally similar. The only difference is an additional double bond that is found in one of the rings in kahweol (4). The cancer chemopreventive activity of these two plant oils was demonstrated in a rat model for mammary carcinogenesis. Repeated exposures to a mixture of kahweol and cafestol followed by a single dose of the carcinogen resulted in a diminished (40%) neoplastic response (5).

Additional studies with kahweol and cafestol indicated that both of these chemicals are blocking agents that induce increases in glutathione S-transferase activity (6). The glutathione S-transferase enzymes form one of the major enzyme systems in the body for the safe removal of complex chemical waste, including carcinogens, from the body (2).

### **Oral Carcinogenesis**

In the late 1980's, we initiated a series of experiments on the antineoplastic activity of green coffee beans, green coffee bean fractions, and coffee chemicals. In each experiment, the hamster cheek pouch model for oral carcinogenesis was utilized (7). The results from the first experiment showed that the addition of 20% green coffee beans to the diets of the experimental animals inhibited by 90-95% the development of the carcinogen-induced oral carcinomas (8).

The second experiment tested the possibility that kahweol and cafestol might be responsible for the antineoplastic activity of green coffee beans seen in this tumor model. With the help of scientists at the Nestle Research Centre in Vers-chez-les-Blanc, Switzerland, we were able to obtain a 50:50 mixture of kahweol and cafestol. Using this mixture a test diet was constructed that approximated the kahweol and cafestol content of a diet containing 20% green coffee beans. The results of this experiment (9) showed that this special diet inhibited the development of carcinogen-induced oral carcinomas by only 35%.

The data from the experiment with the diet containing kahweol and cafestol suggested that green coffee beans might contain other anticancer agents. To test

this possibility an experiment was conducted with green coffee beans and two green coffee bean fractions, green coffee bean oil and defatted green coffee beans. In the Colombian green coffee bean, the oil accounts for approximately 15% of the whole coffee bean. Essentially all of the kahweol and cafestol is in the oil fraction. The residual material accounting for 85% of the green coffee bean is the defatted green coffee bean fraction. The three special diets in this experiment contained either 15% green coffee beans, 12.75% defatted green coffee beans, or 2.25% green coffee bean oil. Each of these special diets significantly inhibited the development of the carcinogen-induced oral tumors. The extent of the inhibition ranged from 70% with whole green coffee beans, to 55% with defatted green coffee beans, to 60% with green coffee bean oil (10,11).

The results of these experiments indicate that the cancer chemopreventive activity of green coffee beans is more complex than originally thought. Instead of two cancer chemopreventive agents, green coffee beans appear to contain multiple chemicals with antineoplastic activity. Some of the chemicals with biological activity are in the oil; others are in the defatted portion of the bean.

The objective of the experiment described in this paper was to see what effect, if any, roasting might have on the cancer chemopreventive activity of coffee beans. During roasting, the beans are heated to 200-230°C and kept at this temperature for 10-15 minutes. This process leads to obvious changes in color and size, as well as, multiple changes in chemical composition. For example, the chemicals that give coffee its unique aroma are formed during the roasting process. A brief report of this research has been published (12).

### **Roasted Coffee Beans and Roasted Coffee Bean Fractions**

For the experiment, 80 female Syrian Golden hamsters (Lak:LVG strain) weighing 80-90 g were purchased from the Charles River Breeding Laboratories (Wilmington, MA). The animals were housed in wire-mesh cages of stainless steel in a temperature-controlled room (22°C) with a 12:12 hour light-dark cycle. Throughout the experiment water and food were furnished *ad libitum*.

After arriving, the hamsters were given 10 days to acclimate. During this time, all of the animals were fed a Purina Lab Chow (St. Louis, MO) specifically formulated for small rodents. After this initial period of adjustment, the hamsters were weighed and randomly divided into one of four equal groups (20 animals per group) and placed on one of four diets. The hamsters in group 1 remained on the Purina Lab Chow. The hamsters in groups 2, 3, and 4 received the same Purina Lab Chow supplemented with either 15% whole roasted coffee beans (group 2), 12.75% defatted roasted coffee beans (group 3), or 2.25% roasted coffee bean oil (group 4). One batch of whole roasted Colombian coffee beans was used to prepare the ingredients used in the diets for groups 2, 3, and 4. On a weight to weight basis, 15% of the Colombian roasted coffee bean is the roasted coffee bean oil fraction and 85% is the defatted roasted coffee bean fraction.

All of the diets were given in powdered form. The diets for the hamsters in groups 2, 3, and 4 were prepared weekly. The animals remained on their respective diets for the rest of the experiment.

The hamsters were given an additional week to adjust to their diets. Seventeen animals were then selected from each group. The left buccal pouches of these animals were painted 3 x weekly with a 0.5% solution of the carcinogen, 7,12-dimethylbenz[a]anthracene (Sigma Chemical Co., St. Louis, MO). Heavy mineral oil was used to prepare this solution. The 3 remaining animals served as controls. The left buccal pouches of these animals were painted 3 x weekly with heavy mineral oil. A #5 camel's-hair brush was used to paint the liquids on the pouches. Each application places approximately 50  $\mu$ l of liquid on the surface of the pouch (9).

After a total of 36 applications, the hamsters were sacrificed by inhalation of an overdose of carbon dioxide. The left buccal pouches were excised and tumors, when present, were counted and measured (length, width, and height). The sum of the three measurements divided by six was used to calculate an average radius for each tumor. This number was then used to determine an approximate volume for the tumor. Since the tumors are exophytic and spherical in shape, the formula for the volume of a sphere,  $4/3\pi r^3$ , was used for this calculation. The sum of the volumes of all of the tumors in a pouch was determined and defined to be the animal's total tumor burden (9,13).

After the gross tumor data were collected, the pouches were mounted on heavy paper and fixed in 10% formalin. The tissues, including the pouches from the control animals, were embedded in paraffin, processed by routine histological techniques, and stained with hematoxylin and eosin. The Student's t-test and Chi-square analysis were used to analyze the significance of the tumor data. These procedures are similar to the procedures used in our earlier experiments with green coffee beans, green coffee bean fractions, and isolated coffee chemicals (8-11).

## Tumor Data

Three hamsters died before the end of the experiment. These deaths occurred relatively early before the animals had a chance to develop tumors. All of these hamsters were excluded from the study.

At the end of the experiment, there were 16 experimental animals in groups 1, 2, and 3 and 17 hamsters in group 4. The tumor incidence data are given in Table I. As indicated, 15 of the 16 animals on the regular chow (group 1) had gross tumors. In 12 of these animals, multiple tumors (2-10) were found. Six of the pouches from the animals on the special diets (groups 2, 3, and 4) were free of visible tumors. Multiple tumors (2-9) were common in two of the three groups (11/16 in group 3 and 13/17 in group 4). In group 2, only 6 of the 16 pouches contained multiple tumors (2-5). The high and low values for the total number of tumors were 70 for group 1 and 29 for group 2.

The data for average tumor number radii, and burden are given in Table II. As illustrated, each of the special diets had an effect on tumor number. Comparing groups 2, 3, and 4 to group 1, it can be seen that the inhibition in average tumor

**Table I. Tumor Incidence**

<i>Group</i>	<i>No. of Animals</i>	<i>No. of Tumor Bearing Animals</i>	<i>Total No. of Tumors</i>
1	16	15 (94%)	70
2	16	13 (81%)	29
3	16	14 (88%)	45
4	17	16 (94%)	66

SOURCE: Adapted from reference 12.

number ranged from a low of 10% for the animals on the roasted coffee bean oil diet (group 4), to 35% for the animals on the defatted roasted coffee bean diet (group 3), to 60% for the animals on the whole roasted coffee bean diet (group 2). Only the differences between groups 1 and 2 were significant (Student's *t*-test). The three diets also had an effect on average tumor radii. Here the extent of the inhibition ranged from a low of 20% for group 4 to 35% for groups 2 and 3.

**Table II. Average Tumor Number, Radii, and Burden**

<i>Group</i>	<i>Avg. No. of Tumors<sup>a</sup></i>	<i>Avg. Tumor Radii (mm)</i>	<i>Avg. Tumor Burden (mm<sup>3</sup>)<sup>a</sup></i>
1	4.4 ± 0.7	2.35	238 ± 65
2	1.8 ± 0.4 <sup>b</sup>	1.55	29 ± 15 <sup>b</sup>
3	2.8 ± 0.6	1.50	38 ± 16 <sup>b</sup>
4	3.9 ± 0.6	1.90	115 ± 33 <sup>c</sup>

<sup>a</sup>Values are means ± S.E.

<sup>b</sup>Statistically different from Group 1, *p*<0.005.

<sup>c</sup>Statistically different from Group 1, *p*<0.05.

SOURCE: Adapted from reference 12.

Since tumor burden takes into account both tumor number and size, the effects of the special diets are magnified in this set of figures. In each case, a significant reduction in tumor burden (Student's *t*-test) was found ranging from 50% for group 4, to 85% for group 3, and 90% for group 2. Another way to compare tumor burden data is to divide the data into two groups. In the past, we have found that the tumor burden data for the control group, in this case group 1, can be split fairly evenly into two subgroups. One subgroup contains the animals with large tumor burden values (greater than 100 mm<sup>3</sup>), while the second subgroup contains animals with small tumor burden values (less than 100 mm<sup>3</sup>). In this experiment, the number of hamsters with large tumor burden values was 8 (group 1), 2 (group 2), 2 (group 3), and 7 (group 4). The number of hamsters with small tumor burden values was 8

(group 1), 14 (group 2), 14 (group 3), and 10 (group 4). From the data, it is obvious that the larger tumor burden values were mainly found in groups 1 and 4. The differences between the four groups were not significant.

Histologically all of the tumors were epidermoid carcinomas. Sections taken from the pouches of the seven experimental animals without any apparent tumors showed signs of dysplasia, ranging from mild to moderate to severe. At the end of the experiment, the average weight of the hamsters was  $223 \pm 4$  g (group 1),  $214 \pm 4$  g (group 2),  $215 \pm 5$  g (group 3), and  $210 \pm 4$  g (group 4). The differences between groups 1 and 2, and groups 1 and 3 were not significant (Student's t-test). The differences between groups 1 and 4 were significant,  $p < 0.05$ . Throughout the course of the experiment the animals in the four groups appeared healthy.

### Comparison of Results: Green and Roasted Coffee Beans

It is difficult to compare the results of two separate experiments; however, in this case, there are two reasons that support a comparison between the earlier results with green coffee beans and green coffee bean fractions and these results with roasted coffee beans and roasted coffee bean fractions. One, the average number of tumors in the control groups were similar, 4.7 in the first experiment and 4.4 in this experiment. Two, the data for the two oil fractions, green and roasted, were similar.

In the first experiment (10,11), the diets containing 15% green coffee beans, 12.75% defatted green coffee beans, and 2.25% green coffee bean oil reduced average tumor number by 45%, 45%, and 25%, respectively, and average tumor burden by 70%, 55%, and 60%, respectively. In this experiment, the diets containing 15% roasted coffee beans, 12.75% defatted roasted coffee beans, and 2.25% roasted coffee bean oil reduced average tumor number by 60%, 35%, and 10%, respectively, and average tumor burden by 90%, 85%, and 50%, respectively. From these results, it is obvious that roasting did not destroy, to any significant extent, the antineoplastic activity associated with the coffee beans and the coffee bean fractions. The data even suggest that roasting increased the anticancer activity of the roasted coffee beans and the defatted roasted coffee bean fraction.

### Potential Relevance for Humans

The potential relevance of this data for the consumers of coffee depends on the coffee bean fraction. For example, the chemicals in the roasted coffee bean oil fraction are usually only found at relatively low concentrations in a cup of coffee. In most forms of brewing, the suspended solids and lipids are removed. In addition, the promising research with kahweol and cafestol (3-6,9) has been negated by results from two recent publications indicating that these chemicals are hypercholesterolemic factors (14,15). With the defatted roasted coffee bean fraction, the situation is different. This fraction contains both the water-soluble chemicals that are extracted during brewing and the coffee solids. There are several reasons to assume that part, if not all, of the anticancer activity of this fraction is due to

chemicals in the water-soluble fraction. One, investigators in two different laboratories have found that a cup of coffee contains one or more chemicals with significant antioxidant activity (16,17). Two, a number of studies have shown that either coffee consumption or chemicals found in a cup of coffee (caffeine, caffeic acid, ferulic acid, and chlorogenic acid) can inhibit the development of carcinogen-induced cancer in animals (18-22).

In a sense, epidemiological evidence on coffee and cancer also supports the idea that the active agents in the roasted defatted coffee beans may have relevance for humans (23-27). For most types of human cancer, the results on coffee consumption and cancer are highly variable; however, a series of epidemiological studies have consistently shown that coffee intake is associated with a significant reduction in colon cancer. The reason for this reduction in relative risk, in some cases approaching 70-80%, is still unknown. Two theories have, however, been advanced to explain this effect. The first is that coffee might inhibit the secretion of bile acids. Bile acids can be metabolized into carcinogens in the lower GI tract. The second is that coffee, like fiber, might speed up the movement of the feces through the lower GI tract. The data on the anticancer activity of roasted defatted coffee beans now suggests a third possibility; the presence of cancer chemopreventive agents.

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## Chapter 8

# Coffee Consumption and Cancer

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Coffee consumption markedly reduces the risk for colon cancer. The incidences of most cancers in the United States, except for lung cancer, have not changed over the last several decades. In experimental chronic animal studies, lifetime coffee consumption did not affect the frequencies of tumors in the test animals (rats and mice). Epidemiology studies have shown no association of coffee consumption with cancer at any organ site. The average odds ratio from epidemiology studies for colon cancer and coffee consumption is 0.78. For studies with benign colon adenomas, the average odds ratio is 0.54. These findings are distributed internationally with reduced colon cancer associated with coffee consumption in Europe, Asia and the United States. Possible mechanisms include the effects of antioxidants in blocking mutations and carcinogen metabolism and increases in glutathione in colonic cells.

Beyond the genetic influences, which can be substantial, the major factors influencing the occurrence of cancer in humans seem to be life style factors (Table I). If one examines the trends of cancer incidences in the United States over the past several decades, it is clear that the trends are not changing substantially, except for lung cancer, for which cigarette smoking is the main cause (Figures 1 and 2) (1). In men, there has been a sharp reduction in the occurrence of stomach cancers, the reasons for which are largely unknown. For women, there is also a reduction in stomach cancers, and also reductions in liver and pancreatic cancers. The occurrences of colon and rectal cancers appear to be stable in both men and women, although there appears to be some reduction in the incidence of the cancers in women over the past several decades (1-3).

Because coffee is such a widely consumed beverage, many experimental and epidemiological studies have been carried out to examine the effects of coffee consumption on cancer occurrence. Among the experimental animal studies, at least four separate studies in both mice and rats receiving coffee at amounts far above that which any human would ever experience reveal no excess of tumors in any of the studies (Table II) (4-8). In fact, in one study in rats there was a significant reduction in breast tumors in the animals consuming coffee (8). Several studies have shown that

caffeine can inhibit tumor formation in experimental systems with mouse skin tumors induced by ultraviolet light (9) and rat lung tumors with a nicotine-derived carcinogen (10)

**Table I. Environmental Contributions to Cancer Mortality**

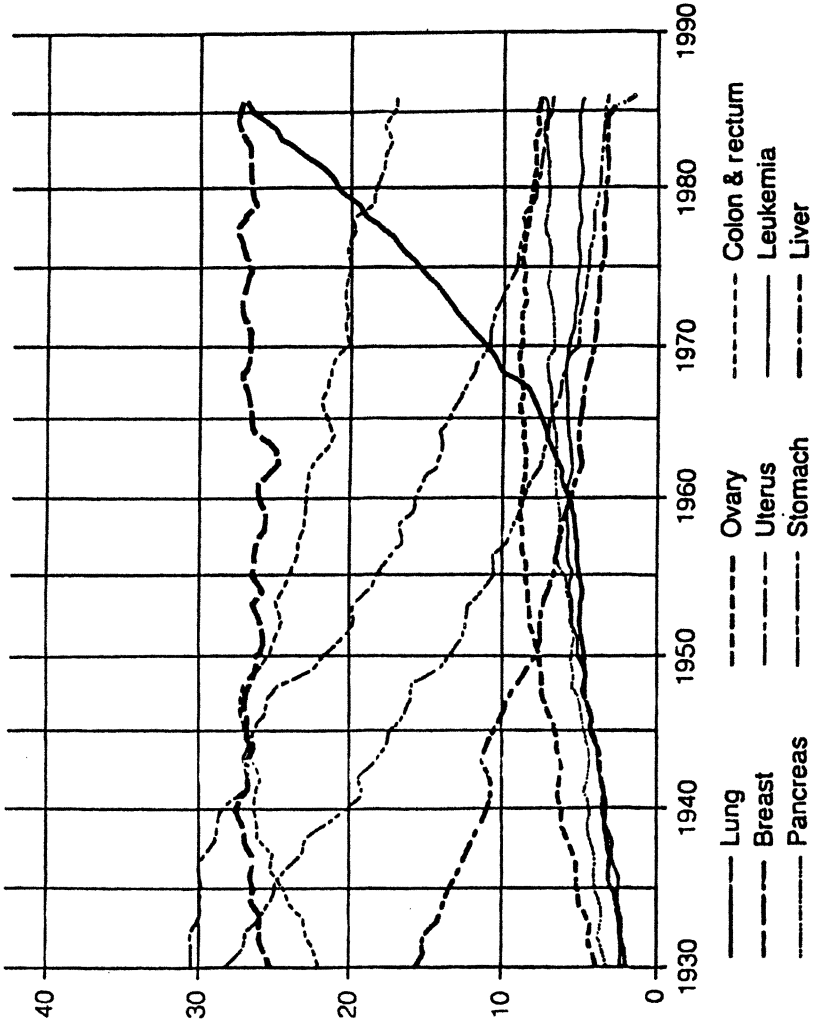
<i>Cause</i>	<i>Percent</i>
Smoking	25-50
Diet	10-70 (35)
Occupation	2-8
Alcohol	2-4
Viruses and parasites	1-10
Radiation	1
Air and water pollution	1

Adapted from ref. 3.

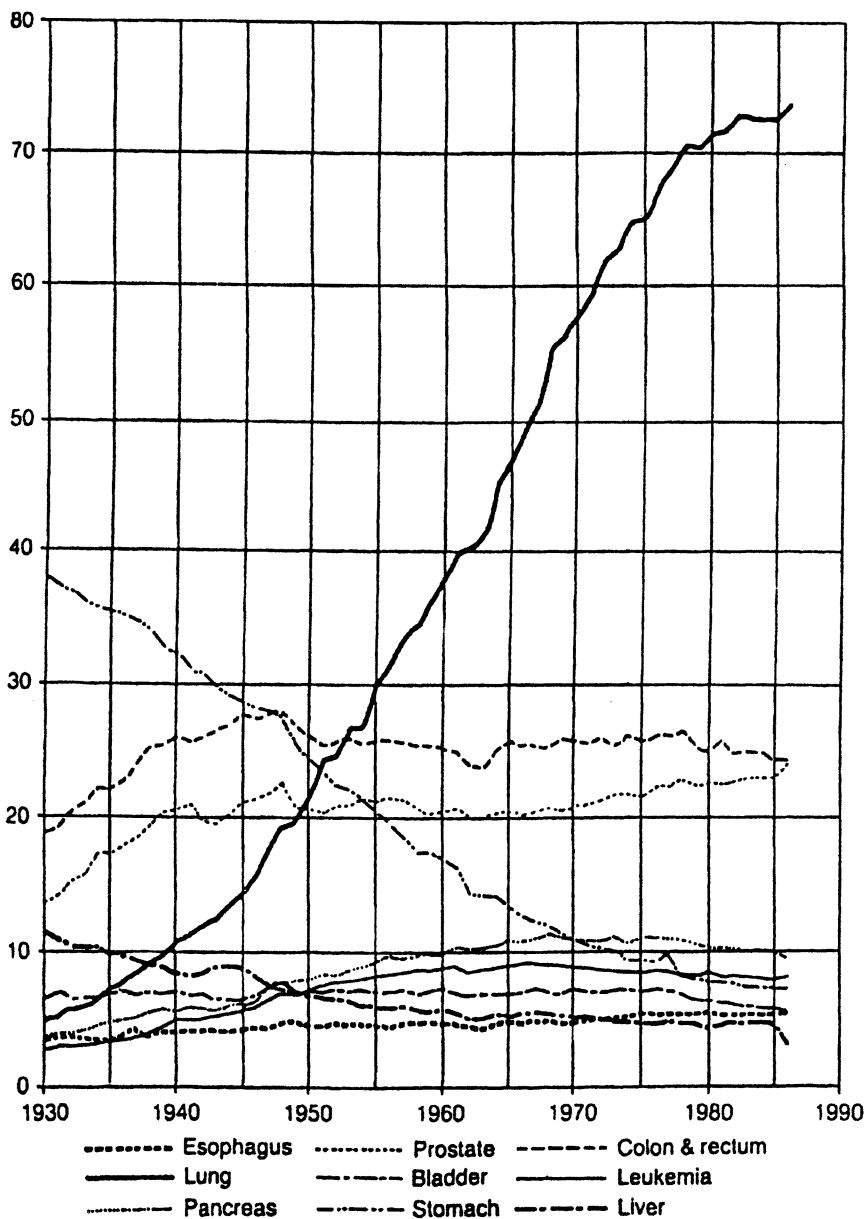
**Table II. Coffee Chronic Animal Carcinogenesis Studies**

<i>Species</i>	<i>Test Substance</i>	<i>Result</i>	<i>Reference</i>
Rats	100% Coffee	No excess tumors	(4)
C57BL6J Mice	100% Coffee	No excess tumors	(5)
Sprague-Dawley Rats	6% in diet, regular and decaffeinated powder	No excess tumors	(6,7)
Sprague-Dawley Rats	100%, 50%, 25% Coffee	No excess tumors	(8)

An examination of the body of epidemiological studies demonstrates that there is little evidence for the increase of cancer associated with coffee consumption at most of the important organ sites (11). An exception to this finding is the repeated observation that the risk of colon cancer is markedly reduced with coffee consumption. These studies have been recently critically reviewed (12). Among the seventeen studies analyzed, all but two showed a substantial reduction in risk of colon cancer as measured by odds ratio. Of these, the case control studies, which would be expected to be the most reliable, showed an average odds ratio of 0.72 for colon cancer associated with coffee consumption (Table III). The two studies that showed some elevated risk were cohort studies in special populations of Mormons and Seventh Day Adventists. The distribution of these findings is worldwide indicating no preference for ethnicity (Table IV). The findings have been demonstrated in populations from the United States, Northern Europe (Belgium, Norway, Sweden), Southern Europe (France, Italy, Spain) and Asia (China, Japan). Figure 3 displays the results of the epidemiology studies in graphic fashion.



1. Age-adjusted cancer rates for women, rates per 100,000 women. (Ref. 1)



**Table III. Colorectal Cancer Study Design**

<i>Study</i>	<i>Number of studies</i>	<i>Number of cases</i>	<i>Odds Ratio</i>	<i>p Value</i>
All Cancer Studies	17	6,192	0.78 (0.66-0.79)	<0.001
Cohort Studies	5	931	0.97 (0.73-1.29)	0.83
Case Control (Total)	12	5,251	0.72 (0.61-0.84)	< 0.001
Case Control (Population)	6	2,244	0.71 (0.53-0.92)	0.01
Case Control (Hospital)	6	3,017	0.74 (0.61-0.90)	0.002
Adenoma Studies	3	883	0.57 (0.44-0.72)	< 0.001

Adapted from ref. 10

**Table IV. Colorectal Cancer - Geographical Location**

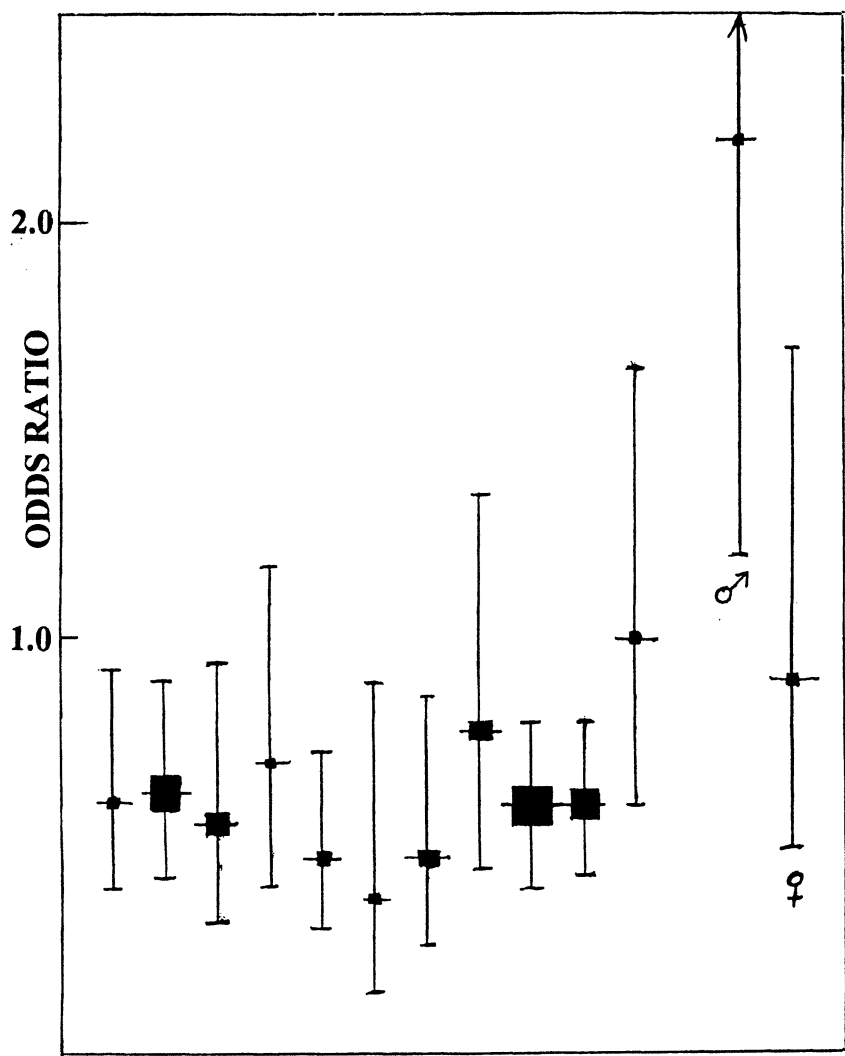
<i>Location</i>	<i>Number of Studies</i>	<i>Number of Cases</i>	<i>Odds Ratio</i>	<i>p Value</i>
Northern Europe <sup>1</sup>	5	1,921	0.65 (0.53-0.79)	<0.001
Southern Europe <sup>2</sup>	6	1,802	0.71 (0.57-0.79)	0.003
United States	3	1,850	0.87 (0.59-1.29)	0.49
Asia <sup>3</sup>	2	424	0.57 (0.44-0.75)	<0.001
Special Populations	2	395	1.45 (0.93-2.28)	

Adapted from ref. 10. <sup>1</sup> Belgium, Norway, Sweden. <sup>2</sup> France, Italy, Spain. <sup>3</sup> China, Japan

The mechanisms for this reduction in colon cancer associated with coffee consumption are not known. Coffee contains a myriad of potentially biologically active substances, among them several antioxidants (13, 14). This class of chemicals has been associated with reduction of carcinogenicity in experimental studies and is a likely class for observations in humans. The mechanisms by which the antioxidants could act are by blocking mutations, inhibiting carcinogen metabolism and inhibiting the proliferation of precancerous cells. A recent report that coffee consumption increases glutathione in colonic mucosa cells offers an additional protective mechanistic prospect.

## Conclusions

The consumption of coffee has been shown not to affect the occurrences of cancer in humans. For colon cancer, there is clear evidence that coffee consumption reduces the risk of colon cancer, reducing the odds ration to about 0.70. This observation has



3. Summary of colorectal cancer epidemiology studies. (Ref. 9). Center of box = odds ratio. Size of boxes is proportional to case number. Length of lines equal to 95% confidence interval.

been found in populations in Europe, Asia and the United States, indicating that ethnicity is not a factor.

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## Chapter 9

# Evaluation of Coffee and Caffeine for Mutagenic, Carcinogenic, and Anticarcinogenic Activity

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The available scientific literature demonstrates that coffee is mutagenic *in vitro* due largely to the presence of methylglyoxal and hydrogen peroxide, but coffee is not mutagenic *in vivo* and its *in vitro* effects are not relevant to humans. Although caffeine induces chromosomal aberrations in plants and in human leukocytes *in vitro*, it is neither mutagenic in several *S. typhimurium* tester strains nor *in vivo*. Both coffee and caffeine have undergone bioassays in rodents for carcinogenicity and are not carcinogenic. Indeed, there is some evidence of a protective effect against cancer in these studies. Also, caffeine in combination with a variety of known rodent carcinogens generally inhibits tumor induction. Furthermore, epidemiologic studies in humans on coffee and total methylxanthine intake and cancer generally suggest no effect or a negative association with various cancers, especially large bowel and female breast cancer.

Beverages made from coffee beans, tea leaves, kola nuts and coca beans have been enjoyed by humans for thousands of years. Because of the long history of use and the wide consumption of these caffeine containing beverages, the potential adverse or beneficial effects of these beverages and caffeine have been studied, especially during the last several decades. Numerous studies have been carried out in several laboratories to evaluate the mutagenic, carcinogenic, and anticarcinogenic activity of caffeine in a variety of *in vitro* tests, in animals and in epidemiologic studies. These studies will be summarized in this paper. Some reviews on this subject have been published (1-3).

### Standard Caffeine Content

A wide range of values exist for the caffeine content of food and beverages. However, based on current information, standard values of caffeine content of various foods has been published and are displayed in Table I (1,4).



Table I. Standard Values of Caffeine Content of Various Beverages

<i>Beverage</i>	<i>Volume (oz)</i>	<i>Caffeine Content (in mg)</i>	<i>mg/oz</i>
<b>Coffee</b>			
ground roasted	5	85	17
instant	5	60	12
decaffeinated	5	3	0.6
<b>Tea</b>			
leaf or bag	5	30	6
instant	5	20	4
Colas	6	18	3
Hot chocolate	5	4	0.8
Chocolate milk	6	4	0.67

Source: References 1, 4.

### In Vitro Evaluation of Coffee and Caffeine for Mutagenic Activity

Both coffee and caffeine have been evaluated for mutagenic activity in a variety of *in vitro* systems (1,5,6). Table II summarizes some of the available data for the effects of coffee in the *S. typhimurium* TA98 and TA100 test system. Both brewed and decaffeinated coffee are mutagenic without activation but this activity is abolished with activation. Both ground and instant coffee are mutagenic in the ARA mutant *S. typhimurium* but catalase abolishes more than 95% of this activity (6). Furthermore, both methylglyoxal and hydrogen peroxide have mutagenic activity in the Ara mutant assay, but caffeine is not mutagenic in this assay. Thus, the evidence indicates that most of the *in vitro* mutagenic effects of coffee are due to the presence of hydrogen peroxide and dicarbonyls and that caffeine is not involved in the *in vitro* mutagenic effects of coffee. Recent studies have also suggested that hydroxyhydroquinone, a component of instant coffee, may be involved in some of the *in vitro* genotoxic effects of coffee (7). In host mediated assays using *S. typhimurium*, in which coffee was administered to Swiss mice by gavage, the results were negative. Also, instant coffee given to Swiss mice showed no significant induction of micronuclei. Coffee had been shown to exert protective effects against genotoxic activities of numerous environmental chemicals in several test systems. Coffee demonstrated protective effects against somatic mutation and mitotic recombination induced by cyclophosphamide, mitomycin C and urethane in both the standard and the high bioactivation crosses of the wing spot test in *Drosophila melanogaster* (8). Another report demonstrated that the administration of coffee with combinations of various dietary constituents, could lead to varying degrees of increases in the *in vivo* anti-genotoxic effects, compared to when these agents were given separately using the mousebone marrow micronucleus test. Although some combinations did not show an enhancement of anti-genotoxic effects, none of the combinations showed an antagonistic effect (9).

Table II. *In Vitro* Genetic Effects of Coffee

<i>Test System</i>	<i>Type of Coffee</i>	<i>Without Activation</i>	<i>With Activation</i>
<i>S. Typhimurium</i> TA 100, reverse mutation	Brewed	+	-
	Instant	+	-
	Decaffeinated	+	-
<i>S. Typhimurium</i> TA 98, reverse mutation	Brewed	+/-	-
	Instant	-	-
	Decaffeinated	+	-

Source: References 1, 5.

In plants, caffeine increases chromosomal aberrations and increases the rate of point mutations. Also, caffeine has been shown to be mutagenic in algae and in some, but not all, fungi. However, caffeine was consistently not mutagenic in six different *S. typhimurium* his<sup>-</sup> reversion tester strains and in *S. typhimurium* forward mutation studies. Caffeine did not induce DNA strand breaks in mammalian cells *in vitro* and was equivocal in inducing sister chromatid exchange in human leukocytes. Caffeine was able to induce chromosomal aberrations in human leukocytes *in vitro*, but numerous studies on the clastogenic effects of caffeine evaluating micronuclei, bone marrow metaphases or dominant lethal mutations were almost uniformly negative, and when positive, the doses were in the toxic range (1).

### Bioassays of Coffee and Caffeine Carcinogenicity

Both instant and brewed coffee have undergone bioassays in rodents. In a life time study on instant coffee in the diet of mice, including the gestation period, no increase incidence of tumors was found (10). In a bioassay of 25, 50, and 100% freshly brewed coffee as the drinking fluid as compared to tap water, an increase in tumors was seen only in the lowest dose group in males but not in other groups of either sex (11). A bioassay at the maximum tolerated dose (6%) of instant coffee in the diet of rats for two years was conducted using both regular, decaffeinated, and decaffeinated plus added caffeine instant coffee. With all groups, the total number of neoplasms was either similar to or significantly lower than the total number in the control group (1). The decrease incidence was seen in the rats fed regular coffee, but was not apparent in the decaffeinated treated group, and in addition there was a tendency toward a decrease in the decaffeinated plus caffeine treated group.

Caffeine also has undergone a bioassay in both mice and rats and these studies are summarized in Table III (13-15). Caffeine did not demonstrate any carcinogenic potential in these studies, but in one study in rats it exhibited a dose dependent

reduction in the number of tumors, but the weight loss in the animals at the highest dose may have contributed to tumor reduction (15).

**Table III. Bioassay of Caffeine in Rodents**

<i>Dose</i>	<i>Species</i>	<i>No. of Animals</i>	<i>Results</i>
5 mg/Kg diet	C57 BL mice	40 M; 40 F	No effect
0.1% or 0.2% drinking wter	Wister rats	50 M; 50 F per group	No effect
0.2-2% drinking water	S. D. rats	50 M; 50 F per group	Dose dependent reduction of tumors

Source: References 13-15.

### **Caffeine Administration to Rodents in Combination with Known Carcinogens**

Following administration of caffeine to mice or rats in combination with known carcinogens, it has been reported that tumor promotion, no effect on tumor development or inhibition in tumor development occurred. However, the majority of studies have shown that caffeine decreased the incidence of tumors that developed. Thus, caffeine decreased the incidence of skin tumors in mice treated with cigarette smoke condensate, ultraviolet light or 4-nitroquinoline-1-oxide and also decreased the incidence of lung tumors in mice treated with urethane (1,16-19).

When administered to rats, caffeine exhibited a biphasic effect on pancreatic tumor incidence by 4-HAQO (4-hydroxyaminoquinoline 1-oxide) inhibiting tumor formation at higher doses and stimulating tumor formation at lower doses. When administered at a concentration of 0.1% in the drinking water, caffeine did not have an effect on urinary bladder cancer in rats treated with BNN (*N*-butyl-*N*-(4-hydroxybutyl)nitrosamine). However, caffeine in the drinking water at various doses did inhibit liver tumor development by 2-AAF (2-acetylaminofluorene), or mammary tumors induced by DMBA (7,12-dimethylbenz[*a*]anthracene) or DES (diethylstilbestrol). Also caffeine, at doses equivalent to that found in 2% black tea (e.g. 680 ppm), reduced, by 79%, the incidence of lung tumorigenesis induced by NNK (4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone) in Fischer 344 rats. These studies have been summarized in Table IV (1, 20-22). Although the mechanism(s) of action of caffeine in inhibiting physical and chemical carcinogens in rodents is unknown, several different mechanisms have been postulated. Among these are effects on cytochrome(s) P450, inhibition of enzymes of purine metabolism, inhibition of cAMP phosphodiesterase, and effects on DNA metabolism, chromatin structure and function (1).

**Table IV. Combination of Caffeine and Carcinogens in Rats**

<i>Dose of Caffeine</i>	<i>Pre or Post Carcinogen</i>	<i>Carcinogen</i>	<i>Target Organ</i>	<i>Maximal Inhibition of Tumors</i>
30-120 mg/Kg	Post	4-HAQO	Pancreas	80% <sup>a</sup>
0.1% drinking water	Same or Post	BNN	Bladder	No effect
0.2% drinking water	Same	2-AAF	Liver	72% <sup>b</sup>
0.1-0.8% drinking water	Pre	DMBA	Breast	58% <sup>b</sup>
0.1-0.2% drinking water	Same	DES	Breast	>99% <sup>c</sup>
680 ppm drinking water	Pre	NNK	Lung	79%

Source: Reference 1, 20-22; <sup>a</sup>Biphasic; <sup>b</sup>Number tumors/rat; <sup>c</sup>Total cancers.

### Epidemiologic Studies

Both coffee and total methylxanthine intake have been studied in a number of epidemiologic studies relative to cancer in humans and these have been reviewed (1,23,24). Epidemiologic studies have involved studies of cancer at all sites and at specific organ sites. Two studies demonstrated no association between coffee consumption and risk of cancer at all sites. The first study evaluated caffeine consumption (coffee, tea, and caffeine containing medications) in over 10,000 persons in the Hypertension Detection and Follow Up study, and no association was found between caffeine consumption and mortality from cancer or any other cause (25). In the second study, a prospective study in Norway, of 21,735 men and 21,238 women age 35-54 followed for 10 years, there was no association between caffeine consumption and overall risk of cancer. There also was no significant association between coffee intake and incidence of cancer of the bladder or pancreas (26). There were some negative associations found with some types of cancer. The organ site at which the literature data is most inconsistent is bladder cancer (23,24). When the association is positive, the relative risk is generally minimal and only a few studies demonstrate an important dose response relationship. Many studies have found no correlation between coffee consumption and bladder cancer (1,23,24). Also, because of bias and confounding factors, especially smoking, occupational factors and diet, the totality of the studies do not implicate coffee as an important risk for bladder cancer. In contrast, several studies of cancer at other organ sites, indicate that coffee or methylxanthine intake may lessen the risk of cancer. Thus as summarized in Table V, in four studies of breast cancer, three of which were case control studies and one an international aggregate study, intake of coffee (or total methylxanthines) was correlated with a negative (protective) association with development of breast cancer (27-30). Also, in three studies, coffee intake was negatively associated with risk of colon cancer (31-34). Thus, in humans, the studies of coffee or total methylxanthine intake in general are supportive of no effect on cancer causation and indeed some studies, especially in large bowel cancer and female breast, are supportive of a protective effect.

**Table V. Epidemiologic Studies of Methylxanthine Intake and Breast Cancer**

<i>Study Type</i>	<i>Cancer</i>	<i>Results</i>
International Aggregate Data	Breast	Negative when fat intake is factored
Case control: 818 cases, 1700 controls	Breast	Negative association $\geq$ 4 cups coffee/day; odds ratio 0.6
Case control: 500 cases, 945 controls	Breast	Negative association $\geq$ 3 cups coffee/day; odds ratio 0.6
Case control: 5984 cases, 5504 controls	Breast	No association with coffee, decaffeinated coffee or tea intake

Source: References 27-30.

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## Chapter 10

# Cancer Prevention Properties of Tea: Biochemical Mechanisms

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The major polyphenols in green tea are catechins especially (-)-epigallocatechin-3-gallate (EGCG), while the major flavanols in black tea are theaflavins including theaflavin, theaflavin-3-gallate, theaflavin-3'-gallate, and theaflavin-3,3'-digallate (TDG). The cancer chemopreventive actions of these tea polyphenols have been demonstrated in many animal models. Previous studies indicated that these polyphenols showed non-specific and broad-spectrum anticarcinogenic effects. Recent studies in our laboratory showed that the EGF receptor gene was overexpressed in human A-431 epidermal carcinoma cells. The binding of epidermal growth factor (EGF) to its receptor and autophosphorylation of EGF receptor were inhibited by theaflavins and EGCG. Furthermore, the inducible nitric oxide synthase was suppressed through down-regulating the activation of nuclear factor  $\kappa$ B (NF $\kappa$ B) by these polyphenols in macrophages. Among these tea polyphenols, TDG was found to be the most active inhibitor for the EGF-binding to its receptor, autophosphorylation of the receptor, and the activation of NF $\kappa$ B. The growth of these cells was significantly inhibited by theaflavins and EGCG. The mechanisms of this inhibition may be due to the blockade of the mitotic signal transduction through modulating EGF-receptor function, MAP kinase cascades, NF $\kappa$ B activation and c-Jun expression.

It has been demonstrated that cancer is the leading cause of death in many countries and it has become, along with cardiovascular and neurodegenerative diseases, the most important issue of modern preventive medicine. Tea is one of the most popular beverages worldwide. Both green tea and black tea have recently attracted attention as naturally occurring cancer preventive agents (1). Tea has three important advantages over some synthetic chemical cancer preventive agents: first, it is nontoxic and available daily to most of the population; second, tea is less expensive and thus is affordable by most people; and third, tea is able to inhibit the development of different types of cancer in various organs according to the results of animal experiments (2).

The anticarcinogenic effects of EGCG and green tea extract on various organs including skin, glandular stomach, duodenum, colon, liver, pancreas and lung in rats and mice have been reported in several laboratories (3-7). The anticarcinogenic effects of black tea extract on skin carcinogenesis and esophageal tumorigenesis in rodents were also reported (8,9). The preventive effects of tea on the cancer development in humans have not been conclusive. Many studies in certain countries had reported no significant association (10,11); in others, a positive association (12,13) and in still others, a negative association between tea consumption and cancer incidence was observed (14-16). A recent study has showed that the slowdown in increase of cancer incidence with age observed among females who consumed more than 10 cups a day is consistent with the finding that increased consumption of green tea is associated with later onset of cancer (17).

## The Principle of Cancer Prevention

The progress of cancer molecular genetics has demonstrated that damage to numerous regulatory genes may result in the development of invasive and metastatic cancer which is the ultimate step of carcinogenesis. It has been established that the biological and pathological processes of multistep carcinogenesis comprises initiation, promotion and progression (18). The natural history of carcinogenesis and cancer provides a strong rationale for a preventive approach to the control of this disease and leads one to consider the possibility of active pharmacological intervention to arrest or reverse the process of carcinogenesis before invasion and metastasis occur. Such intervention is called chemoprevention (19).

Cancer chemoprevention that involves the introduction of synthetic or natural materials, especially polyphenolic compounds, into the diet is attracting the attention of scientists and clinicians worldwide. Although multiple stage carcinogenesis has been established, the promotion stage is generally recognized as the most important step which produces reactive oxygen species (ROS), activates protein kinase C (PKC) activity, elevates mRNA and protein levels of ornithine decarboxylase (ODC), and increases transcription and translation of nuclear oncogenes including c-jun/AP-1, c-fos, c-myc, and NF $\kappa$ B by the stimulation of promoter, 12-*O*-tetradecanoylphorbol-13-acetate (TPA). All these cellular tumor biomarkers can be used to assess the inhibitory efficacy of natural phenolic compounds against tumor promoting action (18).



## Molecular Targets for Chemopreventive Agents

Although many chemopreventive agents have been developed empirically in the past, recent advances in the molecular biology of carcinogenesis suggest that it will be possible to develop new and better agents on a more mechanistic basis. The most striking example is in colon cancer, which is considered a paradigm for understanding the role of multiple genetic lesions in tumorigenesis (20). From the perspective of chemoprevention, the recent discovery that overexpression of the gene for inducible cyclooxygenase (COX-2), a key enzyme for the formation of prostaglandins from arachidonic acid, is an early and control event in colon carcinogenesis provides an important target for drug development (21). It is of interest to mention that the activity of COX-2 is profoundly inhibited by apigenin and several flavonols (unpublished results). Another important gene for the pathogenesis of inflammation and tumor promotion is inducible nitric oxide synthase (iNOS). iNOS is expressed in many cell types, and it catalyzes the production of NO, a ROS that mediates signal transduction and destroys invading pathogens, but may also damage host tissues through inflammation and tumor promotion. Therefore, iNOS also provides another important target for drug development. The inhibition of iNOS biosynthesis by tea polyphenols will be elaborated later (22).

### Cancer Prevention of Tea: Biochemical Mechanisms

It is estimated that about 2.5 million metric tons of dried tea are manufactured annually. About 78% is black tea, mainly consumed in the Western nations and some Asian countries; about 20% is green tea, mainly consumed in Asian countries where tea is a major beverage; and about 2% is oolong tea, mainly produced and consumed in Southeastern China and Taiwan. It has been demonstrated that a tea infusion inhibits the process of mutistep carcinogenesis and the growth of experimental tumors, suppresses the growth of implanted tumor cells and host-bearing malignant tumor invasion and metastasis (23).

The aforementioned inhibitory effects of tea against carcinogenesis and tumor growth have been attributed to the biologic activities of the polyphenols in tea. The major polyphenol in green tea is EGCG, while the major polyphenol in black tea are the theaflavins. The cancer chemopreventive actions of these tea polyphenols have been demonstrated in many animal models(3-9). Previous studies indicated that tea polyphenols showed non-specific and broad-spectrum anticarcinogenic effects. Recent studies in our laboratory showed that the EGF-receptor gene was overexpressed in human A-431 epidermal carcinoma cells. The growth of these cells was significantly inhibited by theaflavins and EGCG. The action mechanisms of this inhibition might be due to the blockade of the mitotic signal transduction through modulating EGF-receptor autophosphorylation, MAP kinase cascades, NF $\kappa$ B activation and c-Jun expression. The biochemical mechanisms of the blockade of these signal transducing pathways by tea polyphenols will be elaborated in the next sections.

## Antiproliferative Effects of Tea Polyphenols

We have examined the antiproliferative effects of tea polyphenols on mouse NIH3T3 fibroblasts and human A-431 epidermal carcinoma cells. The black tea polyphenols theaflavin-3,3'-digallate (TDG) is the most effective at inhibiting the growth of both cells (Table I). The major green tea polyphenol EGCG is also very active, while theaflavin-3-gallate, theaflavin and thearubigin are less active.

**Table I. Inhibition of the Growth of Cancer Cells by Tea Polyphenols<sup>a</sup>**

<i>Tea polyphenols</i>	<i>IC<sub>50</sub> (μM)</i>	
	NIH 3T3 cells	A-431 cells
EGCG	26	28
Theaflavin-3,3'-digallate	15	18
Theaflavin-monogallate	50	> 50
Theaflavin	> 50	> 50
Thearubigin <sup>b</sup>	> 50	> 50

<sup>a</sup> Cells were cultured in 12-well plates with Dulbecco's modified medium (DMEM), which contained 5% fetal bovine serum (FBS). Cells were changed to serum-free DMEM during treatment with various concentrations of polyphenols and then added to FBS to reach a concentration of 5% FBS, and continuously cultured for 2 days. The number of viable cells were determined by trypan blue exclusion method.

<sup>b</sup> The concentration of thearubigin is expressed as μg/ml instead of μM.

The  $IC_{50}$  values for TDG and EGCG were as low as 15 and 26 μM for the growth of NIH 3T3 cells respectively; The  $IC_{50}$  values of these two compounds were 18 and 28 μM, respectively, for the growth of A-431 cells (Table I). Based on results from our previous studies (24), the inhibition of cell growth by EGCG might block EGF ligand binding to its receptor through the inhibition of membrane receptor kinase activity.

## Suppression of Extracellular Signals and Cell Proliferation by Tea Polyphenols

As shown in Table II, when A431 cells were pretreated with tea polyphenols for 30 min; then incubated with <sup>125</sup>I-EGF for 1 h, they showed different degree of inhibition of <sup>125</sup>I-EGF binding to the cells. TDG (10 μM) showed the strongest inhibition (90%), while EGCG was next (60%), followed by theaflavin-monogallate (35%) and theaflavin (25%). After cotreatment of tea polyphenols and <sup>125</sup>I-EGF for 1 hour, most tea polyphenols showed less inhibition, but it is worth noting that TDG still retains most of its inhibitory effect (Table II).

**Table II. Inhibition of the Binding of  $^{125}\text{I}$ -EGF to EGF Receptor in A431 Cells by Tea Polyphenols<sup>a</sup>**

<i>Tea polyphenol (10 <math>\mu\text{M}</math>)</i>	<i>% of Inhibition<sup>b</sup></i>	
	Pretreatment	Cotreatment
EGCG	60	10
Theaflavin	25	15
Theaflavin-monogallate	35	25
Theaflavin-3,3'-digallate	90	80

<sup>a</sup> Human epidermal carcinoma A431 cells were cultured in 24-well plates with DMEM containing 10% FBS. Cells were changed to serum-starved media for 6 h before polyphenol treatment. Serum-starved cells that were pretreated with polyphenols for 30 min were added to  $^{125}\text{I}$ -EGF and incubated with gentle mechanical agitation at 4°C for 1 h. Alternately, cells were treated with polyphenols and  $^{125}\text{I}$ -EGF simultaneously and incubated similar.

<sup>b</sup> % of inhibition = [1-Binding in treated group/Binding in control group] x 100%.

**Table III. Inhibition of EGF- and PDGF-receptor Autophosphorylation in Cancer Cells by Tea Polyphenols<sup>a</sup>**

<i>Tea polyphenol (10 <math>\mu\text{M}</math>)</i>	<i>Inhibition (%)</i>	
	<i>EGF-receptor autophosphorylation in A431 cells<sup>b</sup></i>	<i>PDGF-receptor autophosphorylation in NIH 3T3 cells<sup>c</sup></i>
EGCG	80	70
Theaflavin	20	70
Theaflavin-monogallate	20	90
Theaflavin-3,3'-digallate	100	99

<sup>a</sup> The experimental conditions are as described in Table II.

<sup>b</sup> A-431 cells were treated with 20 ng/ml of EGF for 10 min and used as control.

<sup>c</sup> NIH 3T3 cells were treated with 10 ng/ml of PDGF for 10 min and used as control.

The effects of tea polyphenols on the activation of EGF- and PDGF-receptor autophosphorylations on tyrosine residues by using a specific anti-phosphotyrosine mAb PY-20 had been investigated and the results showed that both TDG and EGCG at 10  $\mu\text{M}$  significantly inhibited the autophosphorylation of EGF- and PDGF-receptors (Table III). Under the described conditions, the black tea polyphenol TDG completely knocked out the autophosphorylation of EGF-receptor (100%) and PDGF-receptor (99%). Theaflavin-3-gallate is also very effective in inhibiting the PDGF-receptor autophosphorylation (90%).

## Blocking the Induction of iNOS by Tea Polyphenols

Nitric oxide (NO) plays an important role in inflammation and also in multistep carcinogenesis. We investigated the effects of tea polyphenols on the induction of inducible nitric oxide synthase (iNOS) in lipopolysaccharide (LPS)-activated murine macrophages, RAW 264.7 cells. TDG was found to be a stronger inhibitor than EGCG for NO generation, iNOS protein and iNOS m-RNA in activated macrophages (Table IV). Western blot and reverse transcriptase-polymerase chain reaction (RT-PCR) analyses demonstrated that significantly reduced 130 Kda protein and mRNA levels of

**Table IV. Inhibition of Inducible Nitric Oxide Synthase in LPS-activated Macrophages by Tea Polyphenols**

<i>Tea polyphenol (10 μM)</i>	<i>Inhibition (%)<sup>a</sup></i>			
	<i>Nitrite<sup>b</sup></i>	<i>iNOS protein<sup>c</sup></i>	<i>iNOS mRNA<sup>d</sup></i>	<i>NFκB<sup>e</sup></i>
EGCG	60	60	42	85
Theaflavin	50	15	15	50
Theaflavin-monogallate	55	40	35	50
Theaflavin-3,3'-digallate	75	75	55	90

<sup>a</sup> The macrophage RAW 264.7 cells were cotreated with lipopolysaccharide (LPS) (50 ng/ml) and tea polyphenol (10 μM) for 18 h. % of inhibition = [ 1-Activity in tested system/Activity in control system ] x 100%.

<sup>b</sup> The nitrite concentration in the culture medium was measured by Griess reaction.

<sup>c</sup> The iNOS protein was determined by chemiluminescence method.

<sup>d</sup> The iNOS mRNA was measured by RT-PCR analysis.

<sup>e</sup> The NFκB binding activity was measured by EMSA.

**Table V. Inhibition of TPA-induced PKC Activity in NIH 3T3 Cells by Tea Polyphenols<sup>a</sup>**

<i>Tea polyphenol</i>	<i>PKC Activity (P mol P/mg protein)</i>	
	<i>Membrane</i>	<i>Cytosol</i>
Control (None)	700	2010
TPA (100 ng/ml)	1930	1040
EGCG (20 μM)	1810	1100
Theaflavin (20 μM)	1900	1070
Theaflavin-monogallate (20 μM)	1600	1070
Theaflavin-3,3'-digallate (20 μM)	870	1930

<sup>a</sup> Cells were treated with TPA (100 ng/ml) alone or TPA plus polyphenol as indicated for 1 h. Cells were then harvested and their subcellular fractions were prepared and PKC activity was estimated using myelin basic protein synthetic oligopeptide (QKRPSQRSKY) as substrate.

iNOS were expressed in LPS-activated macrophages with TDG and EGCG. Electrophoretic mobility shift analysis (EMSA) indicated that TDG and EGCG blocked the activation of nuclear factor  $\kappa$ B (NF $\kappa$ B), a transcription factor necessary for iNOS induction. TDG and EGCG also blocked phosphorylation of I $\kappa$ B from cytosolic fraction and reduced LPS-induced nuclear accumulation of NF $\kappa$ B p65 and p50 subunits ( 22).

### **Inhibition of TPA-induced PKC and AP-1 binding Activities by Tea Polyphenols**

We investigated the inhibition of tea polyphenols on the TPA-induced protein kinase C (PKC) and transcription activator protein-1(AP-1) binding activities in NIH 3T3 cells. TDG (20  $\mu$ M) and EGCG (20  $\mu$ M) showed 94.5% and 9.4% inhibition on TPA-induced PKC activity, respectively (Table V). Translocation of PKC protein from cytosol to membrane was detected in TPA-treated NIH 3T3 cells and both TDG and EGCG were able to block its translocation. By in vitro kinase assay using myelin basic protein (MBP) as a PKC specific substrate, we found that TPA treatment was able to increase PKC activity (shown by detection of phosphorylated MBP protein) and that TDG showed the strongest inhibitory effect on its phosphorylation.

When we analyzed the AP-1 binding activity by EMSA and c-Jun gene expression by northern blot and western blot, the results showed that TDG is the most potent inhibitor on TPA-induced AP-1 binding activity and c-Jun gene expression among these tea polyphenols. These findings might provide new molecular basis for understanding the inhibition of tea polyphenols on TPA-mediated tumor promotion.

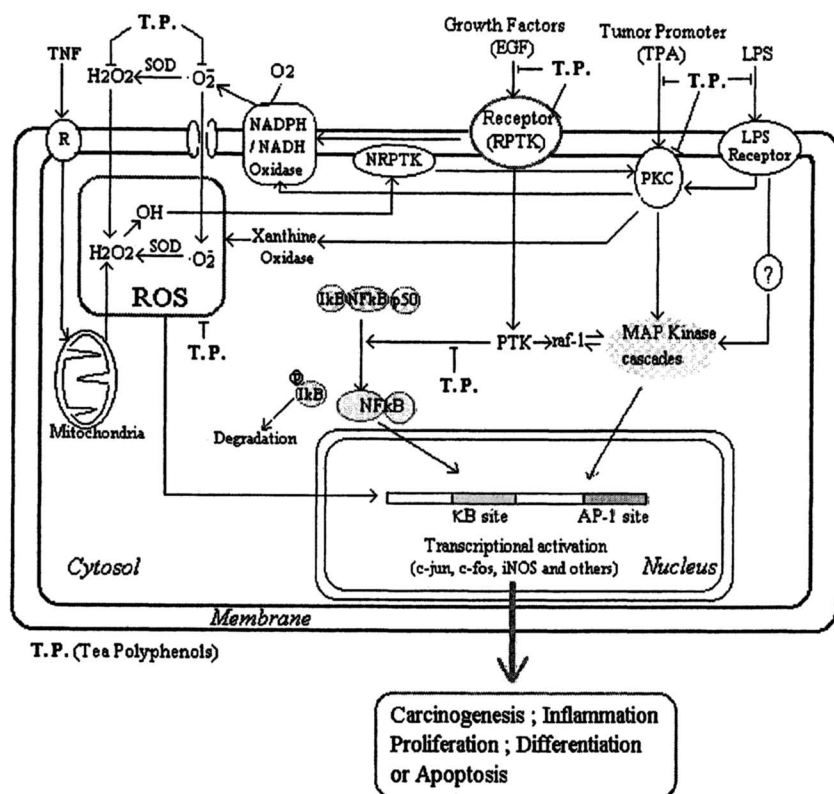
### **General Remark**

During the last few years, a number of molecules have been identified as signal transducers. Among them PKC, protein tyrosine kinase (PTK), receptor protein tyrosine kinase (RPTK) and several growth factors such as EGF, PDGF and CSF may act as signal transducers. Many oncogene and tumor suppressor gene products also belong to this category. There is considerable evidence that these transducers may operate as coordinated or interacting systems that communicate with or control each other. Presumably, disruption of information flow along these transduction pathways would alter normal cell growth that leads to carcinogenesis, inflammation, and apoptosis.

Some of these alterations or abnormality could be inhibited or corrected by tea polyphenols as indicated in Figure 1.

### **Acknowledgements**

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**Figure 1. Cancer Prevention by Tea Polyphenols through Signal Transduction Blockade.** Cell proliferation and differentiation are tightly regulated by a programmed networks of signal transduction pathways through various transducers including extrinsic molecules such as cytokines, growth factors, TGF (transforming growth factor), tumor promoters, TNF (tumor necrosis factor), and LPS (lipopolysaccharide), and intrinsic molecules such as receptor proteins, PTK (protein tyrosine kinase), PKC (protein kinase C), MAP kinase, NFκB, AP-1, c-jun, c-fos, c-myc, iNOS (inducible nitric oxide synthase), ROS (reactive oxygen species) and others. The illegitimate regulation or hyper-function of these signal transductions may lead to the induction of carcinogenesis, inflammation or apoptosis. The tea polyphenols (T.P.) were found to suppress the hyper-function of these transductions in various systems and to block the processes of carcinogenesis.

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## Chapter 11

# Antioxidative Polyphenolic Substances in Cacao Liquor

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We have found antioxidative polyphenolic substances in cacao liquor that is one of the ingredients of chocolate and cocoa. Epicatechin, catechin, clovamide, quercetine, and their glycosides were confirmed as major antioxidants by using several methods. Furthermore, the physiological effects of crude polyphenols derived from cacao liquor (CLP) were examined with experimental animal models. CLP showed 1) antiulceric activity induced by ethanol, 2) inhibition on oxidative stress in vitamin E deficient rats, 3) reduction on LDL oxidative susceptibility in hypercholesterolemic rabbit, 4) antimutagenic effect against heterocyclic amines, 5) inhibitory effect on tumor promotion in two-stage carcinogenesis in mouse skin.

It is well known that free radical induced oxidative stress is an important etiologic factor in many pathological processes. Much attention has been focused on the antioxidants in food. Among the various foods, chocolate and cocoa are stable against oxidative deterioration. This is believed to be because of the fatty acid composition (1) and the polyphenolic substances in cacao beans (2-4). However, in the process of making cacao liquor, where fermentation, roasting, and grinding of raw beans are conducted, it is assumed that these compounds undergo major changes. In this report, we performed the isolation and structural elucidation of polyphenols in cacao liquor which is a major ingredient of chocolate and cocoa. Furthermore, a crude polyphenol fraction derived from cacao liquor (CLP) was prepared, and its physiological effects were evaluated with *in vitro* system and experimental animal models.



## Materials and Methods

### Cacao Liquor

Fermented and dried cacao beans imported from Ghana and roasted and cracked at Meiji Seika Kaisha Ltd.

### Extraction of Polyphenols

Cacao liquor was defatted twice with fivefold n-hexane and extracted twice with fivefold 80 % v/v ethanol for 16 hours. The extract was concentrated and charged on a Diaion HP2MG column (35mm $\Phi$ ×310mm). To remove contaminants, including xanthine derivatives, the column was washed with 20% v/v ethanol, and eluted with 80% v/v ethanol. Eighty percent ethanol fraction was concentrated and freeze-dried. This was cacao liquor crude polyphenols (CLP). CLP contained approximately 50% of total polyphenols by the analysis method of Prussian blue (5), using epicatechin as the standard.

### High Performance Liquid Chromatography

Further purification of the crude polyphenols was carried out by the method described in the previous report (6,7), using preparative HPLC. The column was used an ODS column, and elution solvent was 0.1 % trifluoroacetic acid, containing 40 % v/v methanol /water.

### Antioxidative Activity

#### *Linoleic Acid Oxidation*

Each sample was added at the final concentration of 0.5 mM to 1 g of linoleic acid and incubated at 30°C; the degree of oxidation was measured iodimetry.

#### *Microsomal Lipid Peroxidation*

Microsomes were prepared from rat liver (8). The antioxidants were added to the microsome fraction, and incubated at 37°C with tert-butylhydroxy peroxide (t-BHP) as a radical generator. After incubation, thiobarbituric acid reactive substance (TBARS) was measured (9).

## Effect of CLP on Gastric Mucosal Lesion Induced by Ethanol

### *Experimental Ulcers*

Male 9-week old Sprague-Dawley rats were used. Twenty-four hours before the experiment, the rats were deprived of food. The samples were dissolved or suspended in 0.1% w/v carboxymethyl cellulose (CMC). Five-mL/kg body weight of test solution was intragastrically given to the animals 30 min before the administration of 5 mL/kg ethanol. After 60 min, the animals were sacrificed under anesthesia and their stomachs removed, and opened along the curvature. The degree of mucosal damage was evaluated by a computerized video-scanning system.

### *Measurement of Lipid Peroxide*

The gastric mucosa was homogenized with 1.15 % KCl solution, and the protein concentration of homogenates was measured by the Lowly method (10). The level of TBARS in gastric mucosa was determined (9).

### *Measurement of Xanthine Oxidase (XOD)*

Gastric mucosa homogenized was centrifuged at 4000 g for 10 min at 4°C, and supernatant was collected. The reaction mixture was 0.6 mM xanthine, and 1 mg protein of the sample containing 30 mM phosphate boric acid buffer (pH 8.2). The level of uric acid in the reaction mixture was measured by a Wako uric acid test kit after incubation for 3 hours at 37°C (11).

### *Measurement of Myeloperoxidase (MPO)*

The reaction mixture was 0.4 mM tetramethyl benzidine, 0.3 % H<sub>2</sub>O<sub>2</sub>, and 1 mg protein of the sample. Absorbance at 655 nm of mixture was immediately recorded for 5 min. Activity was calculated from optical density per minute (12).

## Effect of CLP on Oxidative Stress of Vitamin E Deficient Rats.

### *Experimental Procedure*

Male 3-week old Sprague-Dawley rats were fed a normal diet that was reformed AIN-76 diet, a vitamin E (VE) deficient diet that contained less than 6.0 µg/kg α-tocopherol, or they fed 0.25, 0.5, 1.0 % CLP containing VE-deficient diets, for 7 weeks. After the experimental period, animals were sacrificed under anesthesia, and blood and several tissues were removed.

### *Analysis*

α-Tocopherol concentrations in plasma and tissues were analyzed by the HPLC method (13). TBARS in plasma and tissues were determined as described above (9).

## Effect of CLP on LDL Oxidative Susceptibility in Hypercholesterolemic Rabbits

### *Experimental Procedure*

Male 13-week old Japanese white rabbits were fed a high cholesterol diet, which contained 1.0 % cholesterol. After 4 weeks of the feeding, the plasma total cholesterol was raised 12 times, compared with before experiment ( $1,639 \pm 397$  mg/dL), and initial LDL oxidation was measured. One percent CLP-containing diet was fed to the animals after 10 days. LDL oxidation was measured 4, 7, and 10 days after administration.

### *Analysis*

LDL was prepared by the sequential ultracentrifugation method of Havel et al. (14). Thirty-five  $\mu$ g protein/mL of LDL fraction and 200  $\mu$ M 2,2'-azobis (4-methoxy-2,4-dimethylvaleronitrile) (V-70) as radical generators were incubated at 37°C (15). The kinetics of LDL oxidation was determined by monitoring the conjugated-diene formation at 234-nm absorbance. TBARS in LDL fraction induced by V-70 was also determined as described above (9). Plasma lipids were measured by using commercially available kits.

## Antimutagenic Activity Against Heterocyclic Amine of CLP

### *Ames Test of Heterocyclic Amines With S-9 Mix*

The mutation test was carried out by the preincubation method (16), which was a slightly modified method of Ames et al. (17). Test samples, 3-amino-1methyl-5H-pyrido(4,3-b)indole (Trp-P-2) or 2-amino-3,4-dimethyl-3H-imidazo(4,5-f)quinoline (MeIQ), S-9 mix, and bacterial suspension were mixed and preincubated at 37°C for 20 min. Soft agar was added to the bacterial mixture and poured onto the modified Vogel-Bonner medium agar plate. These plates were incubated at 37°C for 2 days, and the revertant colonies ( $his^+$ ) were counted.

### *Ames Test of Activated Heterocyclic Amine*

Trp-P-2 and MeIQ were incubated with S-9 mix at 37°C for 20 min, after incubation cold-acetone was added to the mixture and centrifuged (18). The supernatants were concentrated and used. The mutation test was carried out as described above, except that phosphate buffer was added to the bacterial mixture instead of S-9 mix. The concentrations of activated Trp-P-2 and MeIQ in this study were 20 nmol/plate and 0.1 nmol/plate.

## Effects of CLP on Tumor Promotion in Two-Stage Carcinogenesis in Mouse Skin

Four-week old female ICR mice were shaved and treated with 200 nmol 7,12-dimethylbenz[a]anthracene in 200  $\mu$ L acetone. After 1 week, the animals were treated with 200  $\mu$ L acetone, or 5 nmol 12-*O*-tetradecanoylphorbol-13-acetate (TPA), or 5 nmol with 5 or 10 mg CLP in acetone twice weekly for 20 weeks. Skin tumors more than 1 mm diameter were counted every week.

### Statistical Analysis

Results were expressed as the mean and standard deviations. All analyses were done by using SPSS statistical software. Mean values were calculated by ANOVA and multiple-range comparisons, Student's *t*-test, or paired *t*-test. Values of  $p < 0.05$  were considered significant.

## Results and Discussion

### Polyphenolic Substances in Cacao Liquor

In the analyses of CLP by HPLC, several peaks were detected respectively. Each of them was collected individually by preparative HPLC and analyzed. According to mass and NMR data, these chemicals were identified; (+)-catechin, (-)-epicatechin, clovamide, dideoxy clovamide, quercetine-3-glucopyranoside, and quercetine-3-arabinopyranoside (Figure 1) (6, 7).

### Antioxidative Activity

#### *Linoleic Acid Oxidation*

Antioxidative activity in this method was in the following order: clovamide > epicatechin > catechin > quercetine 3-glucoside, quercetine-3arabinoside, dideoxyclovamide (Figure 2) (7).

#### *Microsomal Lipid Peroxidation*

In this experiment, flavans such as epicatechin and catechin had a potent antioxidative activity, as shown in Figure 3 (7).

### Effect of CLP on Gastric Mucosal Lesion Induced by Ethanol

Five mL/kg of ethanol given intragastrically consistently caused lesions in the mucosa in glandular stomach in the control group. As shown in Figure 4, the groups

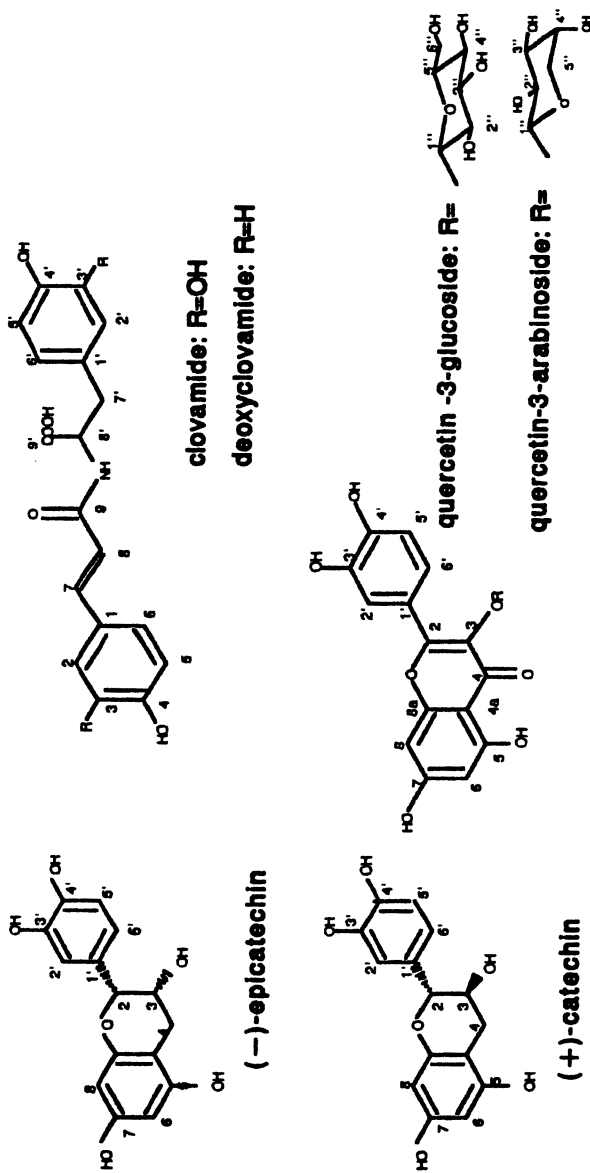


Figure 1. Chemical structure of antioxidative polyphenols in cacao liquor

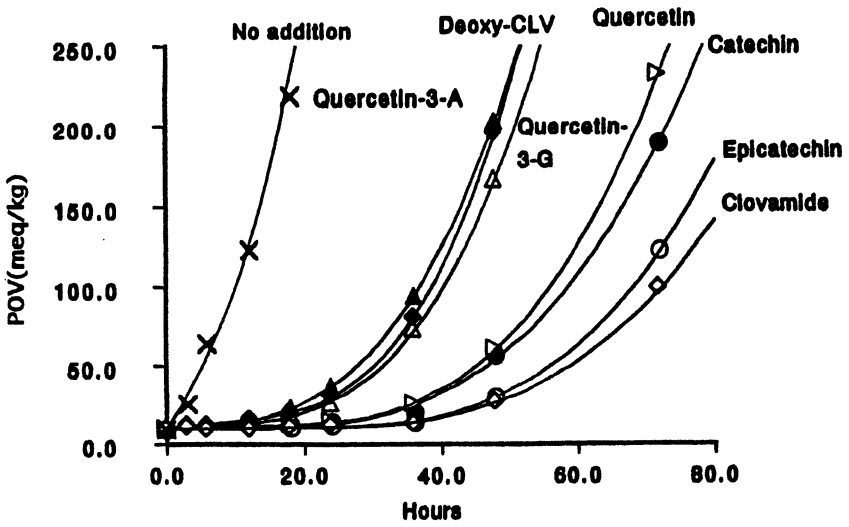


Figure 2. Effects of polyphenolic substances from cacao liquor on linoleic acid autooxidation

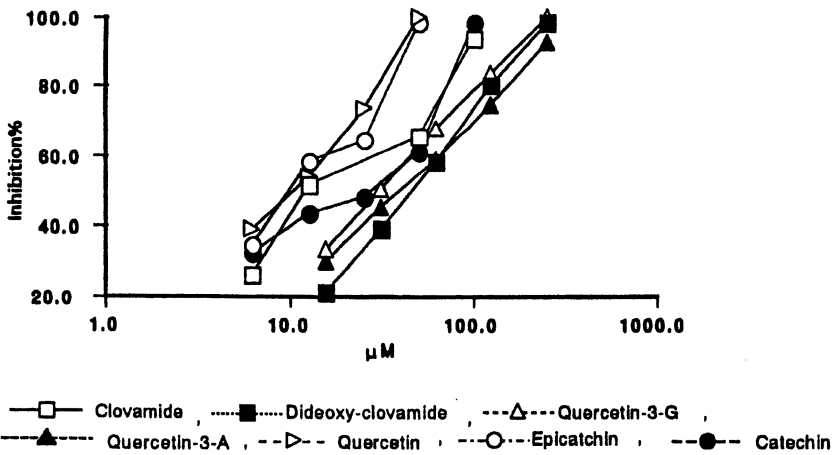


Figure 3. Effects of polyphenolic substances from cacao liquor on t-BHT-induced oxidation of rat microsomes

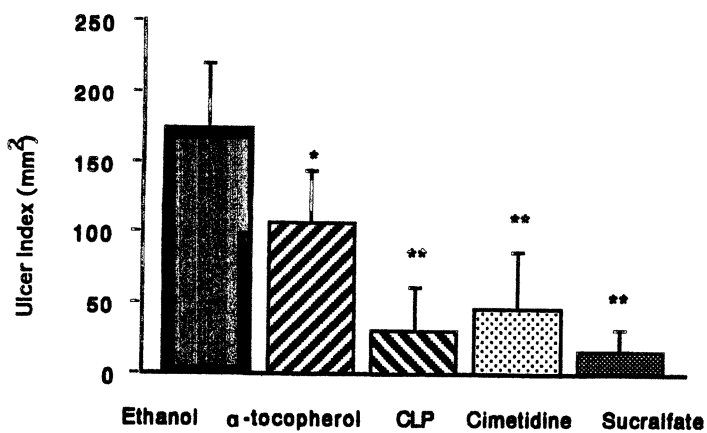


Figure 4. Effects of  $\alpha$ -tocopherol, CLP, cimetidine and sucralfate on gastric mucosal Injury induced by ethanol administration

treated CLP, cimetidine, and sucralfate exhibited a marked reduction of these lesion (19).  $\alpha$ -Tocopherol, a typical antioxidant, slightly reduced lesion formation. The level of TBARS in gastric mucosa, increased 60 min after the administration of ethanol shown in Table I (19). This change was significantly inhibited by the CLP treatment. XOD activity that has been suggested as a major source of oxygen radicals was not increased by ethanol treatment (Table I) (19). The level of TBARS did not correlate with the XOD activity ( $r^2=0.232$ ). MPO, is the marker enzyme of leukocytes, was raised by ethanol treatment, and this change was significantly inhibited by CLP (Table I) (19). The correlation between MPO activity and TBARS is positive ( $r^2=0.634$ ). CLP showed inhibition of leukocytes infiltration in gastric mucosa. In conclusion, the results of this study indicate that the mechanism of antiulcer effect of CLP is reduction of the migration of activated leukocytes to the inflammation area, and so the following attack of oxygen radical generation by these cells.

**Table I. Effect of CLP on TBARs Production, XOD and MPO Activity in Gastric Mucosa Lesions Induced by Ethanol Administration**

	<i>TBARS</i> (nmol/mg protein)	<i>XOD</i> (10-3U/protein)	<i>MPO</i> (U/mg protein)
No treatment	4.26 ± 0.76	1.27 ± 0.49	8.21 ± 1.29
Ethanol	7.64 ± 1.13 +	1.52 ± 0.34	19.62 ± 4.11 +
CLP	3.84 ± 0.64 *	0.38 ± 0.13 **	2.67 ± 2.67 **

Significant difference from no treatment : +,  $p < 0.01$ ;

Significant difference from ethanol : \*,  $p < 0.01$ ; \*\*,  $p < 0.001$ .

### Effect of CLP on the Oxidative Stress of Vitamin E Deficient Rats

In this study, food intake, body weight gain, and food efficiency were equal between normal diet, VE deficient and CLP containing VE deficient-diets group.  $\alpha$ -Tocopherol in plasma and tissues of these animals were markedly decreased. Plasma TBARS was increased in the VE deficient group, compared with control. CLP supplementation showed a slight reduction of TBARS, but not statistically significant (data not shown). TBARS of liver, heart, kidney, and brain was increased in VE-deficient animals, compared with normal rats respectively (Figure 5). This change was reduced by the administration of CLP in a dose-dependent manner. CLP protected animals against oxidative stress by vitamin E deficiency.

### Effect of CLP on LDL Oxidative Susceptibility in Hypercholesterolemic Rabbits

Table II showed the mean and standard deviation of lagtime, the propagation rate, and the TBARS production. CLP supplementation was prolonged the lag time and



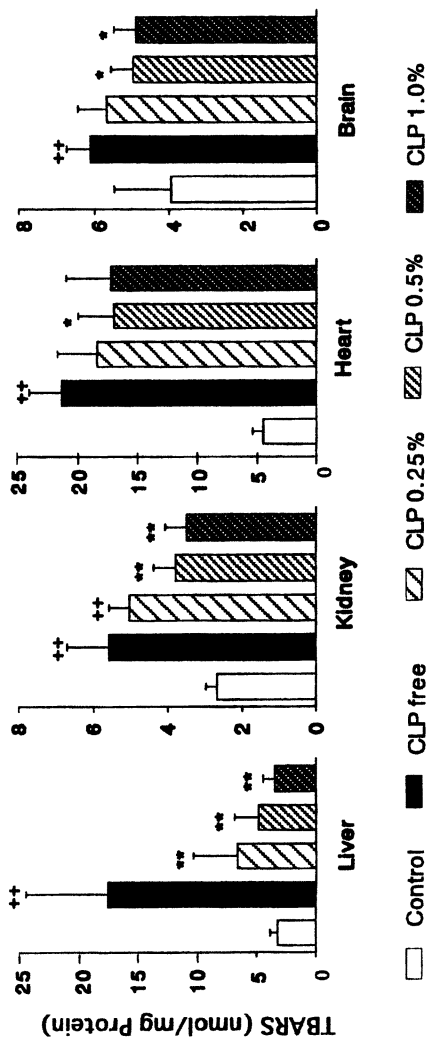


Figure 5. Effect of CLP on lipid peroxide in various tissues of rats fed vitamin E deficient diets

reduced the conjugated diene production. TBARS production in LDL was also inhibited by CLP treatment. The administration of CLP significantly reduced LDL oxidative susceptibility.

**Table II. LDL Oxidizability of Hypercholesterolemic Rabbits After Supplementation of CLP**

	<i>Lag Time (min)</i>	<i>Propagation Rate (nmol/min/mg protein)</i>	<i>TBARS (<math>\mu</math>mol/mg protein)</i>
day 0	37.77 $\pm$ 3.19	12.53 $\pm$ 1.95	1.95 $\pm$ 0.11
day 4	42.93 $\pm$ 4.11 *	9.35 $\pm$ 4.24	1.68 $\pm$ 0.21 *
day 7	44.22 $\pm$ 4.29 *	9.47 $\pm$ 2.82 *	1.59 $\pm$ 0.18 *
day 10	45.78 $\pm$ 4.63 **	6.14 $\pm$ 4.18 **	1.33 $\pm$ 0.37 **

LDL fraction (35  $\mu$ g/mL) derived from rabbits was incubated with 200  $\mu$ M v-70 at 37°C. Conjugated diene production was monitored at 234 nm. TBARS analyzed after 2 hours incubation. Significant difference from ethanol: \*,  $p < 0.01$ ; \*\*,  $p < 0.001$ .

### Antimutagenic Activity Against Heterocyclic Amine(HCA) of CLP

#### *Ames Test of Heterocyclic Amines with S-9 Mix*

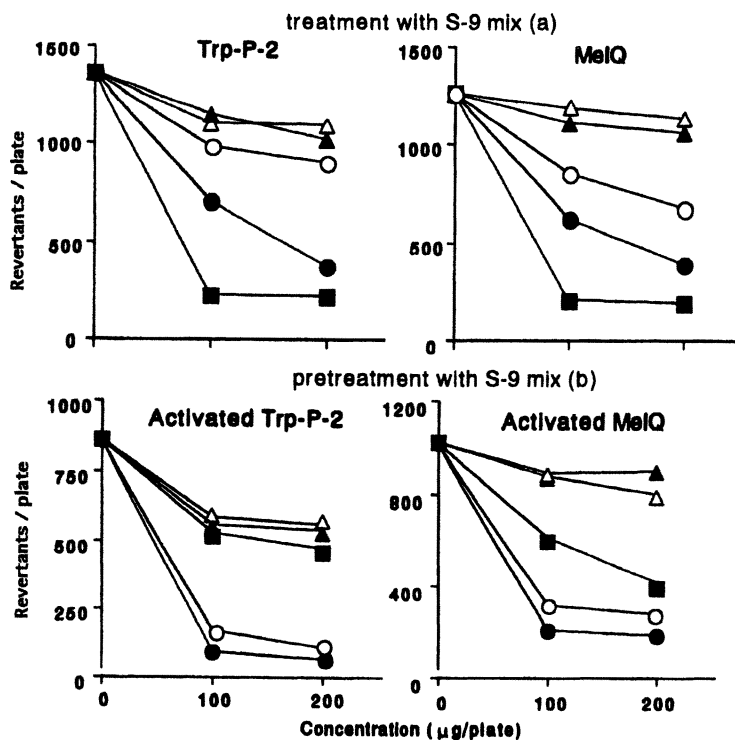
As shown in Figure 6(a), quercetin, CLP, and cacao liquor conjugated tannin fraction (CLT), which was derived from CLP, markedly decreased the revertant colonies induced by Trp-P-2 and MeIQ. Epicatechin and catechin showed slight effects on these.

#### Ames Test of Activated Heterocyclic Amine

CLP and CLT inhibited the mutation induced by activated Trp-P-2 and MeIQ, as shown in Figure 6(b). In this case, quercetin was less effective than these cacao polyphenols. These results suggested that the antimutagenic mechanism of cacao liquor polyphenols was a direct suppression of activated Trp-P-2 and MeIQ, and not the inhibition of enzymatically metabolized heterocyclic amines.

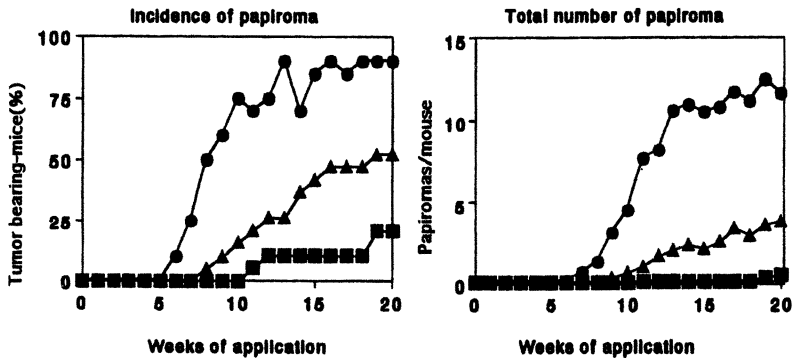
### Effect of CLP on Tumor Promotion in Two-Stage Carcinogenesis in Mouse Skin

As shown in Figure 7, female ICR mice initiated with DMBA and promoted with TPA for 20 weeks developed an average 12.9 skin tumors/mouse, and 95 % of the mice had tumors. Topical application 5 or 10 mg CLP together with TPA, reduced the number of tumors per mouse and the percentage of mice with tumors in a dose-dependent manner, compared with TPA alone.



(△) : epicatechin ; (▲) : catechin ; (○) : CLP ; (●) : CLT ; (■) : quercetin

Figure 6. Effects of polyphenolic substances in cacao liquor on mutagenicity of Trp-P-2 and MeIQ in salmonella typhimurium TA98



Female ICR mice (20 per group) were treated with 200 nmol DMBA.

One week later, the mice were treated with 5 nmol TPA alone (●), 5 nmol TPA with 5mg CLP (▲) or 10 mg CLP (■)

*Figure 7. The prevention of CLP on the promotion of skin pappillomas produced by TPA following DMBA-initiation in mice*

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## Chapter 12

# Antioxidant Capacity and Epicatechin Bioavailability of Polyphenolic-Rich Beverages (Cocoa and Teas)

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The antioxidant capacity of cocoa and tea was investigated by measuring the inhibition of copper- or AAPH-mediated human low-density lipoprotein (LDL) oxidation *in vitro*. The extent of oxidation was determined spectrophotometrically by measuring absorbance (at 234 nm) of conjugated dienes. Both cocoa and teas produced dose-dependent inhibition of LDL oxidation which was similar with both pro-oxidant agents. Cocoa polyphenols extracted with 20% aqueous ethanol showed a stronger antioxidant capacity than those extracted with water. When prepared as ready-to-drink, the polyphenol-rich beverages such as cocoa, green and black teas exhibited a similar antioxidant capacity. In a second stage, the plasma bioavailability of epicatechin from cocoa extract and chocolate was evaluated in man after a single dose administration. Plasma concentration of epicatechin increased markedly after cocoa consumption and the area under the curve of plasma kinetics correlated very well with the dose of epicatechin present in the cocoa product. The epicatechin kinetics were similar with either 20% aqueous ethanol cocoa extract or plain chocolate indicating that the food matrix did not affect the epicatechin bioavailability. Attainable epicatechin plasma values were 0.7  $\mu\text{M}$  from 80 g of plain chocolate representing about 2% of the intake. Similar results have been described for tea. These results suggest that beverages of tea and cocoa make a significant contribution to the daily intake of polyphenolic antioxidants.

The oxidative modification of low density lipoprotein (LDL) is currently viewed as a pivotal step in the pathogenesis of atherosclerosis (1 - 3). Indeed, the uptake of intact LDL by the B,E receptors induces a control of intracellular content of

cholesterol while the uptake of oxidized LDL by scavenger receptor (4) is uncontrolled. Therefore, cholesterol accumulates into macrophages which are progressively transformed into foam cells (5). The detection of products of LDL oxidation in atherosclerotic lesions of rabbit and humans (6) also implicates oxidized LDL in the atherosclerotic process. It is important to find dietary compounds which can inhibit LDL oxidation. The method assessing the susceptibility of LDL towards *in vitro* oxidation is appropriate for determining the influence of dietary treatment with different fatty acid patterns or antioxidants but also for evaluating the antioxidant potential of dietary antioxidant matrices.

Polyphenols constitute an important source of dietary antioxidants, being found widely in fruits, vegetables, cereals and beverages such as red wine, tea, coffee and cocoa. The antioxidant capacity of this diverse group of compounds depends on the individual structure and number of hydroxyl groups.

Polyphenolic derivatives of catechins are found in significant amount in tea, from which they are readily extracted in hot water infusions. Six catechins occur in green tea leaves : catechin, gallocatechin, epicatechin, epigallocatechin, epicatechingallate and epigallocatechingallate. Overall, levels of catechins vary with leaf age, tending to be higher in young leaves. During the manufacture of black tea, enzyme-catalyzed oxidation of the catechins leads to the formation of catechin quinones, which subsequently react to form the more complexly structured pigmented teaflavins and tearubinins. The approximate catechin amounts in fresh leaf, green tea and black tea are in the range of 30-35%, 10-25% and 1-9% of dry matter, respectively (7).

Unfermented cocoa beans are rich in polyphenols, which comprise 12-18% of the whole bean's dry weight. Two major classes of polyphenols are present : catechins (with approximately 35% polyphenols as epicatechin) and anthocyanins, which are responsible for the characteristic purple colour of unfermented cocoa beans (8, 9). During fermentation, the colour of the bean changes from purple to brown. The polyphenols themselves undergo a variety of reactions : epicatechin diffuses from its storage cells and undergoes oxidation and polymerisation to form complex tannins (10, 11) leading to a reduction of epicatechin concentration to approximately 2-3 mg/g fermented cocoa beans (12). Previously, polyphenols were considered only for their colour and aroma but more recently they have been investigated for their biological activities relating to health. Cocoa polyphenols have been shown to exhibit a strong protective effect against LDL oxidation (13, 14), antimutagenic effects have been shown in the Ames test system; and studies in isolated human T and B lymphocytes and granulocytes indicate that cocoa polyphenols have immunoregulatory properties (15).

The objective of this study was to efficiently extract polyphenols from cocoa powder in order to evaluate their antioxidant capacity *in vitro* but also to compare them with food matrix such as cocoa drink, plain chocolate and tea. To produce a biological effect *in vivo*, it is essential that biologically relevant quantities of polyphenols are absorbed. Therefore, the plasma kinetics of epicatechin from cocoa products (20% aqueous ethanol extract and plain chocolate) were evaluated in healthy man and compared with published data for tea.

## EXPERIMENTAL PROCEDURES

### Antioxidant Capacity, in vitro

LDL were isolated from blood of healthy volunteers and oxidized with either 1.7 mmol copper (II) sulfate or 1.25 mmol AAPH in the presence or absence of polyphenolic-rich beverages. After incubation, the extent of LDL oxidation was determined by recording spectrophotometrically the formation of conjugated dienes (16).

Extraction of cocoa polyphenols was performed by refluxing during 1h defatted cocoa powder containing 10 – 12% fat in different solvents i.e. 5% aqueous methanol; 20% aqueous acetone; 20% aqueous ethanol and water. The crude extracts were concentrated and thereafter lyophilized. Their polyphenol content was determined using Folin Ciocalteu method according to Anonymous (17). The results are expressed as gallic acid equivalents (GAE).

Beverages were prepared at different concentrations in order to cover the different habits of consumers from various countries i.e. green and black teas were prepared as 1.5 g, 2 g or 3 g per 220 ml of hot water and infused for 5 min. Cocoa beverages were prepared by diluting cocoa powder (97%) in 220ml hot water at 1.5% up to 3.5%, in order to cover the habits of consumers from different countries.

### Bioavailability of Epicatechin in Plasma

Eight normolipidemic healthy male volunteers were studied on three occasions with one-week interval. Volunteers were asked to refrain from foods rich in polyphenols from the day before the test until its completion (restriction concerned the intake of tea, coffee, wine, fruit juice, cocoa products). They consumed chocolate with bread and water, blood samples were drawn every hour over 8h. Epicatechin glucuronides and sulphates were simultaneously hydrolyzed to aglycones and determined by HPLC (18).

## RESULTS

### Antioxidant Capacity, in vitro

The antioxidant capacity was evaluated by the resistance of LDL towards oxidation. The oxidation of LDL was initiated by copper or AAPH. Conjugated diene lipid hydroperoxides were formed from the oxidation of polyunsaturated fatty acids (PUFAs) present in the LDL particles. The kinetics of oxidation were characterized by three parameters : a) the lag time or the time during which antioxidants were consumed; b) the rate of oxidation ( $R^2$  max) and c) the maximum production of conjugated dienes. The higher the antioxidant potential, the longer the lag time.

In the presence of cocoa extracts, LDL oxidation was delayed as characterized by the increase of lag time whereas the rate of oxidation as well as the maximum production of conjugated dienes remained constant (Fig. 1). This antioxidant potential was dose dependent.



Polyphenols were previously described to have the capacity of chelating transition metal ions. Therefore, in this *in vitro* LDL oxidation model where cupric ions were used as pro-oxidant agent, the protection of LDL against oxidation by cocoa polyphenols may be an artefact due to the chelation of cupric ions rather than a scavenging of the radicals produced. To exclude this possibility, AAPH which generated free radicals at a constant rate over a few hours, was used as an alternative pro-oxidant initiator. The dose dependent ability of cocoa polyphenols to protect LDL against oxidation was similar with both pro-oxidant agents confirming the antioxidant capacity of cocoa polyphenols (Data not shown).

In order to study the effect of the food matrix on the antioxidant capacity of cocoa polyphenols, they were extracted from defatted cocoa powder with various solvents. The extraction yield of cocoa polyphenols increased in the following order : 5% aqueous methanol; 20% aqueous acetone, 20% aqueous ethanol and water with 10, 13, 19 and 32 mg GAE / g cocoa, respectively. The antioxidant capacity of polyphenols present in the 20% aqueous ethanolic and water extracts were evaluated (Fig. 2) when similar amounts of polyphenols were incorporated in the LDL system. At any polyphenol concentration, the ethanolic cocoa extract exhibited stronger antioxidant potential than the water extract, indicating that the solvent used for the polyphenol extraction of cocoa affected not only the polyphenol content of the extract but also the composition of polyphenols, the ones present in the ethanolic extract being more active. Finally, defatted cocoa powder and plain chocolate exhibited similar antioxidant potential indicating that at this stage, the food matrix had no effect (Data not shown).

The antioxidant capacity of polyphenolic-rich beverages such as cocoa and teas were also compared. For this purpose, teas (green and black) and cocoa were prepared at different concentrations in order to cover habits of consumers of different countries. Both beverages protected LDL against *in vitro* oxidation in a dose-dependent manner. The antioxidant potential of cocoa was similar to that of green and black teas (Fig. 3).

### **Epicatechin Bioavailability**

The bioavailability of cocoa polyphenols from plain chocolate (18) was compared to a lyophilized powder of 20% aqueous ethanolic extract of defatted cocoa powder. The aim of using the extract from cocoa powder was to have a simple matrix of polyphenols which would be compared to a more complex food matrix (chocolate). Precise analysis of epicatechin showed 82 mg, 164 mg and 33 mg in 40g, 80 g plain chocolate and 11.45 g cocoa extract.

Cocoa contains a wide variety of polyphenolic compounds. We determined the appearance of epicatechin in plasma as a marker of the cocoa polyphenol bioavailability. Before starting cocoa administration, plasma epicatechin at 0h (Fig. 4) was very low or most of the time undetectable indicating that volunteers did effectively refrain from a polyphenol rich diet. After cocoa intake, plasma epicatechin rose to 111 ng/ml (0.383  $\mu$ M) with 40 g chocolate, to 203 ng (0.7  $\mu$ M) with 80 g chocolate and to 41 ng/ml (0.141  $\mu$ M) with cocoa extract. The clearance of epicatechin from plasma compartment was very fast (half time of 2h, 2.8h and 3h,

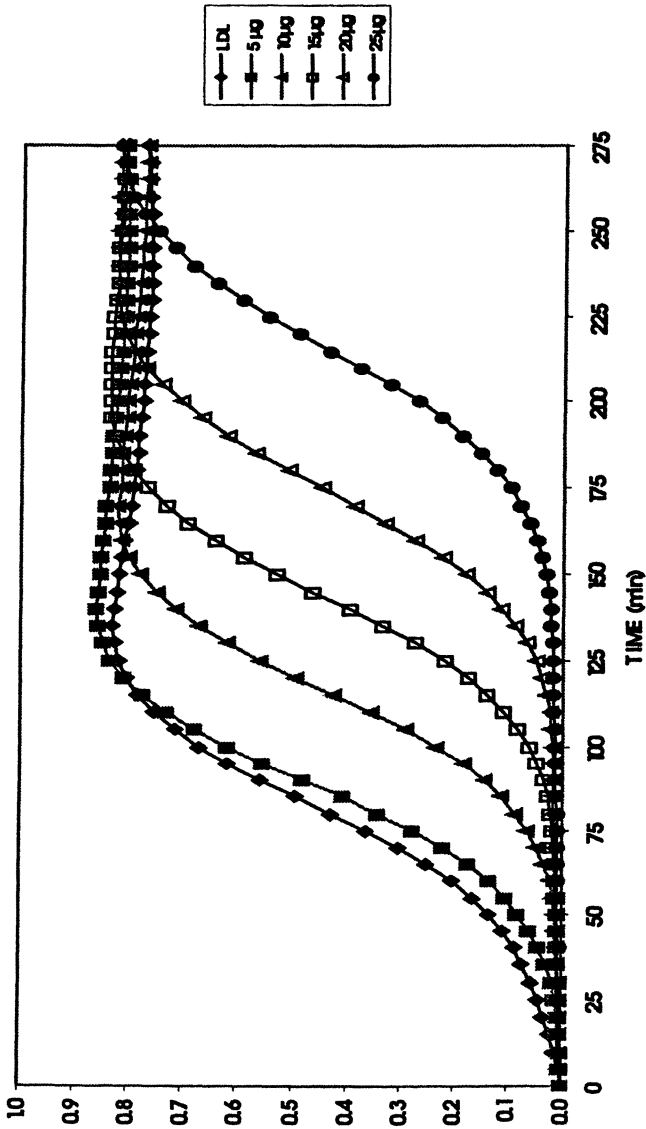
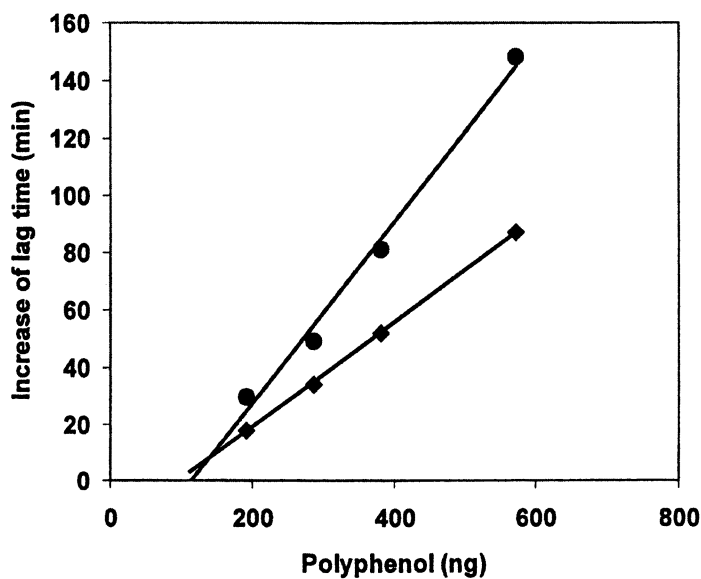


Figure 1: LDL were oxidized with cupric ions (1.7 mM) at 37°C in the absence or the presence of increasing amount of defatted cocoa powder.



◆ Water ● 20% aqueous EtOH

Figure 2: The antioxidant capacity of water and 20% ethanolic cocoa extract was compared at similar polyphenol content. The protection of LDL against oxidation is expressed by the increase of lag time (lag time of LDL with cocoa extract minus lag time of LDL control).

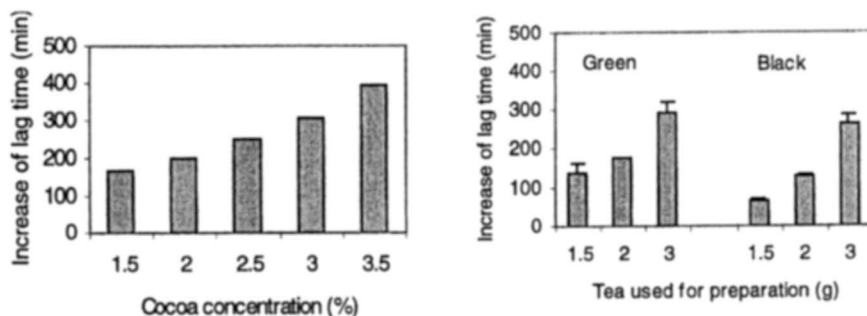
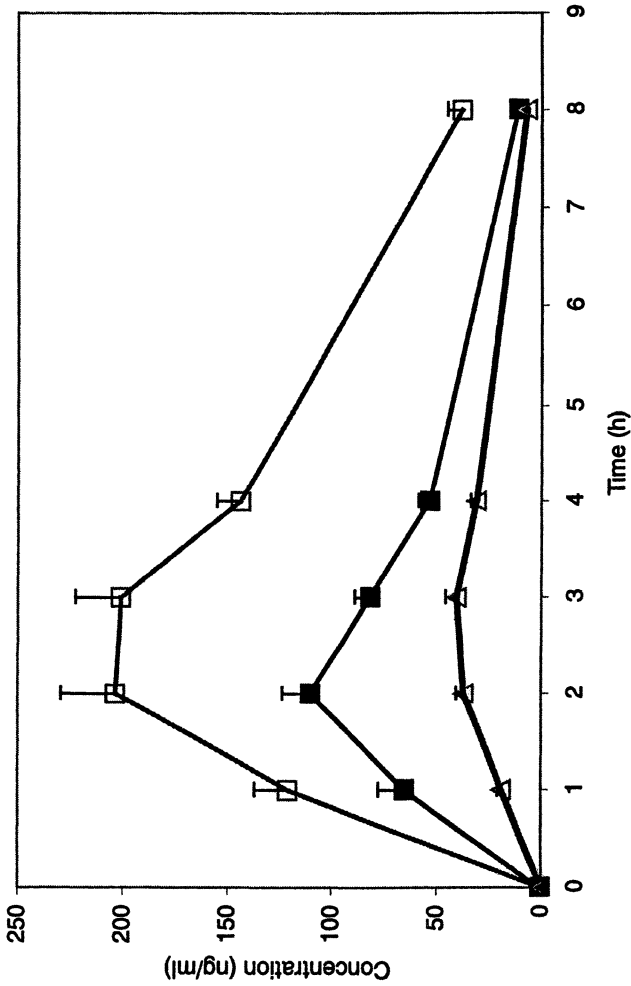


Figure 3: Cocoa and teas beverages were prepared at different concentrations. The protection of LDL against oxidation by cupric ions in the presence of 1  $\mu$ l of each beverage was evaluated. The results mean  $\pm$  SD, n= 3.



—■— 40g plain chocolate —□— 80g plain chocolate —▲— Cocoa extract  
 Figure 4: Plasma epicatechin concentration after ingestion of 40g, 80g plain chocolate or 11.45g 20% aqueous ethanolic cocoa extract. Results are expressed as mean  $\pm$  SD.

respectively). Interestingly, the  $C_{max}$  and area under the curve of the plasma epicatechin kinetics were proportional to the dose of chocolate ingested and was not affected by the food matrix. The relative bioavailability of epicatechin in plasma was 1.45%, 2.12% and 2.05% for 40g, 80g chocolate and cocoa extract, respectively. Interestingly, the bioavailability of epicatechin from cocoa is quite similar to that of tea epicatechins which is 2% (19).

## DISCUSSION

This study demonstrates an antioxidant capacity of cocoa polyphenols towards LDL oxidation. In order to study the effect of the food matrix, cocoa polyphenols were extracted from defatted cocoa powder using different solvents. Water and 20% aqueous ethanol results in a good yield of polyphenols. Although water resulted in more abundant polyphenol content in the extract compared to 20% aqueous ethanol extract, the biological activity of polyphenols present in the water extract is weaker, indicating that the solvent used for the extraction affects not only the total concentration of polyphenols but also the type of polyphenols. The physical and chemical properties of individual phenolic molecules strongly affect their antioxidant potential (20-22). In addition, these molecules could have a synergistic or an antagonistic effect when present in complex mixtures. On the other hand, the antioxidant potential of plain chocolate or cocoa powder is similar indicating that the food matrix has no effect.

Therefore, it is clear from the present data that polyphenols of cocoa and teas (green and black) have an *in vitro* antioxidative capacity. A better understanding of the protective role of dietary antioxidants *in vivo* requires quantitative data on their absorption (23). Indeed after consumption, polyphenols have to cross the intestinal wall but must also resist further catabolism. The metabolism of epicatechin involves two important organs : the liver, where biotransformation enzymes convert epicatechin or their metabolites into conjugated form such as glucuronides or sulphates (24), and, the colon, where microorganisms degrade unabsorbed epicatechins (25). Plasma kinetics of epicatechin from plain chocolate (18) and 20% aqueous ethanol extract were evaluated in man after a single oral administration. These plasma concentration curves represent the net result of two opposite processes : absorption vs elimination i.e. involving catabolism, storage in tissues and excretion in urine). Epicatechin was rapidly absorbed leading to appreciable amount in plasma (up to 0.7  $\mu\text{M}$ ). The maximal plasma concentration as well as the area under the curve were strongly related to the amount of epicatechin present in plain chocolate or extract, indicating that no saturation of the absorption is reached and that the food matrix does not affect the epicatechin absorption. In addition, similar absorption of epicatechin from tea has been previously described by Lee et al (19). Interestingly, plasma concentration of 1  $\mu\text{M}$  which is in the biologically accepted range to achieve a biological effect (26). However, further studies are necessary to elucidate whether polyphenol antioxidants from cocoa show antioxidant *in vivo*.

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## Chapter 13

# Antioxidative Phenolic Compounds in Green–Black Tea and Other Methylxanthine-Containing Beverages

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More than 90 tea samples (green and black, from different origins) have been analyzed for the total polyphenols and for individual catechins, flavonol glycosides, theaflavins, and flavone C glycosides. The contents of total polyphenols in green and black tea samples were about the same while catechins in green tea samples were in average roughly 3 times as in the black tea samples (15 vs 4 %), however, in some Darjeeling samples the catechin amount (ca 10 %) was in the same order of magnitude as in green teas with low catechin contents. The contents of flavonol glycosides (0.58 – 2.11 %) and flavone C glycosides (0.013 – 0.26 %) were only little higher in green teas. - The amounts of theaflavins in black teas were between (0.3 - 2.4 %) for the 4 main theaflavins and 100 – 200 mg/100 g for the 5 minor theaflavins determined. Extraction experiments with tea bags showed that ca 70 % of the total flavonol glycosides were extracted in a tea brewed for 2 minutes (“total” extract was a 2 stage extraction using 70 % methanol).

Health benefits of flavonoids from tea and other sources have been published in a great extent in the past few years. Catechins, especially epigallocatechin gallate (EGCG), theaflavins and flavonol glycosides are thought to be responsible for antioxidative properties in tea (e.g. 1-3). Flavonol glycosides (FOG) have activities against myocardial infarct and stroke. The properties of the compounds, including their absorption characteristics, have been reviewed recently (1-3).

## Newly identified polyphenols

### Proanthocyanidins

In tea many proanthocyanidins have been described by Japanese groups, e.g. [4-6]. Several publications deal with the isolation and identification of proanthocyanidins from tea but only one paper uses HPLC which has been used in other foodstuffs, e.g. [7, 8]. It is likely that the proanthocyanidins contribute to the health benefits but data on the occurrence and the amounts in tea are scarce. In a LC-MS study we found at least 12 proanthocyanidins in a green tea sample which could be tentatively identified (9). More recently the structural elucidation of the compounds in figure 1 was possible using various NMR techniques (10, 11). Three of them (**bold printed in figure 1**) have not been described previously, EAG-4→8-EGCG was identified in tea for the first time. Figure 1 gives a HPLC separation of a green tea sample (RP-18; detection at 280 nm, eluent A was acetic acid (2 %, aq.), eluent B acetonitrile, gradient: 5 – 26 % B in 75 min). The sample pretreatment consists of an extraction from ground tea (75 % acetone) and (after removing acetone, sample dissolved in water) solid phase extraction using polyamide cartridges. The interfering compounds (catechins and flavonol glycosides) are removed from the column by washing with methanol and acidified methanol and finally the proanthocyanidins are eluted using 75 % acetone. A method for the quantification of the compounds is in progress.

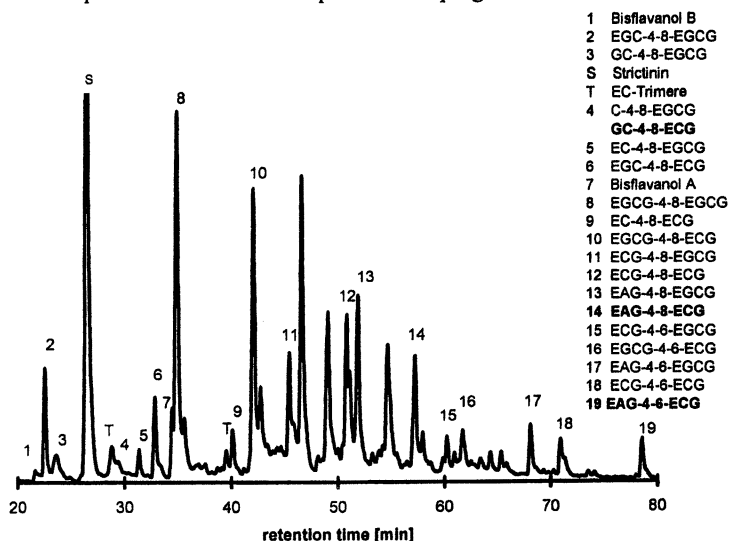


Figure 1. HPLC-separation of proanthocyanidins (details see text). Abbreviations: EGCG: epigallocatechin gallate; EC: epicatechin; GC: galocatechin; ECG: epicatechin gallate; EAG: epiafzelechin gallate; EGC: epigallocatechin



### Flavonol glycosides (FOG)

Recently novel flavonol glycosides have been identified by our group. These are kaempferol-3-O-[ $\alpha$ -L-rhamnopyranosyl-(1 $\rightarrow$ 3)- $\alpha$ -L-rhamnopyranosyl-(1 $\rightarrow$ 6)- $\beta$ -D-glucopyranoside], kaempferol-3-O-[ $\alpha$ -L-rhamnopyranosyl-(1 $\rightarrow$ 3)-(4'''-O-acetyl)- $\alpha$ -L-rhamnopyranosyl-(1 $\rightarrow$ 6)- $\beta$ -D-glucopyranoside] and K-rrgal (kaempferol-3-O-[ $\alpha$ -L-rhamnopyranosyl-(1 $\rightarrow$ 3)- $\alpha$ -L-rhamnopyranosyl-(1 $\rightarrow$ 6)- $\beta$ -D-galactopyranoside]). The structural elucidation was accomplished by NMR after isolation of the compounds by polyamide clean up, gel chromatography and preparative HPLC (12). Figure 2 shows a chromatogram (RP-18; eluent A was 2 % acetic acid (aq.), eluent B was acetonitrile; 6 – 20 % B in 50 min, detection at 354 nm). K-rrgal (peak 12 in figure 2) was present in almost all China samples and only one from Japan. LC-MS data show the presence in Darjeeling and Assam samples of another flavonol trisaccharide (K-rrg) which co-elutes with K-rdg (Peak 13 in fig.2): it contains two rhamnose and one glucose or galactose moiety (possibly branched). A similar observation was made for quercetin trisaccharides (peaks 6 and 9) tentatively identified to contain 2 rhamnose and a glucose or galactose moiety. The compounds from the Assam and the China tea seem to contain the same sugar moieties but their retention behavior is different. The structural elucidation is in progress. Obviously the triglycoside patterns differ characteristically for samples of different origins. It has to be stressed that we did not succeed in resolving all FOG from tea with a single HPLC system. A system for the resolution of quercetin rutinoside and kaempferol glucorhamnogalactoside has been published (13).

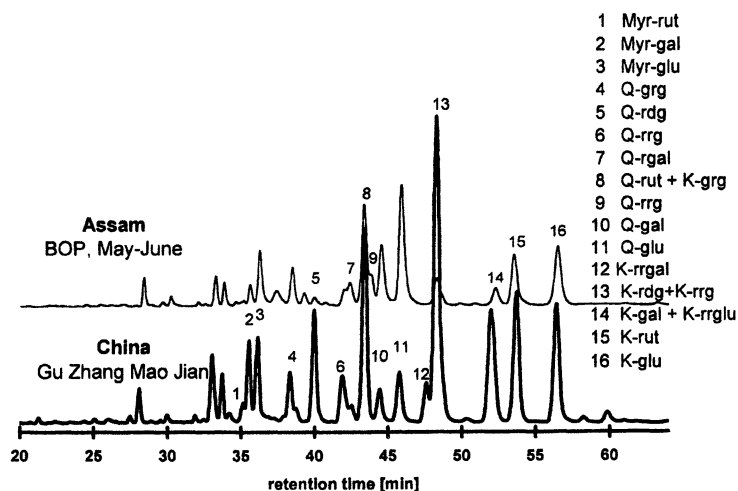


Figure 2. FOG determinations by HPLC. Q: quercetin, Myr: myricetin, K: kaempferol, glu: glucose, gal: galactose, r: rhamnose; rut: rutinoside; rdg: rhamnoglucoside

## Data on polyphenols in green and black tea samples

In a survey study we analyzed green and black tea samples (blends and unblended samples) representing samples typical for the German tea market for total polyphenols and several groups of individual polyphenols (14). The majority of the black tea samples was from India (Darjeeling: 16, Assam: 11, South India: 1, Sri Lanka: 4, Indonesia: 4, Afrika: 3, China: 3), while the green tea samples were primarily from China (China: 38, Japan: 7, Taiwan: 2 along with 1 green Darjeeling, 1 Vietnam and 2 green Assam samples). The methodology for the determinations has in principle been published. Thermospray LC-MS was used to confirm findings if necessary and for method development (15, 16). The final analytical method in case of catechins, theaflavins, flavonol glycosides and flavone C glycosides was RP-HPLC (13, 17 - 19). In case of the catechins only an extraction (2-stage, 70 % methanol, 70 °C) but no clean-up was carried out. The results in detail will be published in the near future (14). Going to details means that catechin data consist of at least 4 individual compounds, theaflavins are up to 11, flavonol glycosides 13, flavone C glycosides 7. An overview of the data generated can be found in table I.

**Table I. Polyphenols in green and black tea samples**

	<i>Green tea</i>	<i>Average</i>	<i>Black tea</i>	<i>average</i>
total polyphenols	10.1 – 22.2	17.0	8.3 – 24.8	16.5
Catechins	8.5 – 20.6	15.1	0.74 – 10.00	4.2
Caffeine	1.5 – 5.2	3.4	2.0 – 5.4	3.5
Theogallin	0.1 – 1.4	0.6	0.1 – 1.0	0.6
Gallic acid	0.01 - 0.19	0.09	0.16 – 0.60	0.26
Theaflavins	n.d.	n.d.	0.30 – 2.41	0.94
Flavonol glycosides *	0.28 – 0.95	0.64 (1.38)	0.24 – 0.87	0.47 (0.89)
Flavone C glycosides*	0.005 – 0.14	0.086 (0.16)	0.02 – 0.12	0.051 (0.09)

Note: Units are g/100 g; n. d. = not determined, \* = calculated as aglycones; in parentheses calculated as glycosides

### *Total polyphenols (TPP)*

The total polyphenols data were determined by the Folin-Ciocalteu method (20) currently under validation for tea by ISO. The data are expressed as gallic acid equivalents as gallic acid was the calibrant. As can be seen, the average levels found were only slightly lower in black than in green tea. More extensive ISO data on green and black tea confirm this result (average levels in green and black teas were 17.5 and 14.4 % respectively).

### Catechins

The data of the catechins given are the sum of EGCG, EGC, EC and ECG. It appears for black teas that these figures are high in Darjeeling samples while those are much lower in Assams (average: 6.6 vs 1.9 %; data not shown). Some of the Darjeeling black teas are higher in catechins compared with some green teas lower in catechins. Table II gives a comparison of black teas high in catechins and green teas low in catechins. In earlier studies we found black teas with even higher catechin levels (up to 12.5 %) and green teas with even lower catechin levels (down to 5%) than those reported in Table II. Even in those black teas high in catechins the comparison of the sum of catechins and the total polyphenols showed that in nearly all cases the proportion of catechins was less than 55 %. In green tea this proportion was more than 55 %, (1 exception). As regards black teas Assams are quite low in catechins (average 1.9 % with a percentage of total polyphenols ca 20 %).

**Table II. Catechins and caffeine in selected green and black teas**

<i>Sample</i>		<i>caffeine</i>	<i>EGC</i>	<i>EC</i>	<i>EGCG</i>	<i>ECG</i>	<i>sum</i>
Darjeeling	black	4.19	0.44	0.20	6.71	2.11	9.46
Japan	green	2.40	2.99	0.67	5.8	1.00	10.46
Japan Bancha	green	1.78	3.57	0.82	4.25	0.78	9.42

Note: Data are given in %. Abbreviations: EGC egigallocatechin; EC epicatechin; EGCG epigallocatechin gallate; ECG epicatechin gallate

### Flavonol glycosides (FOG)

We determined up to 14 FOG (the novel FOG are not included) using two different HPLC systems after polyamide clean-up (13). The FOG concentration (sum, calculated as glycosides) in the tea samples was between 0.6 and 2.1 %. The determination of the individual glycosides is important as the sugar moiety seems to affect the absorption of these flavonoids (21). There was no difference in principle between green and black teas. The average content was higher in the green teas compared with black teas, but this seems at least in part to be due to the origins. The tea with the highest concentration was a China green Pekoe with 2.12 % (calculated as glycosides). Darjeelings had amounts between 0.5 and 1.21 % (average was 0.94 %) while Assam samples were between 0.43 and 0.7 (average was 0.57). Taking a look at the pattern of these flavonoids (calculated as aglycones) there is no significant difference between the average data of green and black tea (kaempferol 30/29 %; myricetin 21/17 %; quercetin 54/50 %) compared with the sum of the aglycones.

### Theaflavins (TF)

Up to 11 theaflavins were determined in extracts of black teas prepared by a

newly developed continuous extraction procedure employing a rotary perforator (22). These are the 4 main theaflavins (theaflavin, the two monogallates and the digallate), theaflavic (only detected in Assams as yet) and epitheafalvic acids, epitheafalvic acid-3-gallate, neotheaflavin, epitheafalagalline, epitheafalagalline-3-gallate. The method is also capable of detecting isotheaflavin but this compound was not detectable in the samples analyzed as yet. The sum of theaflavins was between 0.30 and 2.40 %. It was obvious that the Darjeeling samples had much lower contents of theaflavins (0.30 – 0.62 %) compared with the Assams (1.08 - 1.69 %). Very high amounts were found in African tea samples (1.75 – 2.41 %). The minor theaflavins constituted 5 - 10% of the total found.

#### *Flavone C glycosides (FCG)*

The methodology consists of an extraction, polyamide column chromatography I, enzyme hydrolysis of interfering FOG, polyamide column chromatography II and HPLC (19). The amounts of FCG in are given in table I. There is no obvious difference in the FCG pattern of teas from different origins.

#### **Behaviour of tea polyphenols during decaffeination**

In an experimental study we compared the total polyphenols, the contents of catechins and relative amounts of theaflavins in 5 teas each consisting of 4 samples (1 untreated, 1 decaffeinated using either CO<sub>2</sub>, ethylacetate or dichloromethane). It turned out that ethylacetate extraction gave samples lower in total polyphenols, catechins and theaflavins than both the other decaffeination techniques (23). Details of the technology applied except the solvents used were not available.

### **Polyphenol contents in tea, coffee, and cocoa beverages**

In a study carried out in cooperation with Unilever Research at Colworth House (UK) we analyzed consumer brews of tea (e.g. tea bags containing 3.125 g + 235 mL of water, brewing times between 25 and 240 sec), instant coffee, coffee (different degrees of roast, 20 or 40 g ground coffee x L<sup>-1</sup>), cocoa mixes and dry mixes on cereal base. To get reproducible results, the brewing procedures were standardized. We used 4 types of tea bags (U.K., U.S. and two international type tea bags). The tea bags were placed in a mug, the initially boiling water was added and stirred for 5 sec. Right before the end of the brewing time the bag was removed from the brew and squeezed against the beakers wall. This procedure was repeated six times and the brews combined to form an analytical sample. The methods used were as described above, for chlorogenic acids determination see (24). Chlorogenic acids are the main polyphenolic compounds in coffee and those are also present in tea samples. The data will be published in more detail (25). Selected results from the U.K. tea bags are compiled in table III. Please note that in this investigation, the data for individual

polyphenols have also been determined. In table III e.g. only the sum of flavonol glycosides is given, in fact 13 individual flavonol glycosides have been analyzed. The 70 % methanol extract data give the absolute composition of the tea bag product as sold.

**Table III. Results of the brewing studies using the U.K. tea bag**

<i>Brew time</i>	<i>TSS</i>	<i>TPP</i>	<i>TotalFlavs**</i>	<i>Catechins</i>	<i>FOG</i>	<i>FCG</i>	<i>TF</i>
40sec*	2480	675	584	39	50	5.23	55
120sec*	2990	841	729	56	63	6.65	77
MeOH ext***	n.d.	16.0	14.9	1.34	0.863	0.089	1.54

NOTE: \* Data on aqueous brews are given in mg/liter of brew; \*\* Total flavonoids = TPP - (gallic acid + theogallin + chlorogenic acids) \*\*\* Data on MeOH extract are given in % on weight of leaf. TSS = total soluble solids; n.d. = not determined.

The results for the different types of tea bags were similar. At an average adult daily tea consumption of 600 ml (26), tea contributes (as aglycones) 23.8 mg flavonols and flavones (2 min brew) to the average UK total daily intake of 29.8 mg (27). Tea is the dominant dietary source of these flavonoids (more than 80 % for the 2 min brew) in the UK. - In case of coffee 3 types of coffee (low, medium and dark roast) were brewed in a percolator. The amount of coffee was 20 g per Liter of water, in case of the medium roast sample also a sample with 40 g/L was analyzed. Compared with the total polyphenols in tea brews the figures were higher in coffee. However, no catechins, flavanol or flavone glycosides or other flavonoids could be detected in coffee brews. The group of chlorogenic acids (CGA; 6 main compounds) were the main polyphenols detected in coffee (cf. Table IV).

**Table IV. Coffee-data**

<i>Type</i>	<i>TSS</i>	<i>TPP</i>	<i>CGA</i>	<i>Catechins, FOG</i>
Light-roast (LR)	n.d.	5.41 %	1.69 %	n. det.
Medium-roast (MR)	n.d.	5.25 %	1.92 %	n. det.
Dark-roast (DR)	n.d.	5.70 %	1.55 %	n. det.
LR: 20 g/L	5.64 g/L	0.96 g/L	0.375 g/L	n. det.
MR: 20 g/L	5.74 g/L	1.06 g/L	0.403 g/L	n. det.
MR: 40 g/L	12.11 g/L	2.27 g/L	0.859 g/L	n. det.
DR: 20 g/L	6.08 g/L	1.06 g/L	0.342 g/L	n. det.

Note: n.d.: not determined; n. det.: not detected

In the cocoa drinks (methanol extracts) 6 % of total polyphenols could be detected along with small amounts of chlorogenic acids, catechins and flavonol glycosides. The amounts of total polyphenols in cereal based dry mixes (containing wheat flour, barley among others) was below 1 %. Neither the catechins nor other flavonoids analyzed were detectable.

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## Chapter 14

# Radical Scavenging and Antioxidative Properties of Phenolic Compounds in Relation to Their Chemical Structure

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Many foods of plant origin, especially tea, coffee and cocoa, contain higher amounts of polyhydroxyphenols which show antioxidative effects *in vitro* and *in vivo* prolonging the shelf life of foods and protecting human health. It is shown that there are clear relationships between the antioxidative properties of different polyhydroxyphenols and their reducing and radical scavenging effects. The radical scavenging properties of phenolic compounds were determined by reaction with the stable radical 2,2-diphenyl-1-picrylhydrazyl. It turned out that phenolic compounds with at least one hydroxy or phenoxy group in *o*-position to the phenolic hydroxy group are showing radical scavenging and reducing properties due to their proton-donating activities. There are clear relationships between the chemical structures of phenolic compounds and the degree of their radical scavenging effect. To this catechol and quercetin were the most effective, followed by cinnamic acid derivatives like ferulic acid, sinapic acid and caffeic acid, whereas benzoic acid derivatives were less effective. These compounds are showing the same sequence in their antioxidative activity in lard. In methylinoleate-water emulsions the antioxidative effect of phenolic compounds increases with decreasing droplet size and increasing total surface of the fatty phase.

Caffeine containing beverages like tea, coffee and cocoa drinks are rich in polyphenols which have the capability to prevent cancer and cardiovascular diseases based on their radical-scavenging and antioxidative activity (1 – 5). Polyphenols are recognized as *in vitro* as well as *in vivo* antioxidants prolonging the shelf life of foods and protecting human health (3,4). On the other side, in biological systems like fruits and vegetables, also in tea, coffee and cocoa, polyphenols are oxidized by the enzyme polyphenol oxidase causing enzymatic browning (6).

The most important phenolic compounds in plants can be divided into phenolic carboxylic acids (benzoic acid and cinnamic acid derivatives like chlorogenic acid) and flavonoid compounds like catechins and quercetin.

Figure 1 shows the main representatives of phenolic compounds in foods of plant origin. Hydroxy-benzoic acid derivatives can be found in spices (7) and in smaller amounts in fruits and vegetables (8). Hydroxy-cinnamic acid derivatives like caffeic acid (3,4-dihydroxy-cinnamic acid) or chlorogenic acids (esters between caffeic acid and quinic acid) are present in vegetables and fruits (8, 9). Glycosides of flavons and flavonols like quercetin are occurring in a great variety of fruits, vegetables and spices (8), whereas catechins are present in many fruits and spices (10, 11), in tea and cocoa.

Tea contains several derivatives of catechin (12, a high portion of it being esterified with gallic acid), chlorogenic acids (caffeoyl- and *p*-coumaroylquinic acids,13), 3-galloylquinic acid (1g/100g dry matter,14) and theogallin (15) with a total amount of 20 – 30 % of dry matter. In coffee mainly chlorogenic acids (5-caffeoyl-quinic acid being the main representative) are present (16), whereas in cocoa catechins are prevailing. During roasting the chlorogenic acid content in coffee decreases by 60 % and more. On the other side, during roasting of cocoa a much smaller amount of catechins is lost.

It is well known that cocoa because of its content of catechin and epicatechin shows antioxidative properties used in products like nougat, milk crumb or milk chocolate. It turned out that alcoholic extracts of defatted cocoa or cocoa husks exert antioxidative effects on unsaturated oils (17) which can mainly be attributed to their epicatechin content.

It is also well known that the antioxidative effect of polyhydroxyphenols increases dependent on the amount of OH- or methoxy groups in the *o*- or *p*-position (18). These groups have electron shifting properties (negative sigma values according to the Hammett equation) thus stabilizing the intermediate phenoxy radicals formed within radical chain oxidative reactions; in this way the radical scavenging and antioxidative activity of polyhydroxyphenols is increased.

In order to establish structure-activity relationships of polyhydroxyphenols as shown in Figure 1 the reducing, radical scavenging and antioxidative properties of selected phenolic compounds shall be investigated.



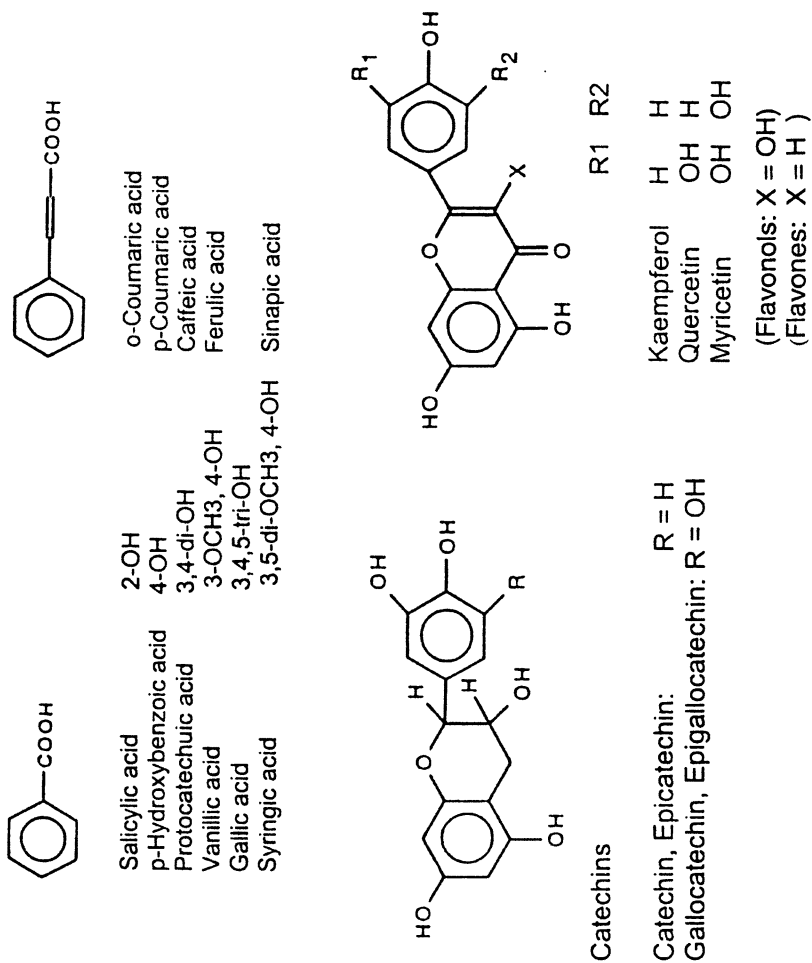


Figure 1. Chemical structures of different classes of polyhydroxyphenols

## Reductive, Radical Scavenging and Antioxidative Properties of Polyhydroxy-phenols in Homogeneous Systems

### Methods

It was demonstrated by Crowe et al. (19) that the overall reducing capacity of milk and milk products can be measured by the potassium-ferricyanide method, where it is reduced to ferrocyanide giving a blue color after addition of Fe-III ions. Because ferricyanide shows stoichiometrical reactions with cysteine and ascorbic acid, the reaction can be calibrated as to the amount of reducing equivalents measured; as expected, one mole of cysteine yielded one reducing equivalent, one mole of ascorbic acid two reducing equivalents.

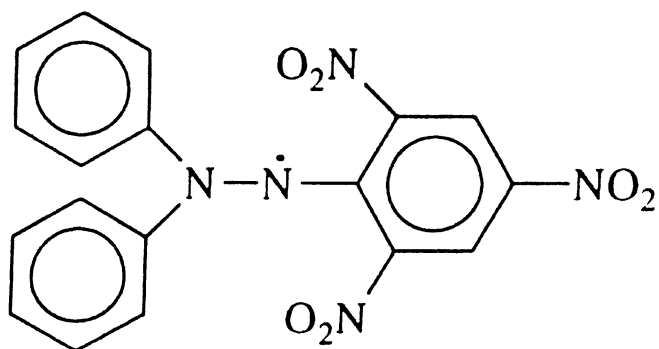
For analyzing the radical scavenging effect of phenolic antioxidants they were reacted with the stable radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Fig.2) showing a blue color. After taking up a hydrogen atom from a hydrogen-donating (reducing) compound, it is transferred to the colorless 2,2-diphenyl-1-picrylhydrazine (DPPHH) in a stoichiometrical reaction (20). DPPH reacts with cysteine, ascorbic acid, tocopherol and polyhydroxyphenols, but not with glucose or phenolic compounds with only one OH-group (20, 21). Figure 3 shows the absorption spectra of DPPH and DPPHH. The reaction can be followed up by measuring the decrease of the absorption at 525 nm (absorption maximum of DPPH). The reaction takes place in ethanolic solution within 60 min at room temperature.

The antioxidative effects of different phenolic compounds were measured by using a modified Swift test at 120 °C (rancimat method) (18). This method is based on a conductometric measurement of volatile acids (mainly formic acid) emerging after the induction period as by-products of fat oxidation. For comparing the antioxidative effects of different phenolic compounds, alcoholic solutions of these compounds were mixed thoroughly with molten lard in amounts of 1 mmol/kg. After that the solvent was evaporated and air passed through aliquots of the lard at 120 °C into a conductivity cell. After the induction period the conductivity increases steeply. There is a good correlation between the induction period measured and the storage stability of fats and oils at room temperature (22).

### Results

The antioxidative effect of polyhydroxyphenols is based on their hydrogen donating effect, by which they inactivate free radicals and interrupt the radical chain of lipid oxidation (23). The question was, if there are structure-related connexions between their antioxidative effect and their reducing and radical scavenging effect.

As it is shown in Figure 4, monohydroxy-benzoic acids and dihydroxy-benzoic acids with the OH-groups in m-position do not exert any reducing or



2,2-Diphenyl-1-picrylhydrazyl (DPPH)

*Figure 2. Chemical structure of 2,2diphenyl-1-picrylhydrazyl*

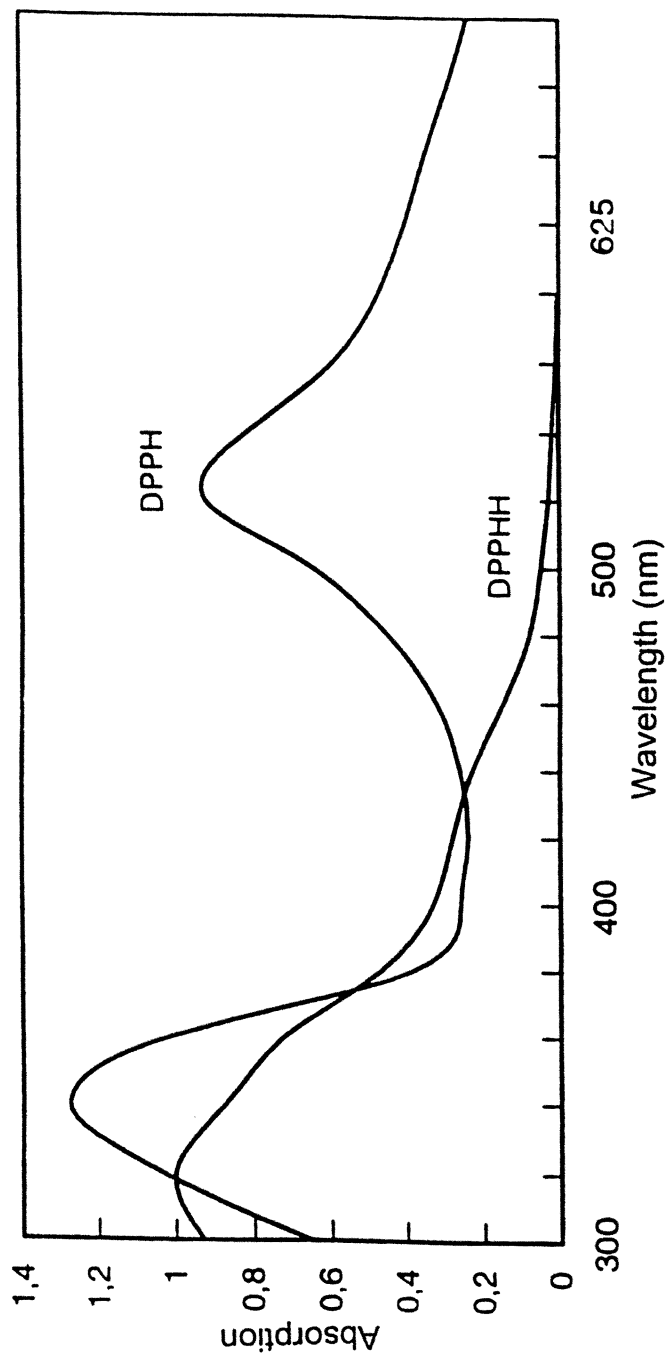


Figure 3. Absorption spectra of 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2-diphenyl-1-picrylhydrazine (DPPHH)

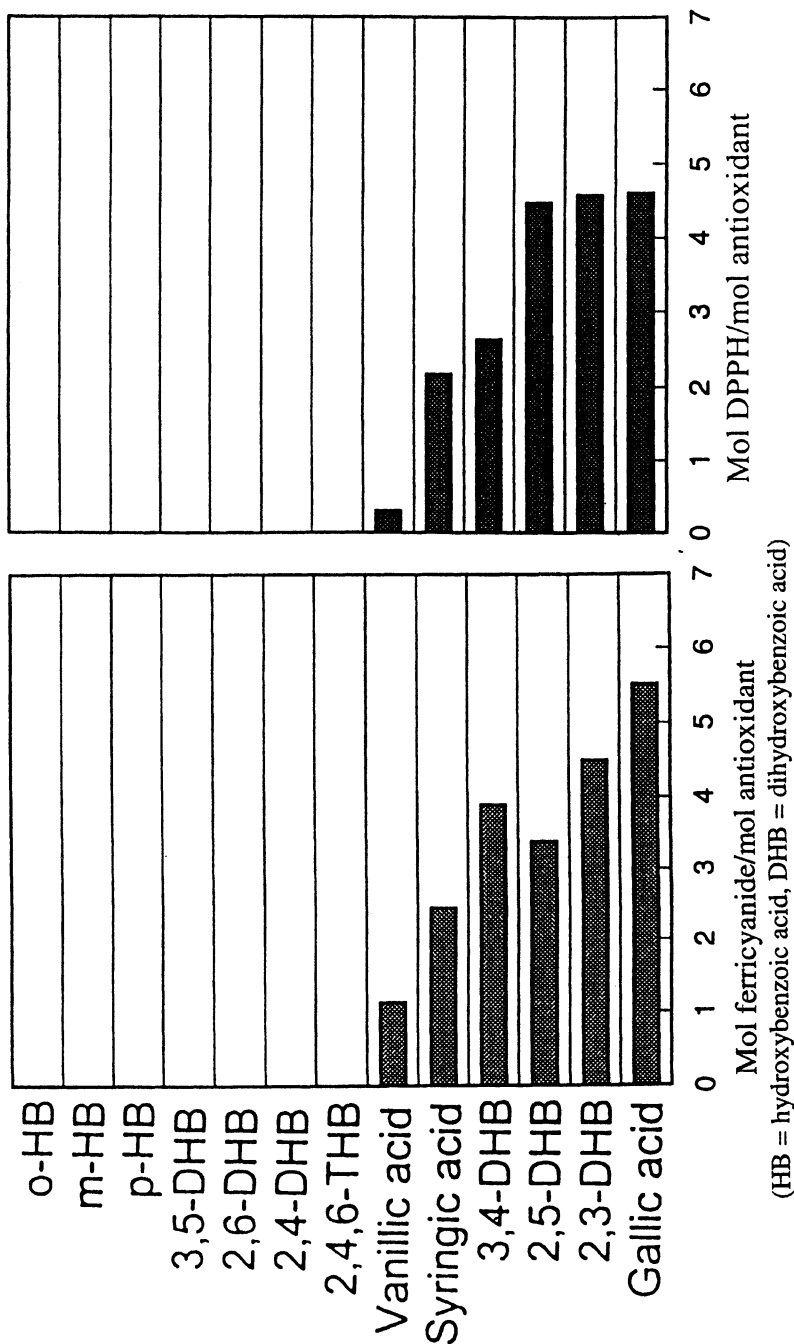


Figure 4. Reducing and radical scavenging properties of hydroxybenzoic acid derivatives

radical scavenging effect. In the presence of one or two electron-shifting methoxy groups in *o*-position to the OH-group there is an increase of both effects from vanillic acid to syringic acid. There is a further increase in the reducing and radical scavenging properties, if there are two or three hydroxy groups in *o*- or *p*-position. Gallic acid shows the strongest reducing capability. It is remarkable that the dihydroxy-benzoic acids and gallic acid have higher reducing and radical scavenging effects than it should be expected from a stoichiometrical point of view. Figure 5 shows the induction periods for the oxidation of lard in the presence of different hydroxy-benzoic acids. It can be seen that monohydroxy- and dihydroxy-benzoic acids with the OH-group in *m*-position have no or almost no antioxidative effects. This is also the case with vanillic and syringic acid (having one or two methoxy groups in *o*-position), whereas they clearly show radical scavenging effects. The antioxidative effect really starts with a second OH-group in *o*- or *p*-position, gallic acid with three hydroxy groups in *o*-position having the highest activity; on the other side, 2,3-dihydroxy-benzoic acid has the lowest effect, possibly because of steric hindrance. Figure 6 demonstrates the reducing and radical scavenging effects of different hydroxy-cinnamic acids. Similar to the monohydroxy-benzoic acids, *o*-, *m*- and *p*-coumaric acid show neither reducing nor radical scavenging capacities. One methoxy group in *o*-position (ferulic acid) introduces reducing as well as radical scavenging properties which are enhanced by a second methoxy group (sinapic acid). As expected from the foregoing results, a second OH-group in *o*-position (caffeic acid) greatly increases both effects. Apparently, esterification of the carboxy group of caffeic acid with quinic acid (chlorogenic acid) does not change these properties. Figure 7 shows a very clear relationship between the antioxidative activities of the above mentioned hydroxy-cinnamic acids and their radical scavenging effects. They have a higher antioxidative potential than the respective hydroxy-benzoic acids.

In Figure 8 the reducing and radical scavenging effects of the flavonoids catechin and quercetin are demonstrated. Catechin having *o*-dihydroxy groups in the B-ring shows a certain similarity to caffeic acid also having *o*-dihydroxy groups. Quercetin which has an OH-group at C3 being in a vinylogical position to the OH-groups in the B-ring has stronger reducing and hydrogen-donating properties than catechin which is comparable to propyl gallate. On the other hand, tocopherol, BHT, BHA and ascorbyl palmitate show smaller effects than catechin and quercetin. Tocopherol and ascorbyl palmitate show the expected reducing capacity of two reducing equivalents per mole. As shown in Figure 9, there again is a good correlation between the radical scavenging effects and the antioxidative effects of all compounds listed in the Figure. It becomes clear that generally phenolic compounds with two or three OH-groups in *o*-position (catechin, quercetin, propyl gallate) have a stronger antioxidative effect than ascorbyl palmitate, BHT, BHA and tocopherol with only one phenolic OH-group. The antioxidative capacity of quercetin may be influenced to a certain extent by its heavy metal-chelating properties caused by the OH-group in C3 position in neighbourhood to the carbonyl function in C4. Hydration of the double bond between C2 and C3 or glycosidation of the OH-group in the C3 position reduces the antioxidative potential of quercetin significantly.

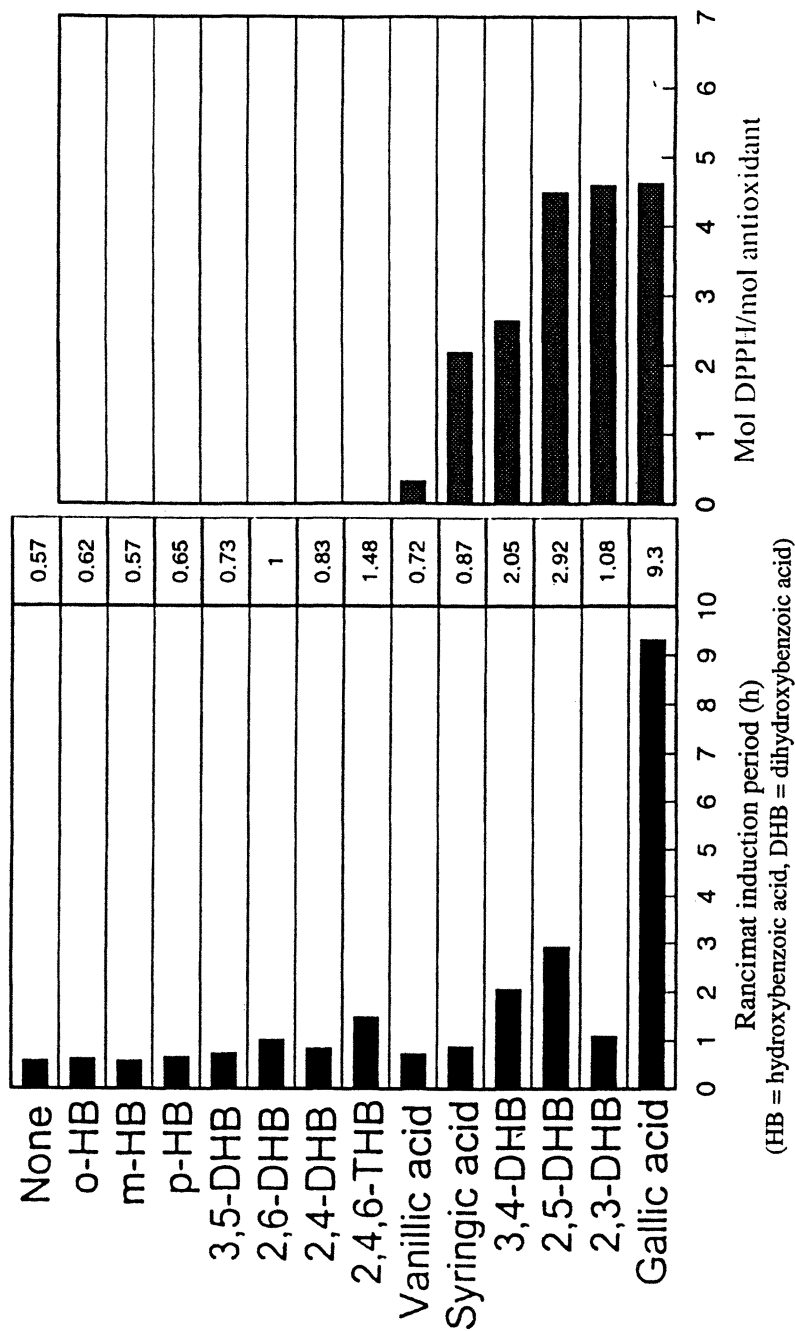


Figure 5. Antioxidative and radical scavenging properties of hydroxybenzoic acid derivatives

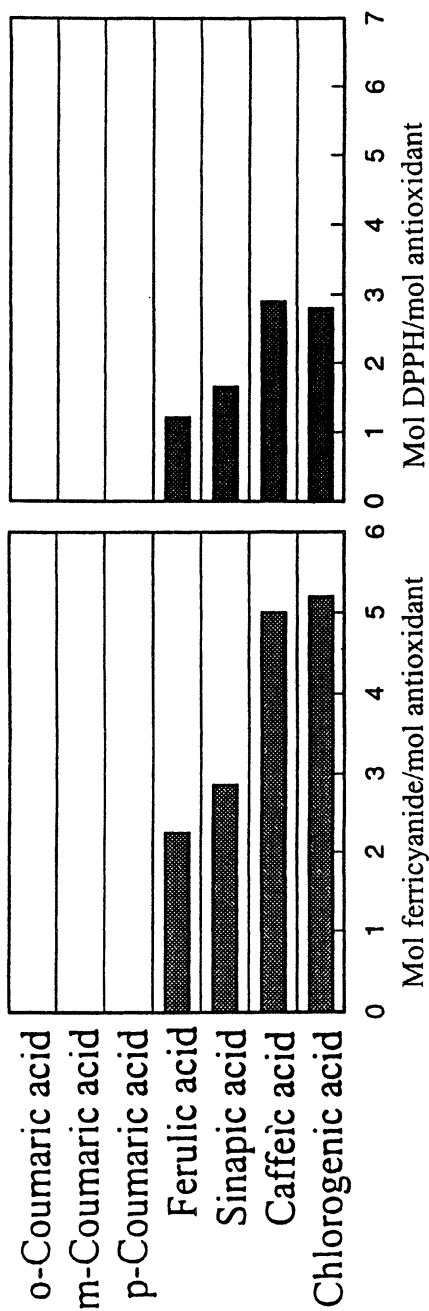


Figure 6. Reducing and radical scavenging properties of cinnamic acid derivatives



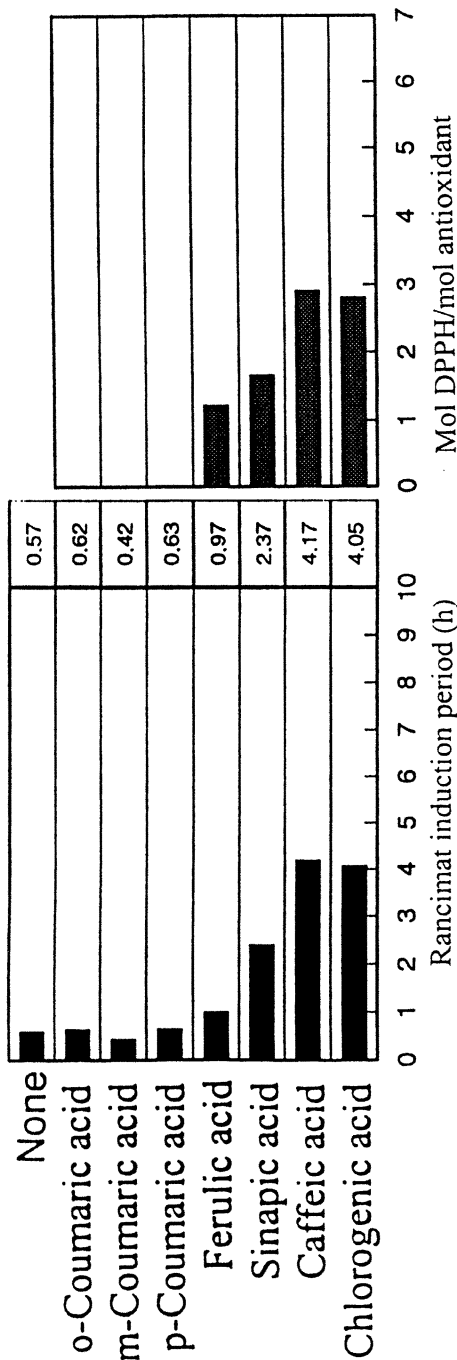


Figure 7. Antioxidative and radical scavenging properties of cinnamic acid derivatives

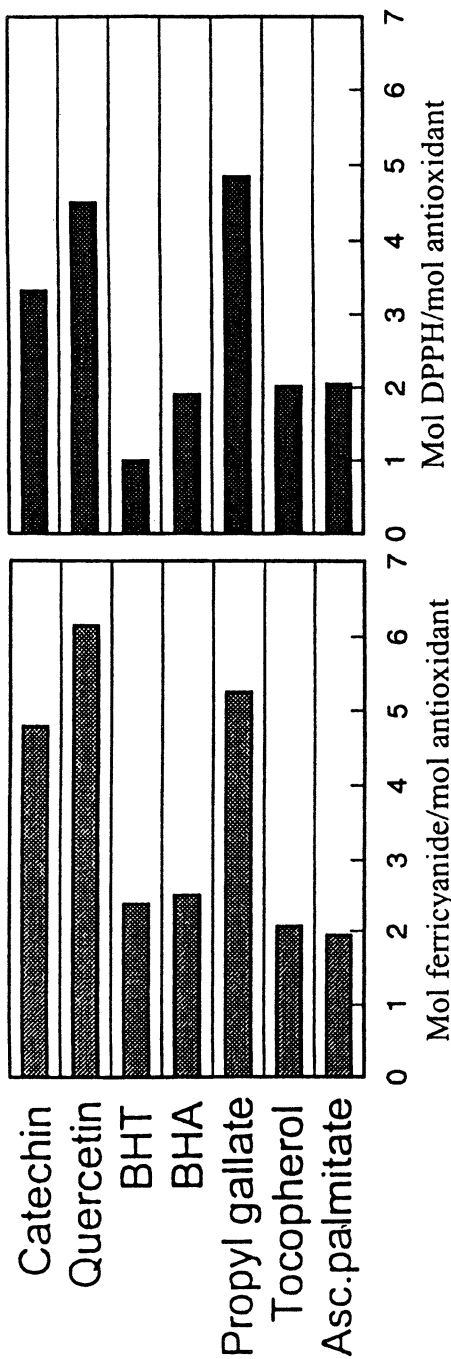


Figure 8. Reducing and radical scavenging properties of some natural and synthetic antioxidants

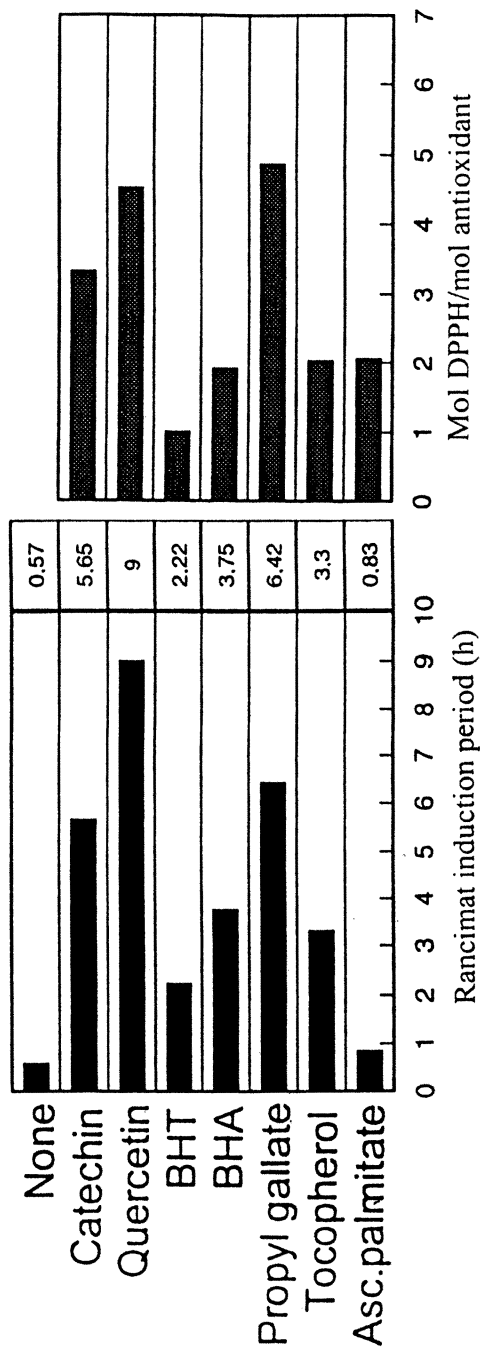


Figure 9. Antioxidative and radical scavenging properties of some natural and synthetic antioxidants

## Antioxidative Properties of Polyhydroxyphenols in Heterogeneous Systems

It is of great practical interest how polyhydroxyphenols exert their antioxidative effect in heterogeneous systems like oil/water emulsions. Therefore methylolinoleate (ML)/water model emulsions (3,4 mmol ML/l) using Tween 20 (1 g/l) as an emulsifier were prepared. Since the solubility of phenolic acids like caffeic acid or gallic acid in the oil or water phase strongly depends on the pH value, the influence of pH on the antioxidative activity of polyhydroxyphenols was investigated. Figure 10 shows the induction periods for different polyhydroxyphenols (0,1 mmol/l each) in ML/water emulsions at different pH values. The induction periods were determined at 30 °C by measuring the oxygen uptake of the emulsions with the Warburg technique. It becomes clear that the antioxidative effects of e.g. ferulic, sinapic or caffeic acid strongly increase by lowering the pH value because of their increased solubility in the fat phase at lower pH values. It is remarkable that polyphenols like gallic acid, caffeic acid or chlorogenic acid showing a strong antioxidative effect in homogeneous systems have a relatively low antioxidative potential in the heterogeneous system, because they are highly soluble in water and part of their antioxidative effect may become effective only at the contact surface between the oil phase and the water phase.

Of great influence on the rate of oxidation is the droplet size of the fatty phase in the emulsions. The droplet sizes were adjusted by addition of different amounts of the emulsifier Tween 20 in the range between 0,1 g/l and 10 g/l. The average droplet sizes dependent on the amount of emulsifier were determined by transmission measurements of the emulsions. It turned out that at an emulsifier concentration of under 1 g/l the droplet size was higher than 1  $\mu\text{m}$ , whereas at a concentration of 10 g/l the emulsion appeared transparent corresponding to a droplet size of less than 0,05  $\mu\text{m}$ . Figure 11 shows the induction periods of the above described ML emulsions at pH 5,5 and different concentrations of Tween 20 in the absence and presence of antioxidants. It can be seen that without antioxidants the induction periods decrease with increasing emulsifier concentrations, because the overall surface of the fatty phase increases with decreasing droplet sizes leading to a better access of oxygen to the substrate. On the other side, in the presence of catechin, which is more soluble in water, the induction periods increase with increasing emulsifier concentrations, because catechin mainly becomes effective at the boarder surface between the fatty phase and the water phase; therefore the antioxidative effect of catechin increases with decreasing droplet size and increasing contact surface between the two phases. Based on the same principle, the prooxidative effect of heavy metal ions becomes more pronounced at higher emulsifier concentrations. However, in the presence of tocopherol, contrary to catechin, the induction periods decrease with decreasing droplet size of the fatty phase, because the concentration of the fat soluble tocopherol in the fatty phase is independent of the droplet size. Therefore, in this case only the increase of the total surface of the fatty phase improving the access of oxygen thus decreasing the induction period remains effective.

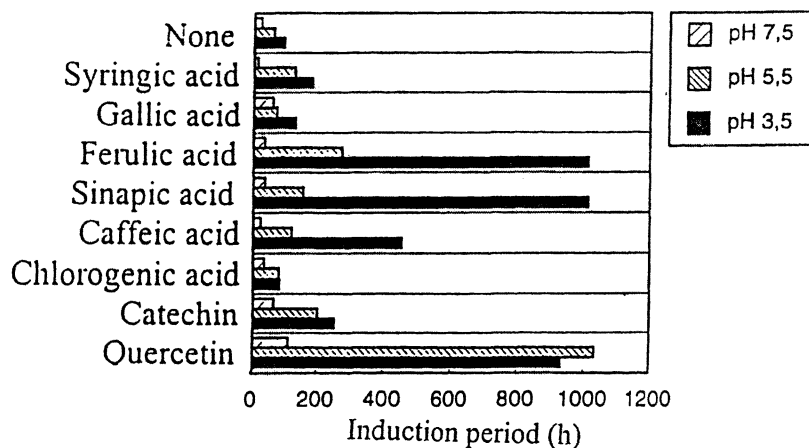


Figure 10. Antioxidative properties of some phenolic antioxidants in methylolinoleate emulsions dependent on the pH value

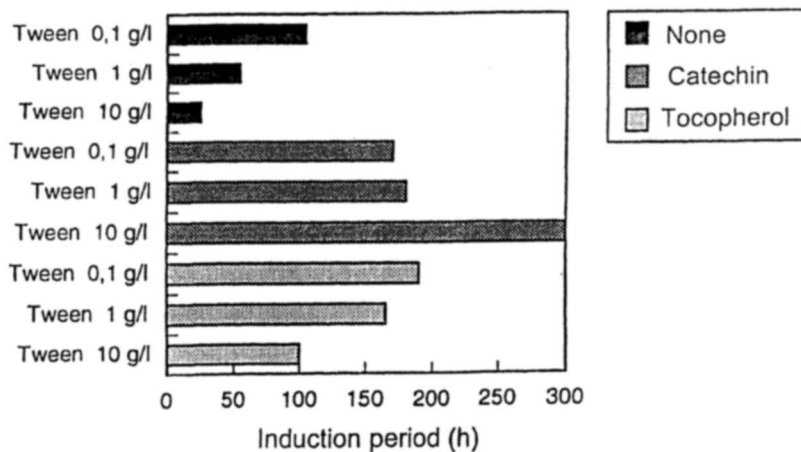


Figure 11. Antioxidative properties of a water soluble and a water insoluble antioxidant in methylolinoleate emulsions dependent on the emulsifier concentration

## Conclusions

The relationship between the chemical structure of polyhydroxyphenols and their antioxidative effect has been pointed out. There are clear correlations between the antioxidative effect of phenolic compounds and their reducing and radical scavenging properties. Therefore, the efficiency of phenolic antioxidants present in foods of plant origin can very easily and quickly be screened by reaction with the stable radical 2,2-diphenyl-1-picrylhydrazyl.

In oil/water emulsions the solubility of polyhydroxyphenols in the fat and water phase and the droplet size of the fatty phase determining the contact area between both phases play an important role with regard to their antioxidative effect.

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## Chapter 15

# Antioxidative Activities of Aroma Extracts Isolated from Various Beans

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Aroma extracts from fresh coffee beans, soybeans, mung beans, kidney beans, whole green peas, and azuki beans obtained by steam distillation under mild conditions (55°C and 95 mm Hg) were examined for their antioxidative activities. The inhibitory effect of these extracts toward hexanal/hexanoic acid conversion was measured by monitoring the amount of hexanal in the test solution over 42-45 days. The aroma extracts from all beans inhibited the oxidation of hexanal for nearly one month at a level of 250  $\mu\text{L}/\text{mL}$ . An extract from mung beans exhibited the greatest activity among the extracts from all beans examined. The antioxidative activity of these extracts was consistent with that of a known natural antioxidant,  $\alpha$ -tocopherol (vitamin E). Gas chromatography/mass spectrometry analysis of the aroma extract from soybeans and mung beans confirmed the presence of C<sub>4</sub>-C<sub>6</sub> lactones as major components. Heterocyclic compounds, such as maltol, already proven to have antioxidative activity, were identified in the extract from soybeans and mung beans. Aromatic compounds, such as eugenol and benzyl alcohol, found in the extracts also had some antioxidative activity.

Since ancient times, humans have enjoyed perfumes which consist of large numbers of aroma chemicals. Also, pharmaceutical activities of aroma chemicals have been known since the time of Hippocrates and people of all cultures have indulged in the soothing warmth of aromatic steam baths. Today, natural leaves and flowers containing numerous aroma chemicals are used in the so-called aroma therapy. In addition to their pleasant smells, aroma chemicals seem to have some beneficial health effects.

Numerous aroma chemicals have been isolated and identified in natural plants as well as in cooked foods. Until recently, aroma chemicals have been investigated from the viewpoint of flavor and fragrance chemistry. However, some medicinal qualities of aroma chemicals have lately been discovered. In particular, the antioxidative

activity of some aroma chemicals have received much attention as the chemicals preventing oxidative damage associated with many diseases, including cancer, cardiovascular disease, cataracts, atherosclerosis, diabetes, arthritis, immune deficiency diseases, aging, and brain dysfunction (1).

There have already been many reports about such naturally occurring antioxidants as vitamin C and vitamin E in fruits and vegetables, as well as phenolic compounds in tea and wine, which reportedly prevent the diseases described above. Recently, we discovered that aroma chemicals found in brewed coffee possess antioxidative characteristics which are comparable to those of vitamin C and vitamin E (2). In the present study, antioxidative activities of aroma extracts isolated from fresh coffee beans, soybeans, mung beans, kidney beans, whole green peas, and azuki beans were tested for antioxidative activity.

## Experimental

### Materials

Fresh coffee beans (*C. arabica*) were donated by Dr. Mike Kawate, Honolulu, Hawaii. Fresh soybeans (*Glycine max*), mung beans (*Vigna radiata*), kidney beans (*Phaseolus vulgaris*), whole green peas (*Pisum sativum*), and azuki beans (*Vigna angularis*) were purchased from a local market. Hexanal, hexanoic acid, undecane, carbon tetrachloride, 5-hydroxy-2-methyl furanone (5-HMF), 2,5-dimethyl-4-hydroxy-3(2H)-furanone (DMHF), and  $\alpha$ -tocopherol (vitamin E) were purchased from Aldrich Chemical Co. (Milwaukee, WI). Butylated hydroxytoluene (BHT) was bought from Sigma Chemical Co. (St. Louis, MO). Authentic chemicals were obtained from reliable commercial sources or as gifts from Takata Co., Ltd. (Osaka, Japan).

### Isolation of Aroma Chemicals by Simultaneous Steam Distillation and Solvent Extraction (SDE)

Coffee beans, soybeans, mung beans, kidney beans, green peas, or azuki beans (200 g) were placed in a 3 L round-bottom flask with 1L deionized water, and the steam distillate obtained at 55°C and 95 mm Hg was extracted with 100 mL carbon tetrachloride simultaneously for 3 h using a modified Likens-Nickerson apparatus (3). The extracts were dried over anhydrous sodium sulfate for 12 h. After sodium sulfate was filtered out, the solvent was removed using a rotary flash evaporator. The distillation was stopped when the volume of extract was reduced to approximately 2 mL. The extract was transferred into a vial and the distillation flask was washed with a minimum amount of dichloromethane and washings were added to the vial. The solvent was further removed under a purified nitrogen stream until the volume was reduced to exactly 2.5 mL.



## Isolation of Aroma Chemicals by Steam Distillation under Reduced Pressure (DRP)

Soybeans or mung beans (200g) were placed in a 3 L round-bottom flask with 1 L deionized water. The solution was steam distilled at 55 °C for 3 h under reduced pressure (95 mmHg). The distillate (900 mL) was extracted with 100 mL dichloromethane using a liquid-liquid continuous extractor for 6 h. After the extract was dried over anhydrous sodium sulfate, the solvent was removed by a rotary flash evaporator. The distillation was stopped when the volume of extract was reduced to approximately 1 mL, and then the solvent was further removed under a purified nitrogen stream until the volume was reduced to 0.2 mL.

### Antioxidative Test

Antioxidative activity of the samples obtained by SDE was tested using their inhibitory effect toward oxidation of aldehyde to acid (4). Test samples (50  $\mu\text{L/mL}$ , 100  $\mu\text{L/mL}$ , and 250  $\mu\text{L/mL}$ ) were added to a 2 mL dichloromethane solution of hexanal (3 mg/mL) containing 0.2 mg/mL of undecane as a gas chromatographic internal standard. The oxidation of the sample solution was initiated by heating at 60 °C for 10 min in a sealed vial and stored at room temperature. The headspace of each vial was purged with pure air (1.5 L/min, 3 seconds) every 24 h for first 10 days. The decrease in hexanal was monitored at 5-day time intervals.

The authentic chemicals of caffeine, 5-HMF, DMHF, BHT, and  $\alpha$ -tocopherol, as well as the major chemicals found in the extracts from soybeans and mung beans, were also examined for their antioxidative activity using the same testing method.

### Quantitative Analysis of Hexanal

The quantitative analysis of hexanal was conducted according to an internal standard method previously reported (5). A Hewlett-Packard (HP) model 5890 gas chromatograph (GC) equipped with a 30 m x 0.25 mm i.d. ( $d_f = 1 \mu\text{m}$ ) DB-1 bonded-phase fused-silica capillary column (J & W Scientific, Folsom, CA) and a flame ionization detector (FID) was used. The linear velocity of the helium carrier gas was 30 cm/sec. The injector and the detector temperatures were 300°C and 280°C, respectively. The oven temperature was programmed from 40°C to 180°C at 4°C/min and held for 10 min.

### Identification of Aroma Chemicals in the Extracts from Soybeans and Mung Beans

Aroma chemicals obtained by DRP were identified by comparison with the Kovats gas chromatographic retention index I (6) and by the MS fragmentation pattern of each component compared with those of authentic chemicals.

An HP 5890 series II gas chromatograph interfaced to an HP 5791A mass selective detector (GC/MS) was used for mass spectral identification of the GC

components at MS ionization voltage of 70 eV. A 30 m x 0.25 mm i.d. ( $d_f = 1 \mu\text{m}$ ) DB-WAX bonded-phase fused-silica capillary column (J & W Scientific, Folsom, CA) was used for the GC analysis. The linear velocity of the helium carrier gas was 30 cm/sec. The injector and the detector temperatures were 250 °C. The oven temperature was programmed from 50 °C to 180 °C at 3 °C/min and held for 40 min.

## Results and Discussion

### Testing Methods for Antioxidative Activity

The most commonly used method for testing the antioxidative activity of a chemical or a group of chemicals has been the thiobarbituric acid assay (TBA). The TBA method involves measurement of malonaldehyde formed from a lipid upon oxidation. However, this method is not specific for MA and often overestimates MA levels because some chemicals such as aldehydic compounds react with TBA to produce absorbance spectra similar to that of the TBA-MA complex (7). Recently, a capillary GC method for specific determination of MA has been developed in our laboratory. This method involves that MA formed from lipids upon oxidation is derivatized to 1-methylpyrazole which is subsequently determined by a gas chromatograph with a nitrogen-phosphorus detector [8, 9, 10, 11).

The aldehyde/carboxylic acid assay developed in our laboratory (4) is a simple method to measure the antioxidative activity of chemicals or a group of chemicals. This method is based on the autooxidation of aldehydes to carboxylic acids with active oxygen species such as a hydroxy radical (12). Fatty aldehydes are readily converted to the corresponding fatty acid in the oxygen-rich dichloromethane solution through a radical-type reaction (13). This method has been validated using known antioxidants. Figure 1 shows the results of the antioxidative activity test on  $\alpha$ -tocopherol (vitamin E), BHT, DMHF, 5-HMF, maltol and caffeine.  $\alpha$ -Tocopherol and maltol inhibited hexanoic acid formation by 100% at the level of 250  $\mu\text{g/mL}$ . BHT inhibited hexanoic acid formation by 100% at the level of 50  $\mu\text{g/mL}$ . The results indicate that the aldehyde/carboxylic acid system used in the present system is a useful method for antioxidative test of chemicals which are soluble in dichloromethane.

The methods involved in lipid/MA are useful for quick tests of samples but they may not well represent actual systems of *in vivo* and *in vitro* oxidative processes. Although the aldehyde/carboxylic acid assay used in the present study requires prolonged time periods, it is probably closer to the actual oxidation process that occurs in foods and beverages.

### Results of Antioxidative Tests on Aroma Extracts from Various Beans

Figure 2 shows the antioxidative activities of different amounts of extracts from coffee beans, soybeans, mung beans, kidney beans, green peas, and azuki beans throughout a storage period of 40 days. All extracts exhibited a dose-dependent activity. Hexanal in a control sample was completely oxidized to hexanoic acid after

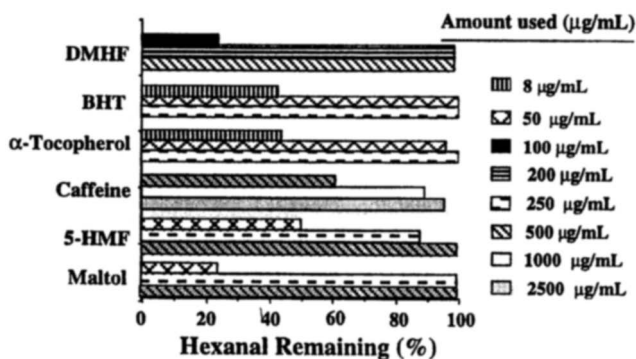


Figure 1. Antioxidative activity of chemicals over 40 days tested by aldehyde/carboxylic acid method.

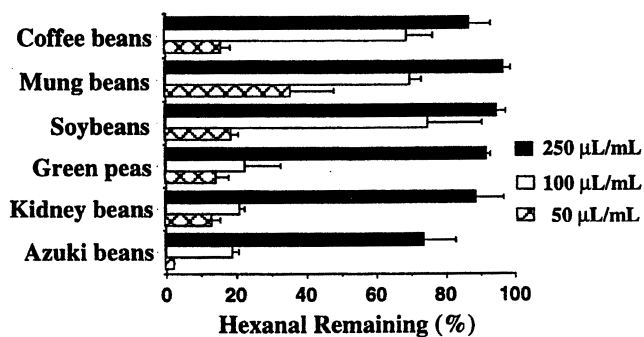


Figure 2. Antioxidative activity of aroma extracts from various beans over 40 days.

30 days. At a 250  $\mu\text{L}/\text{mL}$  level, extracts from mung beans and soybeans inhibited the aldehyde/carboxylic acid conversion for more than 45 days. The extract from fresh coffee beans inhibited hexanal oxidation by nearly 90% over a 43 day period. This sample contained caffeine which inhibited hexanal oxidation by 60% over a 40 day period at a level of 500  $\mu\text{g}/\text{mL}$  (Figure 1). However, the antioxidative activity of a coffee extract is not only due to caffeine but also to other aroma chemicals (2). The extracts from kidney beans and azuki beans inhibited hexanal oxidation by 90% and 80% at the same level, respectively. At a 50  $\mu\text{L}/\text{mL}$  level, extracts from mung beans and soybeans inhibited oxidation for 20 days and 15 days, respectively. Overall results indicate that extracts from mung beans and soybeans exhibited the greatest antioxidative activity among the beans tested.

### **Aroma Chemicals Identified in Extracts from Mung Beans and Soybeans**

Table I shows aroma chemicals identified in extracts from mung beans and soybeans. Over 300 GC peaks were observed in chromatograms of both soybean and mung bean extracts. Among those, 57 aroma chemicals were identified in the soybean extract and 37 aroma chemicals were identified in the mung bean extract. Aroma chemicals identified in the soybean extract are 14 alcohols, 12 aldehydes and ketones, 6 aromatic compounds, 7 lactones, and 10 miscellaneous compounds. Aroma chemicals identified in the mung bean extract are 11 alcohols, 5 aldehydes and ketones, 4 lactones, 6 aromatic compounds, and 9 miscellaneous compounds.

### **Results of Antioxidative Tests on Aroma Chemicals Found in Extracts from Soybeans and Mung beans**

The chemicals marked with an asterisk (\*) in Table I were tested for antioxidative activity. Figure 3 shows the antioxidative activity of the major aroma chemicals found in soybeans and mung beans. BHT,  $\alpha$ -tocopherol, and DMHF were not found in the extracts, but used as known standard antioxidants. All chemicals exhibited a dose-dependent activity. Among the chemicals identified in the extracts from soybeans and mung beans, eugenol exhibited the most potent activity; it inhibited hexanal oxidation by 100% at a 200  $\mu\text{g}/\text{mL}$  over 20 days. The antioxidative activity of maltol in the present study was consistent with a previous report (2). DMHF inhibited hexanal oxidation by 100% at levels higher than 200  $\mu\text{g}/\text{mL}$  over 20 days. DMHF, which has been found in many natural plants (14) and cooked foods (15), was proven to have strong antioxidative activity by the method with blood plasma (16). The results indicate that the aldehyde/carboxylic acid assay used in the present study is effective to measure the antioxidative activity of chemicals. Among aroma chemicals, heterocyclic compounds—such as pyrroles and thiazoles—are known to possess an antioxidative activity (17, 18). On the other hand, 1-octen-3-ol exhibited a slight effect at the 500  $\mu\text{g}/\text{mL}$  level, suggesting that compounds possessing alkyl double bond(s) have some activity.

Lactones, which have been known as a chemical that possesses butter-like flavor (19), were one of the major chemical groups found in both extracts. However, lactones did not exhibit any antioxidative activities in the testing system used in the

**Table I. Aroma Chemicals Identified in the Extracts from Soybeans and Mung beans**

Compound	GC Peak Area % <sup>a</sup>	
	Soybeans	Mung beans
<b>Alcohols</b>		
isobutanol	0.167	0.451
butanol	0.190	0.775
1-penten-3-ol	0.348	-
2-methyl-1-buten-3-ol	0.249	-
2-methylbutanol	1.344	1.483
3-methylbutanol	1.868	2.122
pentanol	2.281	2.163
(Z)-2-pentenol	0.374	-
hexanol	10.064	8.309
(Z)-3-hexenol	0.304	-
3-octanol	0.260	-
1-octen-3-ol*	7.634	-
heptanol	0.174	-
2-ethylhexanol	1.706	2.828
acetoin	0.468	0.272
2-hydroxy-2-methyl-4-pentanone	-	0.349
2-hydroxy-3-methyl-2-cyclopenten-1-one	0.182	-
2-hydroxy-2,6,6-trimethylcyclohexanone	-	0.411
2-phenyl-2-propanol	0.911	0.960
benzyl alcohol*	6.072	25.976
phenylethyl alcohol*	2.183	12.969
styrallyl alcohol	-	0.560
β-phenoxyethanol	0.142	0.933
phenol	0.573	1.550
eugenol*	-	1.593
methyleugenol	-	0.708
<b>Aldehydes and Ketones</b>		
acetone	0.028	0.051
hexanal	0.411	-
hydroxyacetone	0.258	-
(E)-2-heptenal	0.192	-
3-hydroxypentan-2-one	0.152	-
(E,E)-2,4-heptadienal	trace	-
benzaldehyde	0.942	-
(Z,E)-3,5-octadien-2-one	0.828	-

*Continued on next page.*

Table I. *Continued*

Compound	GC Peak Area % <sup>a</sup>	
	Soybeans	Mung beans
(E,E)-3,5-octadien-2-one	0.326	-
2-hydroxyoctane-3-one	0.175	-
acetophenone	0.230	-
<b>Lactones</b>		
$\alpha$ -methyl- $\gamma$ -butyrolactone	2.515	-
$\beta$ -methyl- $\gamma$ -valerolactone	0.317	-
$\delta$ -valerolactone*	2.602	0.924
$\beta$ -methyl- $\gamma$ -butyrolactone	7.122	-
$\gamma$ -butyrolactone*	17.580	3.677
$\gamma$ -hexalactone	1.813	0.308
$\gamma$ -nonalactone	0.426	0.295
<b>Heterocyclic Compounds</b>		
2-methylpyrazine	0.339	-
2,5-dimethylpyrazine	0.125	-
2,6-dimethylpyrazine	0.123	0.576
1-methyl-2-pyrrolidone*	4.076	-
3-methyl-2(5H)-furanone	0.219	-
5-ethyl-2(5H)-furanone	0.314	-
3-furfuryl alcohol	-	0.771
maltol*	4.968	2.741
2-acetylpyrrole	trace	-
5,6-dihydro-4-methyl-2H-pyran-2-one	0.252	-
octanoic acid	0.082	-
nonanoic acid	0.310	-
3-ethyl-4-methyl-1H-pyrrole-2,5-dione	0.540	0.353
<b>Miscellaneous Compounds</b>		
octadecyl acrylate	0.664	-
hexanoic acid	0.507	-
nonanoic acid	-	0.783
dodecanoic acid	-	trace
dimethyl sulfone	0.194	-
N-methylsuccinimide	0.337	-
ethylene glycol monbutyl ether	-	2.303

Table I. Continued

Compound	GC Peak Area % <sup>a</sup>	
	Soybeans	Mung beans
2-(methylthio)ethanol	-	1.534
isophorone	-	1.296
N,N-dimethylacetamide	0.262	0.498
dipropylene glycol monomethyl ether 1st	0.170	1.367
dipropylene glycol monomethyl ether 2nd	0.525	0.386
2-amino-benzonitrile	-	0.209

<sup>a</sup>Solvent peak was excluded

\*Tested for antioxiditive activity

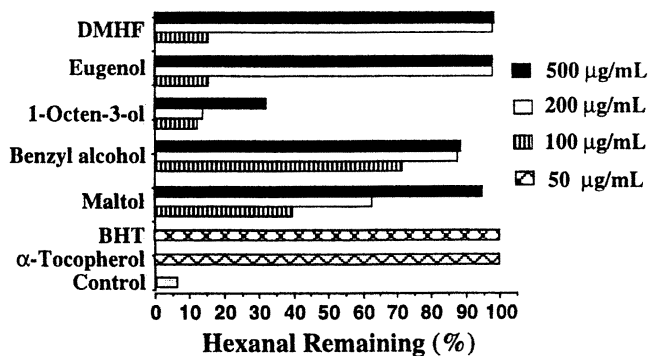


Figure 3. Antioxidative activity of aroma chemicals found in extracts from soybeans and mung beans over 20 days.

present study. Except as shown in Figure 3, the other aroma chemicals tested did not show any appreciable activity.

## Conclusions

As mentioned the introduction, the physiological activities of aroma chemicals have been known since ancient times and aroma therapy, using natural plant extracts, has long been a popular health treatment. The antioxidative activities of the aroma chemicals studied here, as well as those of heterocyclic compounds previously reported, are not as strong as those of vitamin C or  $\alpha$ -tocopherol. However, the aroma chemicals are present in natural plants or cooked foods in tremendous numbers. For example, nearly 1,000 aroma chemicals have been found in brewed coffee extracts which reportedly possess antioxidative activity. Therefore, the total antioxidative activity of these aroma chemicals may be comparable to that of vitamin C or  $\alpha$ -tocopherol. Since humans are exposed to reactive oxygen species 24 hours a day, it is extremely important that antioxidant supplements be taken constantly and consistently. Exposure to these aroma chemicals may help to prevent oxidative damages, which are a factor in many diseases.

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## Chapter 16

# Radical Scavenging Mechanisms of Catechins on 2,2-Diphenyl-1-picrylhydrazyl Radical

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Catechins, which are the main constituents of tea, have been shown to have strong scavenging activity against many kinds of radicals. In this study, the radical scavenging mechanisms of catechol type catechin on 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging were evaluated by identifying the structure of the reaction products. 4-Methylcatechol (4-MC) which is a B-ring model of the catechol type catechin was reacted with DPPH radicals in EtOH. 4-Methyl-5-ethoxy-benzoquinone was isolated as a reaction product, and 4-methyl-*ortho*-benzoquinone was identified as an intermediate of this reaction in EtOH. Next, (+)-Catechin (+C) was reacted with DPPH in EtOH under dark conditions. The B-ring of +C showed the same type of reaction as 4-MC. While under irradiation of fluorescent or day light, new C-C linkage between 6' and 4a-carbon of +C was formed. The conversion of 4-MC and B-ring of +C to *ortho*-quinone form consumed 2 molecules of DPPH radical which agreed with the stoichiometric factors of 4-MC and +C.

Catechins (shown in Figure 1.) , are the main constituents of tea and protect from many diseases. One of their main protective roles is the antioxidative effect against many kind of radicals (1, 2). We have previously reported the radical scavenging activity of catechins (3). In this report, the numbers of radical scavenging of

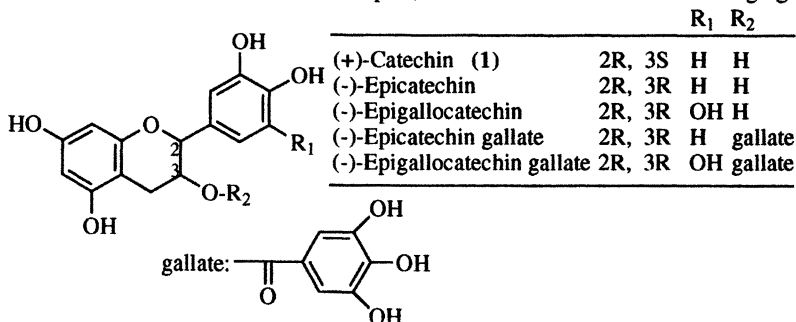


Figure 1. Structure fomulae of tea catechins.

catechins and related compounds at the initial stage of the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging reaction were measured as stoichiometric factors, for the purpose of comparing the structure and radical scavenging activity. These stoichiometric factors were found to depend on the structure of their B-ring and existence or lack of the galloyl group. The stoichiometric factor 2 was shown by the catechol moiety, and 5 was shown by the pyrogallol moiety. However, after the rapid reaction of catechins and related compounds with DPPH radicals in the early stage, a slow reaction was observed to proceed.

In this report, we examined the mechanisms of radical scavenging reaction of 4-methylcatechol (4-MC) and (+)-catechin (+C) on the DPPH radicals for the purpose of elucidating the complex radical scavenging reaction of catechins.

## Materials and Methods

### Reagents

2,2-diphenyl-1-picrylhydrazyl (DPPH), 4-methylcatechol (4-MC), ascorbic acid, acetonitrile, ethyl acetate, trifluoroacetic acid and phosphoric acid were purchased from Wako pure chemical (Tokyo, Japan). Deuterated solvents were purchased from Merck. (+)-Catechin (+C) was obtained from the reagent group of our laboratory. Water was purified through a Milli-Q system (Millipore).

### Spectral Measurement

$^1\text{H}$ - ,  $^{13}\text{C}$ - and 2D NMR spectra were measured with JNM-Lambda 500 NMR spectrometer (JEOL, Tokyo). Chemical shifts are expressed as delta values using TMS as an internal standard.

FAB mass spectra were measured with JMS DX-303 mass spectrometer (JEOL, Tokyo). Glycerol was used as matrix and xenon gas was used as reactant gas.

### HPLC Condition for Analysis

HPLC was carried out with Waters 996-16 photodiode array HPLC system and Millennium chromatography manager (Waters, Millford IL). The conditions of HPLC were as follows: Mobile phase A:  $\text{CH}_3\text{CN}:\text{EtOH}:\text{0.05\% phosphoric acid in distilled water} = 12:0.6:90$ , B : 100%  $\text{CH}_3\text{CN}$ , gradient conditions: start A 100% (hold 5 min), 5- 15 min from 0% B to 90% B, Scan from 220 nm to 500 nm, and the changes of the peak area were detected at 280 nm. The column was heated to 40°C .

### Preparation and Identification of 3

56 mg (0.2 mmol) of 4-MC was reacted with 256 mg (0.6 mmol) of DPPH in EtOH at room temperature for 3 days. Ten volumes of distilled water was added to the reacted solution. The diluted solution was centrifuged at 3000 rpm for 15 min. The supernatant was evaporated *in vacuo* at 40°C. The concentrate was purified by

HPLC with Capcell-Pak C18 20 mm ID x 250 mm (Shiseido, Tokyo Japan) and 12% CH<sub>3</sub>CN in 0.01% trifluoroacetic acid (TFA) solution. The purified Peak II (compound 3) was lyophilized. 1 μL of 3 in methanol (2 mg/ml) was mixed with glycerol as a matrix, and FAB mass spectra of 3 was measured. 3 was dissolved in CD<sub>3</sub>CN for measuring NMR spectra.

#### Preparation and Identification of 5

29 mg (0.1 mmol) of +C was reacted with 118.3 mg (0.3 mol) of DPPH in 1 L EtOH at room temperature for 4 days under dark conditions. Excess amount of ascorbic acid was added to reacted solution. The mixture was concentrated. EtOH was removed and 40 mL of distilled water was added to the mixture. The suspension was centrifuged at 3000 rpm for 15 min. The supernatant was filtered by paper filter, then filtered by 0.45 μm membrane filter. The filtrate was evaporated *in vacuo* at 40°C. The concentrate was purified by HPLC with Capcell-Pak C18 20 mm ID x 250 mm (Shiseido, Tokyo Japan) and 12% CH<sub>3</sub>CN in 0.01% trifluoroacetic acid (TFA) solution. The purified reduced Peak IV (compound 5) was lyophilized. 1 μL of 5 in methanol (2 mg/ml) was mixed with glycerol as a matrix and the FAB mass spectra of 5 was measured. 5 was dissolved in CD<sub>3</sub>CN for measuring NMR spectra.

#### Preparation and Identification of 8

90 mg (0.3 mmol) of +C was reacted with 360 mg (0.9 mmol) of DPPH in 1.8 L EtOH at room temperature for 4 days under light irradiation. Ten volumes of distilled water was added to the reacted solution. The diluted solution was centrifuged at 3000 rpm for 10 min. The supernatant was evaporated *in vacuo* at 40°C. The concentrate was purified by HPLC with Capcell-Pak C18 20 mm ID x 250 mm (Shiseido, Tokyo Japan) and 12% CH<sub>3</sub>CN in 0.01% Trifluoroacetic acid (TFA) solution. The purified Peak V (compound 8) was lyophilized. 1 μL of 8 in methanol (2 mg/ml) was mixed with glycerol as matrix for measuring of FAB mass spectra of 8. 5 mg of 8 was dissolved in CD<sub>3</sub>CN and Methanol-*d*<sub>4</sub> for measuring NMR spectra.

#### NMR Experiment of the Reaction Mixtures

10 mg of 4-MC was reacted with 60 mg of DPPH in CD<sub>3</sub>CN at room temperature. 10 mg of +C was reacted with 30 mg of DPPH in CD<sub>3</sub>CN at room temperature. Each reacted solution was measured by <sup>1</sup>H-, <sup>13</sup>C- and heteronuclear multiple bond connectivity (HMBC) spectra.

#### Time Course Experiments of the Changes of the Compounds in the Reaction Mixture.

2 mL of 1 mM 4-MC solution was reacted with 20 mL of 0.34 mM DPPH solution, and 2 mL of 1 mM +C solution was reacted with 20 mL of 0.34 mM DPPH solution under dark conditions. Each solution was analyzed by HPLC with auto sampler and PDA under the scheduled program.

## Results and Discussion

### DPPH radical Scavenging Reaction of 4-Methylcatechol (4-MC)

4-MC was examined as a B ring model system of the catechins which have catechol type B-ring. 4-MC was reacted with same mole of and DPPH radicals in EtOH. After complete extinction of DPPH radicals, the reacted mixture was analyzed by HPLC equipped with photodiodearray detector. In the chromatogram of the reacted solution, in addition to the peak of unreacted 4-MC, two new peaks appeared (shown in Figure 2). Peak I had lambda maximum at 403 nm, while on the other hand peak II had lambda maximum at 427 nm in the UV spectra. We attempted to isolate these two peaks by preparative HPLC with ODS column. However, peak I could not be isolated because of its instability.

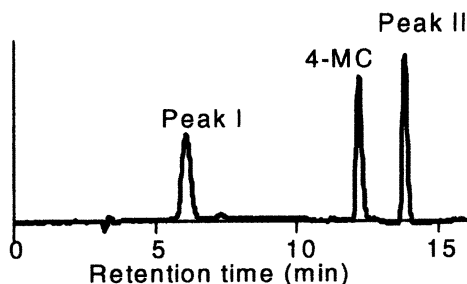


Figure 2. HPLC chromatogram of reaction mixture of 4-MC and DPPH radical in EtOH

The structure of purified peak II was elucidated by measuring FAB-MS and NMR spectrum. The structure of peak II was identified as 4-methyl-5-ethoxy-*ortho*-benzoquinone (3). It was

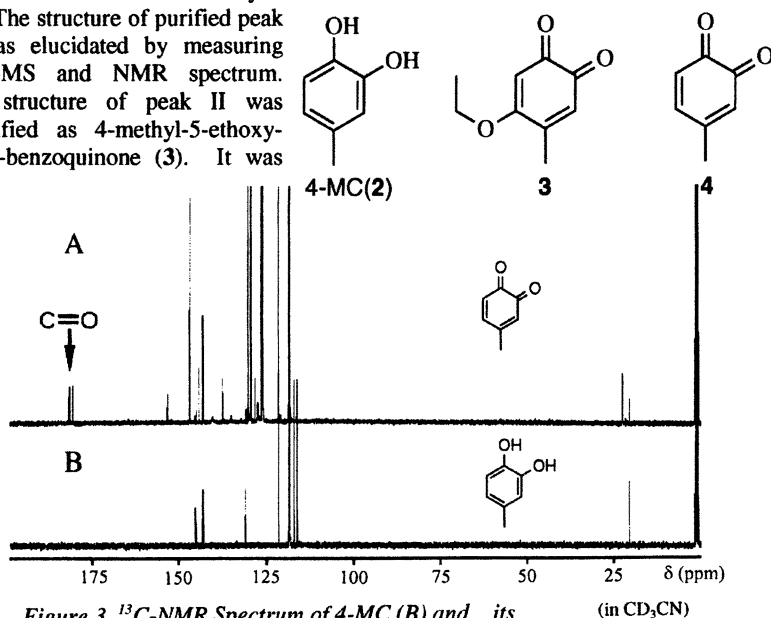


Figure 3.  $^{13}\text{C}$ -NMR Spectrum of 4-MC (B) and its reaction Peak I in reaction solution with DPPH (A)

speculated that the compound **3** was produced by nucleophilic addition of EtOH to oxidized form of 4-MC. The formation of compound **3** may be related to the donation of a proton from the EtOH. To eliminate the effect of the proton donation from the solvent, 4-MC was reacted with DPPH radicals in CH<sub>3</sub>CN which is aprotic polar solvent. In the HPLC chromatogram of the reacted solution, in addition to the peak of 4-MC, another peak which had same retention time and UV spectra as Peak I appeared. By the addition of excess amounts of ascorbic acid to the reaction mixture in CH<sub>3</sub>CN, Peak I was decreased and only the peak of 4-MC appeared. By the addition of EtOH to the reaction mixture in CH<sub>3</sub>CN, the peak which had same retention time and UV spectra as Peak II (compound **3**) appeared as a single peak in the chromatogram of the reacted solution. From this result, we conclude that Peak I is the first product of the DPPH radical scavenging reaction of 4-MC and compound **3** is created from Peak I with EtOH.

For the purpose of structural elucidation of Peak I, 4-MC was reacted with DPPH radicals in CD<sub>3</sub>CN. The reaction mixture was measured by <sup>13</sup>C-NMR by using the modified method reported by Sawai *et al.* (4). In the NMR spectrum of the mixture, two signals of the carbonyl carbons were observed at 180.4 and 181.3 ppm (shown in Figure 3.). By the measurement of the HMBC spectra of the reacted solution, 4-methyl-*ortho*-benzoquinone (**4**) were determined to be present in the solution.

Next, 4-MC was reacted with 2X excess mole of DPPH radicals in EtOH for the time course experiment of the radical scavenging reaction. The reacted solutions were analyzed by HPLC with PDA. Formation of the reaction intermediate (**4**) began to occur along with the rapid decrease of 4-MC immediately after initiating the reaction. Subsequent reaction in which the formation of compound **3** and the regeneration of 4-MC took place was observed along with the decrease of **4** (shown in Figure 4). On the basis of the result, the radical scavenging mechanism of 4-MC on the DPPH radicals in EtOH was proposed as shown in Figure 5.

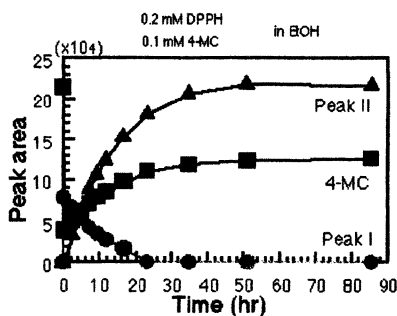


Figure 4. Changes of peak area of 4-MC, Peak I and Peak II in reaction mixture.

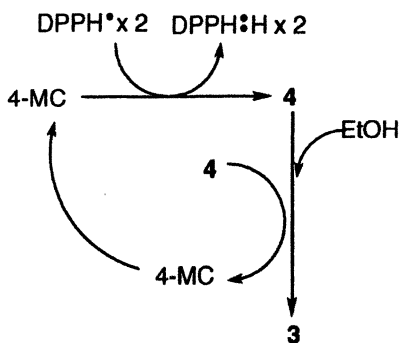


Figure 5. Reaction mechanism of 4-Methylcatechol with DPPH radicals

#### DPPH radical Scavenging Reaction of (+)-Catechin (+C)

The stoichiometric factor of (+)-catechin (+C) was two, which was the same as 4-MC. The fact that +C and 4-MC have the same stoichiometric factor, leads us to the

assumption that the B-ring of +C should react with DPPH radicals via the same mechanism as 4-MC.

+C was reacted with the equal mole of DPPH radicals in EtOH under dark conditions. The reaction mixture was measured by HPLC with PDA. The chromatogram of the reaction mixture is shown as Figure 6A. In this chromatogram two new peaks appeared. Peak III has the lambda maximum at 384 nm and Peak IV at 427 nm in its UV spectra. Isolation of Peak III and IV was attempted but these two peaks could not be isolated due to their instability. The reacted fraction which contained Peak IV was reduced by an excess amount of ascorbic acid. The reduced peak IV was purified by preparative HPLC with ODS column. The  $^1\text{H}$ -,  $^{13}\text{C}$ -, 2D-NMR spectra and FAB mass spectra of this isolated compound were measured. On the

basis of the results, reduced Peak IV was determined as 6'-ethoxy-(+)-catechin (5). From observation of the structure of compound 3 formed from 4-MC reacted with DPPH radicals in EtOH, and the structure of compound 5, we determined the structure of Peak IV as structure 6.

Further, +C was reacted with DPPH radical in  $\text{CD}_3\text{CN}$ . Then, the  $^{13}\text{C}$ -NMR and HMBC spectrum of the reacted solution were measured. On the basis of the NMR experiment, the production of compound 7, which was the *ortho*-benzoquinone form of +C, was confirmed in the reaction solution. The Peak III had the lambda maximum at 384 nm and was reduced to +C by ascorbic acid. From these results, the Peak III was identified as compound 7. Next, the time course experiment of the DPPH radical scavenging reaction of +C was carried out. +C was reacted with 3X excess mole of DPPH radicals in EtOH. The reaction mixture was measured by HPLC with PDA. The time course changes of the peak areas of +C, Peak III (7) and Peak IV (6) are shown in Figure 7. The pattern changes were similar to that of 4-MC. Peak III (7) was produced immediately with a decrease of +C in the earlier stage of the reaction, then it was decreased slowly with the passage of time. On the other hand, +C and Peak IV increased with the decrease of Peak III. From calculations of the changes of the peak area, the regeneration of one molecule of +C and the production of one molecule of Peak IV (6) were occurred with decrease of two molecules of Peak III (7).

On the other hand, when +C was reacted with DPPH radicals under light irradiation, the chromatogram of the reacted solution was different from that under

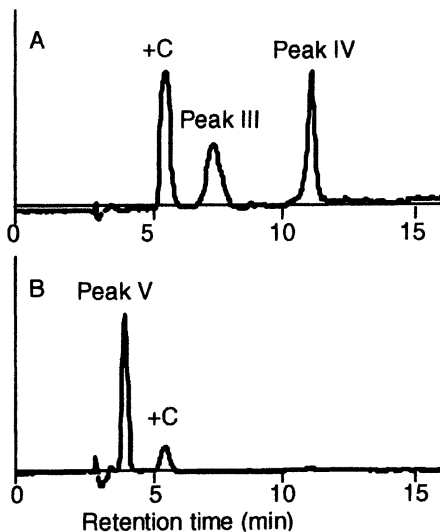


Figure 6. HPLC chromatogram of reaction mixture of +C and DPPH radical in EtOH. A : under dark conditions  
B : under light irradiation

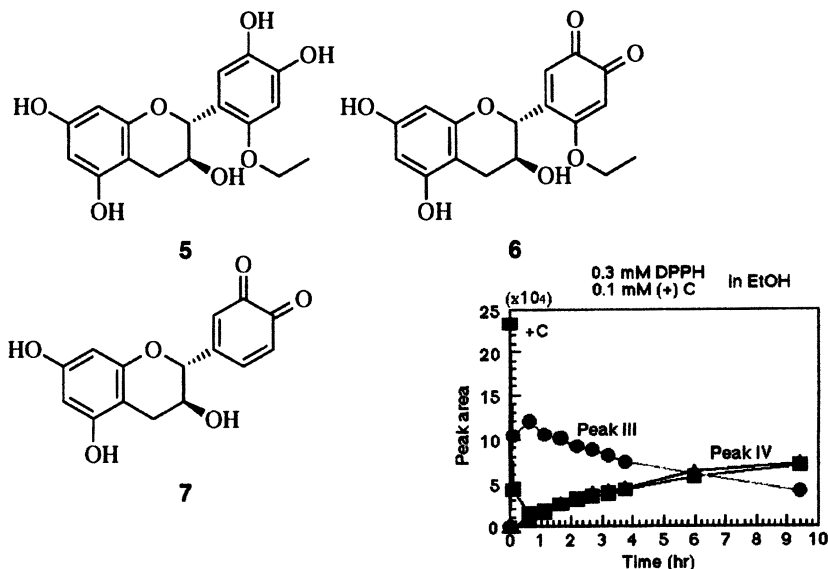
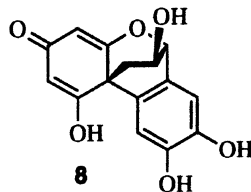


Figure 7. Changes of peak area of +C, Peak III and Peak IV in reaction mixture.

dark conditions. The chromatogram of the reaction mixture under light irradiation is shown in Figure 6B. Peak V which had a different retention time and UV spectrum from those of Peak III (7) and IV (6) was detected in the reacted solution. The Peak V was elucidated by HPLC with ODS column and the structure of the isolated Peak V was elucidated by measuring of FAB-MS and NMR spectrum. The structure of Peak V was determined as compound 8 which had the new C-C bond between 6' and 4a carbons of +C. The compound 8 has been reported by Hirose *et al.* (5), as the main product from the reaction of +C with 2, 2'-azobis [2-methyl-propanenitrile] (AIBN) radicals in EtOAc-MeOH mixed solvent under light irradiation. In present study Peak V (8) was not produced from +C reacted with DPPH radicals in CH<sub>3</sub>CN, but when EtOH was added to the reacted mixture in CH<sub>3</sub>CN, the Peak V (8) was formed. The formation of Peak V(8) requires light irradiation and protonic solvent.

On the basis of these results, the pathway of the DPPH radical scavenging of +C in EtOH is shown in Figure 8. One molecule of +C reduced two molecules of DPPH radicals and changed into compound 7 which had *ortho*-benzoquinone type B-ring. The reaction of two molecules of compound 7 with EtOH leads to the formation of one molecule of compound 6 and the regeneration of one molecule of +C. In this reaction, two molecules of hydrogen were donated to one molecule of compound 7 from one molecule each of EtOH and compound 7. On the other hand, compound 8 was formed through 7 with the formation of a new C-C bond under light irradiation. Compound 8 may have the ability to reduce DPPH radicals.





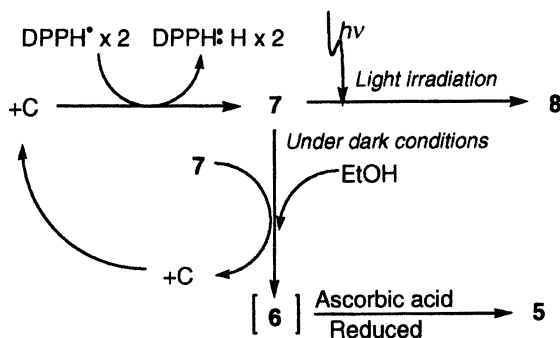


Figure 8. Reaction mechanism of (+)-Catechin with DPPH radicals.

$+C$  and 4-MC were found to have initially a rapid scavenging activity followed by a slower scavenging ability. On the basis of the results obtained in this study the rapid scavenging activity was thought to be maintained until the catechol moiety in  $+C$  and 4-MC was converted to *ortho*-benzoquinone form. This reaction consumed two molecules of DPPH radicals which agreed with the stoichiometric factors of  $+C$  and 4-MC. Although the DPPH radical may be scavenged rapidly by 4-MC and  $+C$  which were regenerated in the process of formation of compound **3** and compound **5** from compounds compound **4** and compound **6** respectively, the apparent slow scavenging activity observed may be due to the rate-limiting reactions of compounds compound **4** and compound **6** with EtOH.

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## Chapter 17

# Hypolipidemic Activity of Green Tea Epicatechins

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Previous epidemiological studies and animal experiments in rodents have demonstrated that drinking green tea is associated with decreased levels of serum total cholesterol (TC) and triacylglycerols (TG). However, the mechanisms involved remain poorly understood. We have studied the hypolipidemic activity of jasmine green tea epicatechins (GTE) and tested the various mechanisms which might be responsible for this lipid-lowering effect using hamsters as an animal model. The results showed that GTE either added in diet or in drinking water lowered significantly the levels of TC and TG in both serum and liver. GTE had no effect on the activity of liver fatty acid synthase, 3-hydroxy-3-methyl glutaryl coenzyme A (HMG-CoA) reductase and intestinal acyl CoA:cholesterol acyltransferase (ACAT). However, GTE increased significantly the fecal excretion of total fatty acids, neutral and acidic sterols. It is most likely that the hypolipidemic activity of GTE is mediated by its inhibition of absorption of cholesterol and TG.

High plasma cholesterol is one of the greatest risk factors in contribution to the prevalence and severity of coronary heart disease (1-2). Consumption of green tea has been shown to be associated with decreased serum total cholesterol (TC) and triacylglycerols (TG) in both humans and rodents (3-4). The effect of drinking green tea on plasma lipoproteins appears to be characterized by decreasing low-density lipoprotein (LDL) cholesterol while increasing high-density lipoprotein (HDL) cholesterol (5-6). However, biochemical mechanisms remain unknown.

We have tested the hypothesis that the green tea epicatechin (GTE) derivatives are the major active compounds attributable for the hypolipidemic activity of green tea by using hamsters as an animal model. The hypolipidemic activity of green tea water extract (GTWE) was therefore compared with that of

isolated GTE. We also sought to ascertain whether supplementation of GTE would lead to any changes in liver fatty acid synthase (FAS), 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, a key enzyme in cholesterol synthesis, and intestinal acyl CoA: cholesterol acyltransferase (ACAT), which is believed to play important role in intestinal cholesteryl esterification before cholesterol is absorbed and assembled in the chylomicrons. Part of this study has been published elsewhere (7).

## Hypolipidemic Activity of Jasmine GTWE and GTE

### *Preparation of GTWE and GTE*

The GTWE was prepared by adding 15 g jasmine tea (*Camellia sinensis*) leaves into 1 L of freshly boiled water (80°C). After 15 minutes, the infusion was filtered and then saved as 15 g GTWE/L at 4°C until it was used next day. Jasmine GTE was isolated and individual derivatives were analyzed by high performance liquid chromatography (HPLC) as previously described (8). Jasmine tea contained 75 g GTE/kg. The purity of GTE isolated was 95% in which (-)-epigallocatechin gallate, (-)-epigallocatechin, (-)-epicatechin gallate and (-)-epicatechin accounted for 62%, 19%, 8% and 5%, respectively. GTE solution was prepared by dissolving 5 g GTE in 1 L distilled water.

### *Animals and Diet*

The hypercholesterolemic diet described by Sanders & Sandaradura (9) was modified. The diet was high in fat and cholesterol and was prepared by mixing the following ingredients: casein, 200 g; lard, 200 g; corn starch, 418 g; sucrose, 100 g; AIN-76 mineral mix, 40 g; AIN-76A vitamin mix, 20 g; DL-methionine, 1 g; and cholesterol, 1g. All hamsters were fed this hyperlipidemic diet. One group of hamsters received the distilled water while the other two groups received either 5.0 g GTE/L solution or 15 g GTWE/L solution as the only source of drinking water. For all three groups, sucrose was added to the fluid at the concentration of 15 g/L to overcome the bitterness of green tea solutions. At the end of 4 wk, all fluids were withdrawn and the distilled water was given instead. After the food was withheld for 14 h, the hamsters were sacrificed and the blood was collected via the abdominal aorta. After clotting, the blood was centrifuged at 1300 g for 10 min, and serum was then collected.

### *Results*

No significant differences in body weight gain and food intake were observed among the control, GTE and GTWE groups. The GTE and GTWE solutions had similar concentrations of total tea epicatechin derivatives. Compared with the control, serum TG, TC and Apo B were significantly lower in both GTE and GTWE groups ( $p < 0.05$ , Table I). In contrast, the concentration of serum HDL-C and Apo A-1 did not differ among the three groups. The results demonstrated that jasmine GTWE solution and the isolated GTE possessed similar hypolipidemic

activities (Table I), indicating that GTE are the major active components or at least partially contribute to the hypolipidemic activity of green tea.

### Dose-Dependent Hypolipidemic Activity of GTE

#### *Animals and Diet*

The GTE supplemented diet was prepared by adding jasmine GTE extract to the above hyperlipidemic diet (powder form), which was then mixed with 1 L gelatin solution (200 g/L). Once the gelatin had set, the food was cut into approximately 20 g cubed portions and stored frozen at -20°C. Male hamsters were randomly divided into four groups (n=9). All animals received the tap water as the only drinking fluid. One group of hamsters was fed the control diet while the other three groups were given one of the three GTE-supplemented diets (1.1, 3.4 and 5.7 g GTE/kg). At the end of 4 wk, all of the hamsters were sacrificed after food-deprivation for 14 h. The blood was collected via the abdominal aorta and the serum was obtained as above.

**Table I. Effects of Green Tea Water Extract (GTWE) and Green Tea Epicatechins (GTE) supplemented in Drinking Distilled Water for Four Weeks on Serum Lipid and Apolipoprotein Profiles in Hamsters<sup>1,2</sup>**

<i>Analyte</i>	<i>Control</i>	<i>5.0g GTE/L</i>	<i>15g GTWE/L</i>
TG, mmol/L	3.39±1.17 <sup>a</sup>	1.94±0.65 <sup>b</sup>	2.36±0.75 <sup>b</sup>
TC, mmol/L	5.38±0.59 <sup>a</sup>	4.35±0.67 <sup>b</sup>	4.81±0.36 <sup>b</sup>
HDL-C, mmol/L	1.94±0.25	1.94±0.23	2.04±0.26
Apo A-1, g/L	0.80±0.04	0.80±0.02	0.81±0.03
Apo B, g/L	0.52±0.01 <sup>a</sup>	0.37±0.04 <sup>b</sup>	0.39±0.07 <sup>b</sup>
Apo A-1/Apo B	1.6±0.3 <sup>b</sup>	2.3±0.5 <sup>a</sup>	2.1±0.4 <sup>a</sup>

<sup>1</sup>Values are means±SD, n=12. <sup>2</sup>Means in a row with different letters differ significantly, p<0.05. TG, triacylglycerols; TC, total cholesterol; HDL-C, high-density lipoprotein cholesterol; Apo B, apolipoprotein B; Apo A-1, apolipoprotein A-1.

SOURCE: Reproduced with permission from reference 7. Copyright 1999 American Society for Nutritional Sciences.

#### *Results*

The objective was to examine the dose-dependent hypolipidemic activity of jasmine GTE extract supplemented in diet. Significantly lower levels of serum TG and TC were observed in all GTE-supplemented groups compared with that of the control group (p<0.05, Table II). The hypolipidemic effects of dietary GTE were

dose-dependent among the control, 3.4 and 5.7 g GTE/kg diet groups ( $p < 0.05$ ) although serum TG and TC did not differ in the 1.1 and 3.4 g GTE/kg diet groups. Dietary GTE did not affect serum HDL-C.

**Table II. Effect of Varying Levels of Green Tea Epicatechin (GTE) Supplemented in Food for Four Weeks on Serum Lipids in Hamsters<sup>1,2</sup>**

Analyte	Control	1.1g GTE/kg	3.4g GTE/kg	5.7g GTE/kg
TG, mmol/L	8.25±1.79 <sup>a</sup>	5.87±1.97 <sup>b</sup>	6.02±1.97 <sup>b</sup>	4.56±1.65 <sup>c</sup>
TC, mmol/L	5.04±0.54 <sup>a</sup>	4.55±1.16 <sup>b</sup>	4.53±1.01 <sup>b</sup>	4.27±0.62 <sup>c</sup>
Serum HDL-C, mmol/L	1.42±0.15 <sup>a</sup>	1.40±0.23	1.40±0.15	1.50±0.15

<sup>1</sup>Values are means±SD, n=9. <sup>2</sup>Means in a row with different letters differ significantly,  $p < 0.05$ . TG, triacylglycerols; TC, total cholesterol; HDL-C, high-density lipoprotein cholesterol.

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### Time-Course Changes in Serum TC and TG in Hamsters Fed the GTE-supplemented Diet

#### *Animals and Diet*

The time-course changes in serum TC and TG in hamsters given a GTE-supplemented diet were monitored. In brief, male hamsters were randomly divided into two groups (n=7). One group was fed the control diet containing no GTE while the other group was fed the same diet but supplemented with 5.7 g GTE/kg diet, a level previously shown to significantly reduce serum TG and TC concentrations. All animals were allowed free access to tap water. On d-2, 14, and 28, after food-deprivation for 14 h, the hamsters were bled from the retro-orbital sinus into a heparinized capillary tube under light ether anesthesia (10). On d 36, all the hamsters were sacrificed and the blood was collected as described above.

#### *Results*

After 14 d of feeding the high fat and cholesterol diet, serum TG and TC levels were elevated in the controls and in hamsters fed the 5.7 g GTE/kg diet (Figure 1). After 28 d, the differences between the control and 5.7g GTE/kg diet groups was significant ( $p < 0.05$ ) with the latter having lower serum TG and TC concentrations. At the end of the experiment, serum TG was 35% ( $p < 0.05$ ) lower while serum TC was 31% lower ( $p < 0.05$ ) in the GTE-supplemented group compared with that of the control.

### Effect of Dietary GTE on Fecal Excretion of Neutral and Acidic Sterols

A possible mechanism for the hypocholesterolemic activity of GTE may be its inhibition of cholesterol and bile acid absorption. In fact, GTE supplemented hamsters had higher fecal excretion of total neutral sterols and cholesterol during the first 20 d of the experiment compared with those fed the control diet ( $p < 0.05$ , Table III). The output of total fecal bile acids in the GTE-supplemented group was not different from that in the control during the first 20 d but it was greater ( $p < 0.05$ ) during d 21-34 (Table III). Among the bile acids, deoxycholic acid and chenodeoxycholic acid plus cholic acid were significantly higher in the feces of hamsters fed the diet supplemented with GTE while the concentration of lithocholic acid did not differ between the groups (Table III). Nevertheless, the reduced absorption of dietary cholesterol during d 0-20 was directly associated with a lower serum cholesterol concentration in the GTE-supplemented group. In addition, the greater synthesis and excretion of acidic sterols (the major end products of cholesterol catabolism) in hamsters fed GTE during d 21-34 would also serve to lower the level of serum cholesterol.

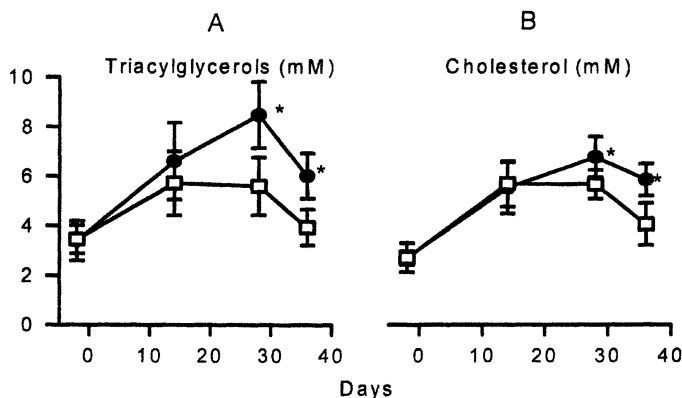
### Effect of Dietary GTE on Hepatic HMG-CoA Reductase

Cholesterol homeostasis is a delicate balance among dietary intake, synthesis and catabolism. Serum total cholesterol can be lowered if cholesterologenesis is inhibited. HMG-CoA reductase mediates the first committed step in the *de novo* synthesis of cholesterol from its precursor, acetate. The partial inhibition of this rate-limiting enzyme by dietary plant sterols and hypocholesterolemic drugs such as lovastatin and simvastatin is a typical example of reducing plasma cholesterol (11). However, it was found that the activity of liver HMG-CoA reductase in the hamsters who drank the fluid containing 5.0g GTE/L ( $26.4 \pm 2.8$  pmol $\cdot$ min $^{-1}$  $\cdot$ mg protein $^{-1}$ ) was not different from that of the control ( $27.3 \pm 2.0$  pmol $\cdot$ min $^{-1}$  $\cdot$ mg protein $^{-1}$ ), suggesting that inhibition of this enzyme is not part of GTE's hypocholesterolemic mechanism.

### Effect of Dietary GTE on Intestinal ACAT

Intestinal ACAT plays a key role in the intestinal absorption of cholesterol by esterifying cholesterol into chylomicrons before its absorption (12). It has been shown that tea catechins decrease micellar solubility and intestinal absorption of cholesterol in rats (13). We hypothesized that GTE may interfere the absorption of cholesterol by inhibiting the ACAT activity. However, no difference in the activity of intestinal ACAT was obtained between the control ( $171.9 \pm 118.9$  pmol $\cdot$ min $^{-1}$  $\cdot$ mg protein $^{-1}$ ) and GTE group ( $142.8 \pm 69.6$  pmol $\cdot$ min $^{-1}$  $\cdot$ mg protein $^{-1}$ ), suggesting that GTE increases the fecal output of cholesterol mainly due to its binding capacity and acceleration of cholesterol and bile acid excretion. In fact, GTE has

been shown to form insoluble coprecipitates with cholesterol and thus decrease cholesterol absorption (14).



**Figure 1** Effect of dietary green tea epicatechins (GTE) on serum total triacylglycerols (panel A) and total cholesterol (panel B) in hamsters (□, control; ●, GTE). Values are means  $\pm$  SD,  $n=7$ . \*Means at a time point differ significantly,  $p<0.05$ . (Reproduced with permission from reference 7. Copyright 1999 American Society for Nutritional Sciences.)

### Effect of Dietary GTE on Body Lipid Composition

Dietary GTE may significantly affect the body lipid composition. Hamsters fed GTE had lower liver total cholesterol concentrations and lower carcass and liver TG concentrations than controls ( $p<0.05$ , Table IV). In contrast, carcass and liver phospholipids did not differ in the two groups (data not shown). The carcass but not the livers of the GTE-supplemented hamsters were characterized by a lower TG/FFA ratio compared with that of the control. The activity of liver FAS did not differ in the control and GTE-fed groups ( $8.2 \pm 1.6$  versus  $8.3 \pm 0.7$   $\text{nmol}\cdot\text{min}^{-1}\cdot\text{mg protein}^{-1}$ ). The GTE-supplemented group had a higher concentration of total fecal fatty acids ( $240 \pm 9.5$   $\mu\text{mol}\cdot\text{g feces}^{-1}$ ) than the control ( $203 \pm 9.0$   $\mu\text{mol}\cdot\text{g feces}^{-1}$ ) during the first 20 d of the experiment ( $p<0.01$ ). Afterwards, there was no difference in the fecal total fatty acids between the control ( $203 \pm 27$   $\mu\text{mol}\cdot\text{g feces}^{-1}$ ) and GTE-supplemented groups ( $201 \pm 16$   $\mu\text{mol}\cdot\text{g feces}^{-1}$ ).

**Table III. Effect of Green Tea Epicatechins (GTE) on Fecal Output of Neutral and Acidic Sterols in Hamsters (mg/g feces)<sup>1,2</sup>**

Analyte	Day 0-20		Day 21-34	
	Control	5.7g GTE/Kg	Control	5.7g GTE/kg
<b>Neutral Sterols:</b>				
Cholesterol	3.90±0.88 <sup>a</sup>	4.86±0.99 <sup>b</sup>	1.87±0.99	2.15±0.62
Coprostanol	0.53±0.47	0.49±0.45	3.87±1.45	3.94±2.08
Coprostanone	0.35±0.08 <sup>b</sup>	0.46±0.10 <sup>a</sup>	0.34±0.17	0.40±0.12
Dihydrocholesterol	0.46±0.11	0.50±0.13	0.78±0.21	0.82±0.25
Total	5.24±0.88 <sup>c</sup>	6.31±0.55 <sup>d</sup>	6.86±1.93	7.31±2.22
<b>Acidic Sterols:</b>				
Lithocholic acid	1.03±0.34	1.12±0.40	1.33±0.46	1.37±0.36
Deoxycholic acid	1.21±0.44	1.33±0.60	1.20±0.13 <sup>b</sup>	2.07±0.41 <sup>a</sup>
Chenodeoxycholic + Cholic acids	0.32±0.20	0.21±0.10	0.43±0.17 <sup>d</sup>	0.68±0.22 <sup>c</sup>
Total	2.56±0.83	2.66±0.40	2.96±0.66 <sup>d</sup>	4.12±0.73 <sup>e</sup>

<sup>1</sup>Values are means±SD, n=4 cages with 2 hamsters per cage. <sup>2</sup>Means in a row with different letters differ significantly; <sup>ab</sup> p<0.05; <sup>cd</sup> p<0.01

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Suppression of body TG accumulation in the GTE-supplemented group deserves further investigations. Greater excretion in fecal total fatty acids may contribute partially to a lower accumulation in body TG of hamsters supplemented with GTE. GTE may enhance the hydrolysis of TG to FFA for oxidation. This was supported by the observation that carcass FFA content was significantly higher in hamsters supplemented with GTE (Table IV). This was also reflected in the significantly lower TG/FFA ratio of GTE-fed hamsters. In fact, a previous study by Sano et al. (4) had demonstrated that adrenaline-induced lipolytic activity in abdominal adipose tissue was significantly elevated in rats given green tea for 8 or 16 wk. It is unlikely that lower body TG accumulation in the GTE supplemented group was associated with suppression in fat synthesis because no difference in the activity of liver FAS was observed between the control and GTE-supplemented groups.

## Conclusion

Hypolipidemic activity in hamsters fed a high fat diet either in the form of GTWE or GTE solutions is in agreement with the two recent epidemiological studies which showed an inverse correlation between tea consumption and the concentration of serum TC and TG in Japanese (3,6). However, this is in contrast



to two other studies in which no correlation between tea consumption and plasma cholesterol was found (15-16). Chinese and Japanese mainly consume green tea whereas Caucasians mainly drink black tea. The beneficial effect of drinking green tea over black tea may be attributed to its higher content of GTE because in the former, GTE remain relatively unchanged compared with that of the fresh tea leaves while the GTE are mostly oxidized during the fermentation process in the latter (17).

**Table IV. Effects of Dietary Green Tea Epicatechins (GTE) for Five Weeks on Carcass and Liver Lipid Concentration in Hamsters<sup>1,2</sup>**

<i>Analyte</i>	<i>Control</i>	<i>5.7g GTE/kg</i>
<b>Carcass</b>		
TG, $\mu\text{mol/g}$	296.0 $\pm$ 63.8 <sup>a</sup>	209.8 $\pm$ 19.9 <sup>b</sup>
FFA, $\mu\text{mol/g}$	207.0 $\pm$ 35.5 <sup>b</sup>	262.2 $\pm$ 15.6 <sup>a</sup>
TG/FFA	1.4 $\pm$ 0.3 <sup>a</sup>	0.8 $\pm$ 0.1 <sup>b</sup>
<b>Liver</b>		
TG, $\mu\text{mol/g}$	16.8 $\pm$ 2.1 <sup>a</sup>	9.4 $\pm$ 3.0 <sup>b</sup>
FFA, $\mu\text{mol/g}$	10.9 $\pm$ 1.2 <sup>a</sup>	7.0 $\pm$ 1.2 <sup>b</sup>
TG/FFA	1.5 $\pm$ 0.2	1.3 $\pm$ 0.4
Cholesterol, $\mu\text{mol/g}$	30.5 $\pm$ 5.2 <sup>a</sup>	13.5 $\pm$ 5.4 <sup>b</sup>

<sup>1</sup>Values are mean $\pm$ SD, n=7. <sup>2</sup>Means in a row with different letters differ significantly,  $p < 0.05$ . TG, triacylglycerols; FFA, free fatty acids.

GTE not only reduced serum TG and TC but also Apo B with Apo A-1 being unaffected. Apo B is the principal protein in LDL, comprising approximately 90% of total LDL protein mass (18). It plays a major role in the recognition of cellular receptors for the catabolism of LDL (19). Numerous studies have indicated that Apo A-1 and Apo B measurements are useful in assessing the risk of cardiovascular disease (19-21). It has been reported that they are more specific and sensitive biochemical markers of cardiovascular disease risk than HDL-C and LDL-C. People with a low Apo A-1/Apo B ratio may have a higher risk of cardiovascular disease (22). The present results clearly demonstrated that supplementation of GTE either in diet or drinking water favorably modified the balance of these two apolipoproteins (Table I). If drinking green tea in humans is associated with a significantly lower risk in cardiovascular disease, part of the mechanism may involve an increase in Apo A-1/Apo B ratio.

The results obtained in hamsters, although not directly transferable to humans, may have some implications for individuals who often consume a high fat and cholesterol diet. The reduction in serum TG and cholesterol by dietary GTE is not associated with inhibition of liver FAS, HMG-CoA-R and intestinal ACAT but it is most likely mediated by its inhibition of absorption of dietary fat, cholesterol and reabsorption of bile acids.

## Acknowledgement

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## Chapter 18

# Health Benefits of Green Tea Catechins: Improvement of Intestinal Conditions

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Green tea catechins were confirmed to have a marked antioxidative as well as antibacterial and antiviral activity. When green tea beverage or catechin capsules are consumed, these materials will contribute to the prevention of various life style related or age related diseases. Green tea catechins have roles in the prevention of these diseases as well as keeping the entire digestive tract healthy. In order to make the best use of the health promoting functions of tea catechins, it is imperative to confirm the safety as well as the distribution and metabolism of green tea catechins after oral intake.

While the benefits of tea drinking have long been cited, there has been no mention of tea polyphenols until a few decades ago. Tea catechins are the major component in tea (*Camellia sinensis* L.), and approximately 100 mg may be consumed in one cup. Drinking a cup of green tea is somewhat synonymous with consuming tea catechins. Tea catechins have been fractionated and purified in significant quantity to pursue animal experiments at our laboratory over the last 20 years. Various *in vitro* and *in vivo* experiments have been carried out with these compounds, the results of which have led us to conclude that tea catechins are most likely effective in preventing a number of life-style related, age-related diseases of humans, such as cancer, hyperlipidemia, hypertension, hyperglycemia, etc. At present, the actual fate of catechins after oral intake has been only partly elucidated. It is assumed that a portion of the catechins taken orally are absorbed from the small intestine into the portal vein and subjected to metabolism in the liver, undergoing methylation or forming conjugates of glucuronide or sulfate (1,2,3). The majority of catechins, presumably more than 80%, are thought to remain in the intestinal tract and to be excreted into the feces (4). While in the large intestine, catechins influence the intestinal flora in a very favorable way.

## The Importance of Intestinal Flora on Human Health

Intestinal flora are influenced by the diet or various exogenous factors and in a similar fashion the flora will influence the health of the host in various ways. Unhealthy dietary habits will induce unhealthy flora by which various undesirable compounds will be produced. These decomposition products (ammonia, sulfide, amine, phenol, indole, etc), bacterial toxins, carcinogenic compounds (nitroso compounds, epoxides etc.) or secondary bile acids may eventually take their toll on the host in the form of cancer, aging, atherosclerosis, hypertension, liver disfunction, auto-immune diseases, lowered immune response, if such undesirable exogenous factors are not rectified. In various human studies with fecal specimens, it was shown to be likely that in the intestines of sick people the so-called 'good' bacteria such as *Bifidobacteria* or *Lactobacilli* tend to decrease and the so-called 'bad' bacteria such as *Clostridium* tend to increase as compared to those of healthy people. The increase of *Bifidobacteria* or *Lactobacilli* in the intestines of human host occurs along with the concomitant decrease of *Clostridium* microorganism. Recently, various kinds of oligo-saccharides have become popular as dietary supplements since oligomeric sugars are mainly utilized by *Bifidobacteria* and not by other intestinal bacteria. Based on these findings, we are now encouraged to consume yogurt, oligo-saccharides or the powder of *Bifidobacteria* on a daily basis in order to maintain intestinal health. How tea catechins influence the intestinal flora is the topic of this paper.

### Antibacterial Potency of Tea Catechins

The minimum inhibitory concentrations (MIC) of the catechins in green tea against well known strains of foodborne pathogenic bacteria and 6 strains of lactic acid bacteria are shown in Table I. Results showed that tea catechins act antibacterially against *Staphylococcus aureus*, *Clostridium botulinum*, *Clostridium perfringens*, *Bacillus cereus*, *Vibrio* strains, *Plesiomonas shigelloides* and *Aeromonas sobria*. The growth of those bacteria was inhibited at a catechin concentration less than that contained in a normal cup of tea, that is ranging from 500 to 1,000 ppm. The foodborne pathogenic bacteria which are inhibited by tea catechins are largely those which have been responsible for a number of incidences of food poisoning. Consequently, tea catechins may possibly be used to prevent bacterial foodborne diseases. Moreover, tea catechins showed virtually no activity against such lactic acid bacteria as *Bifidobacteria* or *Lactobacilli* at the concentration of 1,000 ppm. The fate of tea catechins in the digestive tract after oral intake and their activity in the gut is of interest at present.

### The Fate of Tea Catechins in the Digestive Tract

Among the four kinds of tea catechins, (-)-Epigallocatechin gallate (EGCg) is quantitatively the major component (more than 50%) and physiologically the most potent. Therefore, we traced the fate of EGCg in the digestive tract in rats. Fifty mg of

**Table I. Minimum Inhibitory Concentrations of Tea Catechins Against Foodborne Pathogenic and Enteric Bacteria**

Bacteria	MIC (ppm)				
	Polyphenon	EC	ECg	EGC	EGCg
<i>Staphylococcus aureus</i> IAM 1011	450	>800	800	150	250
<i>Vibrio fluvialis</i> JCM 3752	200	800	300	300	200
<i>V. parahaemolyticus</i> IFO 12711	200	800	500	300	200
<i>V. metschnikovii</i> IAM 1039	500	>1000	>1000	500	1000
<i>Clostridium perfringens</i> JCM 3816	400	>1000	400	1000	300
<i>Clostridium botulinum</i> A, B mix.	<100	>1000	200	300	<100
<i>Bacillus cereus</i> JCM 2152	600	>1000	600	>1000	600
<i>Plesiomonas shigelloides</i> IID No. 3	100	700	100	200	100
<i>Aeromonas sobria</i> JCM 2139	400	>1000	700	400	300
<i>Lactobacillus brevis</i> subsp. <i>gravesensis</i> JCM 1102	>1000	>1000	>1000	>1000	>1000
<i>L. brevis</i> subsp. <i>brevis</i> JCM 1059	>1000	>1000	>1000	>1000	>1000
<i>L. brevis</i> subsp. <i>otakiensis</i> JCM 1183	>1000	>1000	>1000	>1000	>1000
<i>Bifidobacterium bifidum</i> JCM 1255	>1000	>1000	>1000	>1000	>1000
<i>B. adolescentis</i> JCM 1275	>1000	>1000	>1000	>1000	>1000
<i>B. longum</i> JCM 1217	>1000	>1000	>1000	>1000	>1000

Polyphenon: mixture of individual catechins (purity>80%); EC: (-)-epicatechin; ECg: (-)-epicatechin gallate; EGC: (-)-epigallocatechin; EGCg: (-)-epigallocatechin gallate (major catechin in tea).

EGCg was administered orally to fasted rats, then after 1, 2, 5, 8, 12, 16 and 20 hours the residual content of EGCg in the stomach, small intestine, large intestine as well as in the feces was determined. As shown in Figure 1, within a few hours after administration the level of EGCg in stomach decreased rapidly and increased in the small intestine. The amount of EGCg in the large intestine began to increase sharply as the amount in the small intestine decreased, and was at its highest about 8 hours after ingestion, at which time only a trace amount remained in the other organs. EGCg appeared in the feces 12 hours after ingestion and increased gradually thereafter. Two

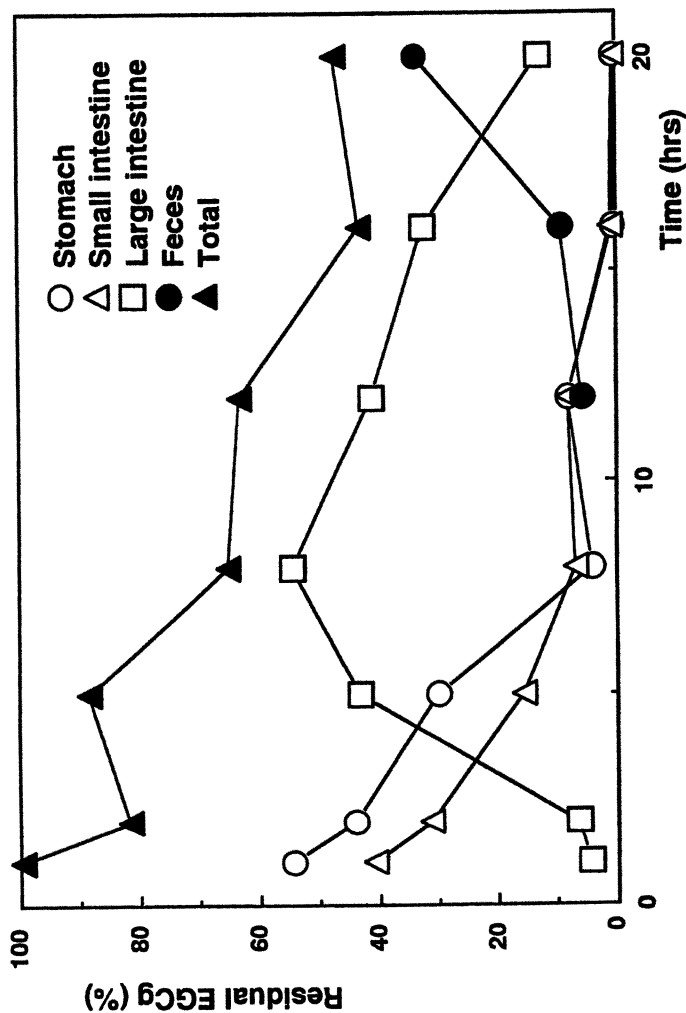


Figure 1. Residual EGCG in different section of the digestive tract of rats administered 50 mg of EGCG. (Reproduced with permission from reference 6. Copyright 1995 MultiMedia Healthcare/Freedom, LLC.)

hours after administration, when a small amount of EGCg was already in the large intestine, about 20% of the EGCg was lost (ie. unrecoverable). We concluded that this 20% disappearance of EGCg was the amount absorbed into the body through the small intestine. This assumption is supported by a previous study which confirms that EGCg does not undergo any degradation in the stomach or small intestine (unpublished data). In addition the experimental results of Hattori *et al.* (5) show that when gallated catechins (EGCg, ECg) are incubated anaerobically with the content of the large intestine of rats or with those of humans, there is no degradation of catechins for 48 hours with rats, and some appreciable degradation with human feces. These data imply that the majority of catechins, at least 50%, will remain intact or undergo reactions with microflora in the large intestine and pass into the feces. In light of these factors an investigation is warranted into the influence of tea catechins on the intestinal flora in humans.

## Experimental

### Feeding of Catechins by Gastroenteral Tube Feeding

In order to evaluate the effects of tea catechins on the intestinal flora and the fecal metabolites in humans, residents in long-term care facilities in and around Hamamatsu, Japan, who were receiving gastroenteral liquid alimentation via nasogastric or gastric tubes were administered tea catechins and their fecal specimens were analyzed (6). The subjects studied were 10 females and 5 males, ranging in age from 51 to 93 years (average, 70.3 years) and their body weight ranged from 28 kg to 56 kg (average, 41kg). All subjects received 1,000 kcal of the same nutrition daily, supplemented with 300 mg of catechins, which was divided into three doses a day and mixed in the liquid alimentation for a period of three weeks. Tea catechins used were "Polyphenon 60" provided by Mitsui Norin Co. (Fujieda City, Japan), which contains 62% catechins. A daily dose of 300 mg of catechins is equivalent to 484mg of "Polyphenon 60" powder, which will correspond to 4 to 6 cups of green tea. Freshly voided fecal specimens were collected on days 0 (before administration), 7, 14, and 21 of administration and day 7 postadministration. The fecal measures of microfloral populations, pH, moisture content, ammonia, sulfide, other odorous metabolites, and organic acids, were analyzed at each collection. Results indicated remarkable improvement of bowel conditions. As shown in Figure 2, following the administration of tea catechins, levels of *Bifidobacteria* and *Lactobacilli* increased significantly whereas those of *Bacteroidaceae*, *Eubacteria*, *Enterobacteriaceae* and *Clostridia* decreased significantly. Figure 3 shows other parameters. Fecal pH and ammonia decreased significantly whereas sulfide and fecal odorous metabolites (phenol, cresol, ethyl phenol, indole, and skatol) increased at day 7 and then significantly decreased at

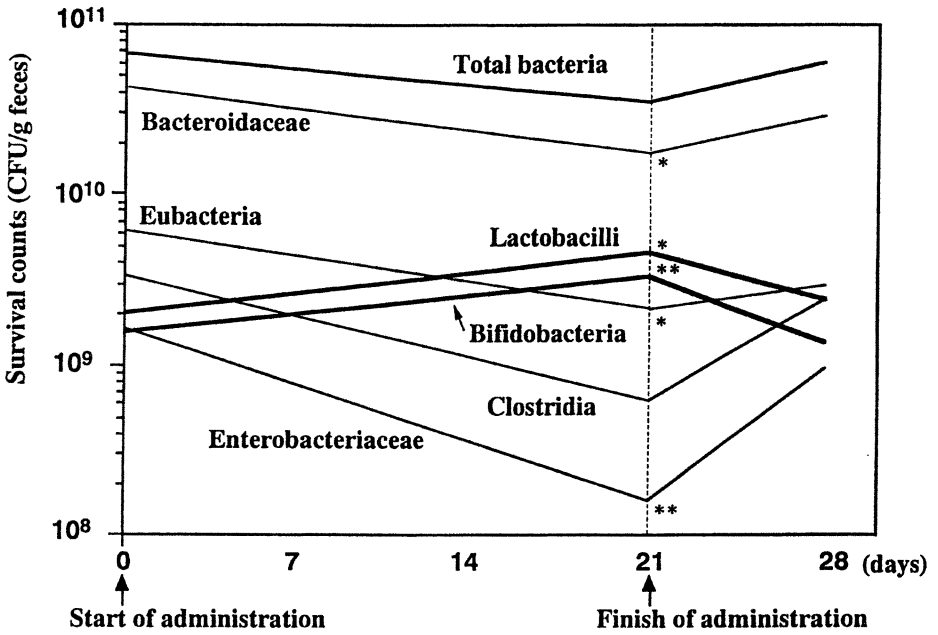
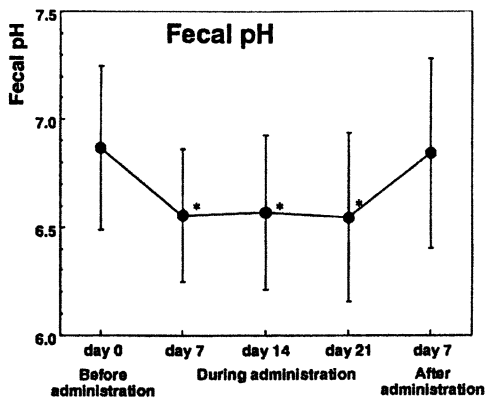
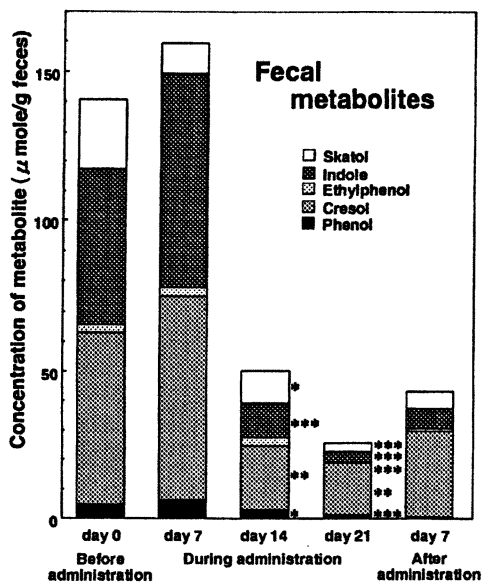


Figure 2. Effect of green tea catechin administration (300 mg/day) on fecal flora of 15 human volunteers.



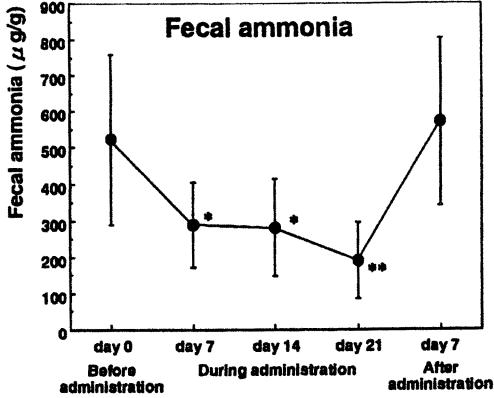


Graph is expressed as mean  $\pm$  SD. \*: Significant difference ( $p < 0.05$ ) from the pH of day 0 (before the administration).

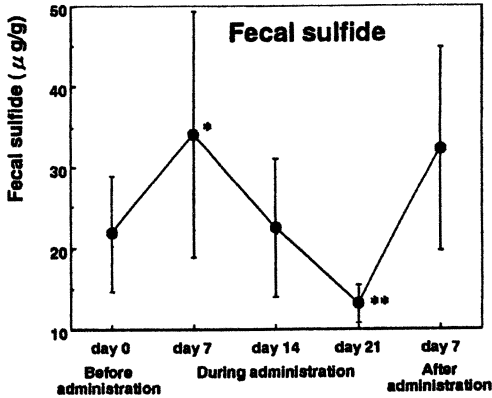


\*, \*\*, \*\*\*; Significant difference (\*= $p < 0.05$ , \*\*= $p < 0.01$ , \*\*\*= $p < 0.001$ ) from the value of day 0 (before the administration).

Figure 3. Changes of fecal parameters by oral tea catechin. Continued on next page.

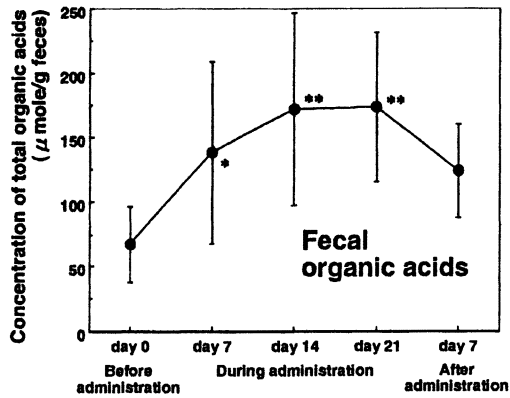


Graph is expressed as mean  $\pm$  SD. \*\*, Significant difference ( $*=p<0.01, **=p<0.001$ ) from the value of day 0 (before the administration).



Graph is expressed as mean  $\pm$  SD. \*\*, Significant difference ( $*=p<0.05, **=p<0.001$ ) from the value of day 0 (before the administration).

Figure 3. *Continued.*



Graph is expressed as mean  $\pm$  SD. \*, \*\*; Significant difference (\*= $p < 0.01$ , \*\*= $p < 0.001$ ) from the value of day 0 (before the administration).

Figure 3. *Continued.*

day 21. Total organic acids (acetic, propionic, butyric etc.) increased significantly throughout the catechin administration. Fecal moisture content ranged from 76% to 72% and tended to decrease by catechin administration. All these measures, as well as the populations of fecal bacteria, tended to reverse after the end of catechin administration.

### Feeding of Catechins by Oral Administration

In the first experiment, tea catechin was administered to the subjects by way of enteral feeding. In the second experiment, we further studied the effects of tea catechins with a larger number of elderly inpatients on solid food diet (7). The subjects were 35 residents in a nursing home in Shizuoka, Japan: 31 females and 4 males from 66 years to 98 years of age (average 85.4 years). As was the case in the previous study, most of them had some kind of illness and were bedridden but none had complications of the gastrointestinal tract or endocrine organs and none were receiving antibiotics. The same amount of tea catechins as in the previous study, 300mg of tea catechin or 484 mg of "Polyphenon 60", was administered daily for a period of 6 weeks. Two tablets each containing 50 mg of catechins were taken by the subjects after each meal. Fecal specimens were collected by the nursing staff immediately or within 2 hours after being voided. Specimens were collected on day 0 (before administration, period 0), days 9-11 of administration (period I), days 23-25 (period II), days 37-39 (period III), and days 16-18 of postadministration (period IV). Such fecal parameters as pH, moisture content, ammonia and sulfide were measured. Results are shown in Figure 4. Comparing values before administration with those during tea catechin administration, the pH values decreased significantly in period I and III, and fecal moisture content decreased significantly in period III. Fecal concentrations of both ammonia and sulfide decreased significantly in period I, II and III. On days 16-18 postadministration (period IV), all the above data showed a tendency to return toward the levels of period 0 (before administration).

### Discussion and Conclusion

The intestinal microflora of neonatals is dominated by *Bifidobacteria*. After weaning, the dominance of *Bifidobacteria* is replaced by gram-negative rod bacteria, which is typically the flora of adulthood. With the onset of old age, marked decrease of the counts of *Bifidobacteria* and the increase of those of *Clostridia* is observed. With respect to the composition of intestinal flora, the increase of the counts of *Bifidobacteria* and the decrease of those of *Clostridia* is regarded to be beneficial for human health. Tea catechins, which are extracted from green tea, have selective antibacterial activity, but not against lactic acid bacteria. The hypothesis that tea catechins will remain mostly in the digestive tract without being absorbed led us to examine the effect of tea catechins on the intestinal microflora. Tea catechins in an amount equivalent to 4-6 cups, ie. 300mg, were administered daily to tube fed

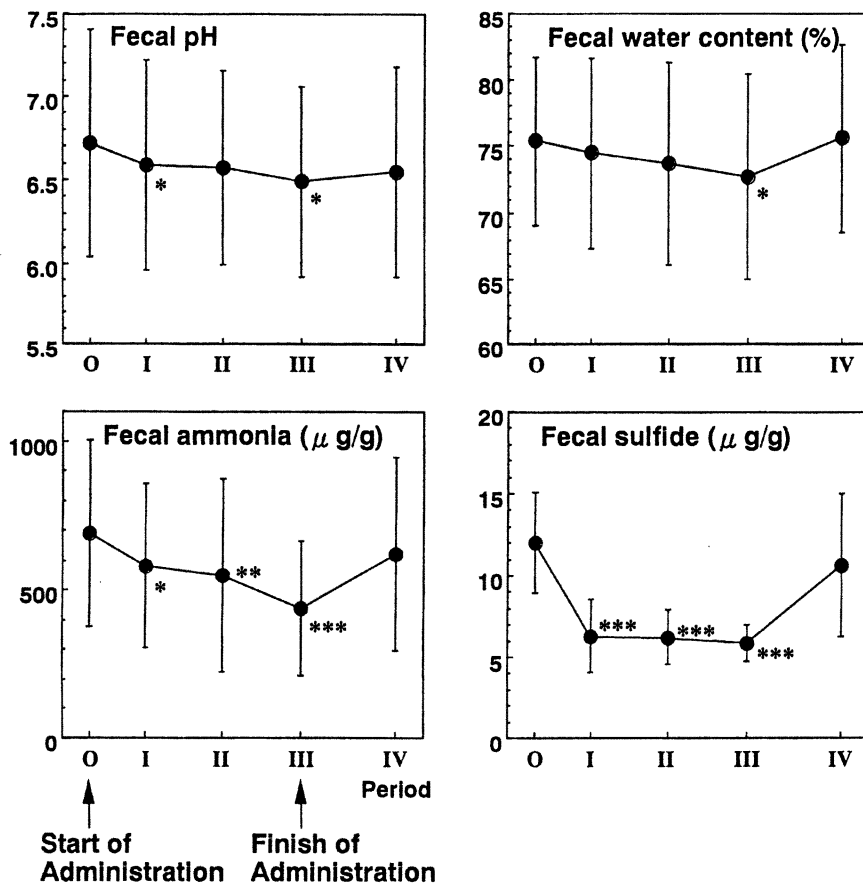


Figure 4. Effect of tea catechin administration on fecal pH, water content, ammonia and sulfide. Graph is expressed as mean  $\pm$  SD.

\*; Significant difference ( $p < 0.05$ ) from the value of day 0 (before administration).

\*\*; Significant difference ( $p < 0.01$ ) from the value of day 0 (before administration).

\*\*\*; Significant difference ( $p < 0.001$ ) from the value of day 0 (before administration).

subjects and to elderly people who were on solid food. The analyses of the feces of those subjects revealed that, in three weeks, there was a significant increase of *Bifidobacteria* and *Lactobacilli* and a decreasing level of *Clostridia*. This indicates very favorable floral conditions in the intestine (First Experiment). The favorable improvement of bowel conditions was confirmed by the significant decrease of pH, the concentration of ammonia and sulfide, and the significant increase of total organic acids. The significant decrease of malodorous fecal metabolites was also confirmed by tea catechin feeding. The group of those who were on the solid food diet (Second Experiment), showed the same tendencies. The nursing staff's sensory records in both experiments endorsed the reduction of fecal odors by tea catechin administration. From these human data, it is inferred that a certain amount of tea catechin will suppress the production of putrefactive compounds and improve the health of the intestine by influencing the intestinal flora in a very favorable way.

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## Chapter 19

# Potential Cardiovascular Health Benefits of Procyanidins Present in Chocolate and Cocoa

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Recent epidemiological research indicates that diets rich in flavonoid-containing foods may be associated with a reduced risk for cardiovascular disease. This protective effect is attributed, in part, to the ability of flavonoids to act as antioxidants. Certain chocolates and cocoas contain substantial amounts of procyanidins, and thus belong in the category of flavonoid-rich foods. Recent advancements in the identification and isolation of procyanidins, especially oligomeric procyanidins, from chocolate and cocoa have facilitated the investigation of individual procyanidin fractions with regard to their potential cardiovascular health benefits. In the following paper, we report on the antioxidant capacity of a cocoa as determined by the Oxygen Radical Absorbance Capacity (ORAC) assay, and the ability of individual procyanidin fractions from this same cocoa to inhibit low-density lipoprotein (LDL) oxidation *in vitro*. In addition, mechanisms are discussed by which flavonoids in chocolate and cocoa may enhance cardiovascular health.

*Theobroma cacao*, described by the ancient Mayans as “the food of the Gods,” is primarily consumed today as chocolate. There is evidence dating back to the first and second centuries A.D. of cacao usage by the Olmec Indians in the Gulf Coast Regions of Mexico (1). Following the Spanish conquest of Central and South America during the 16<sup>th</sup> century, Cortes introduced chocolate to the Royal Court in Spain. It was described as a nutritious drink that allowed one to travel without any further food in hot weather (1). Cacao soon became popular among the upper echelon of European society, and European physicians recommended the cacao beverage as a nutritious food, particularly when mixed with milk (1). Recent studies suggest that many of the

ancient beliefs about the health benefits of chocolate consumption may have some validity (2,3,4).

Chocolate, of plant origin, contains vitamins, minerals and phytochemicals. Interestingly, this is often overlooked because of the “unhealthy” stigma associated with foods containing saturated fat and caffeine. In this context, it should be noted that milk chocolate has been found to have a neutral effect on cholesterol levels (5). In addition, the caffeine contributed to the diet by chocolate is negligible compared to popular beverages such as coffee, tea, and colas (6,7). Milk chocolate can range from 2-30 mg caffeine/50g and dark chocolate from 10-60 mg caffeine/50g .

### Procyanidins in Chocolate and Cocoa

Prominent among the phytochemicals found in chocolate is the flavan-3-ol (-)-epicatechin and, perhaps of most interest, the related series of complex procyanidin oligomers built upon this monomeric unit (8) (Figure 1). It is important to note that these polyphenolic compounds contribute to the reaction chemistry during the manufacture of chocolate and also contribute to chocolate’s characteristic flavor (9).

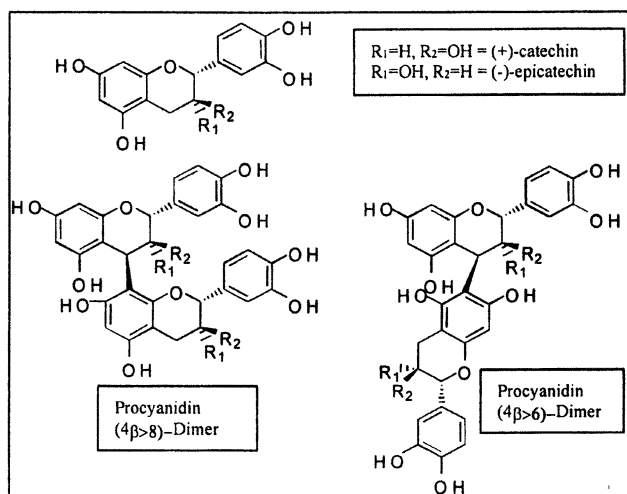


Figure 1. Flavan-3-ol monomeric and dimeric procyanidins (8).

Efforts to identify and separate polyphenols in cocoa beans began with Forastero beans in the 1950’s using paper chromatography (10). More advanced techniques, such as high-performance liquid chromatography (HPLC), have led to an increased understanding of the occurrence of these compounds in chocolate and cocoa.



Hammerstone *et al.* (8), recently identified and separated monomeric and oligomeric procyanidins through the decamer fraction in raw cocoa and in select chocolates using a modified normal-phase HPLC method, coupled with on-line mass spectrometry (MS) using an atmospheric pressure ionization electrospray chamber (Figure 2). This technique allows for rapid separation and identification of the oligomeric procyanidins present in chocolate as well as other foods (11). Prior to the development of this method, the highest oligomeric unit identified in cocoa had been octamer as described by Clapperton *et al.*, using column chromatography, reverse-phase HPLC and positive ion fast-atom bombardment mass spectrometry (9). These analytical advances now provide an opportunity to study the potential health benefits of specific procyanidins present in chocolate and cocoa.

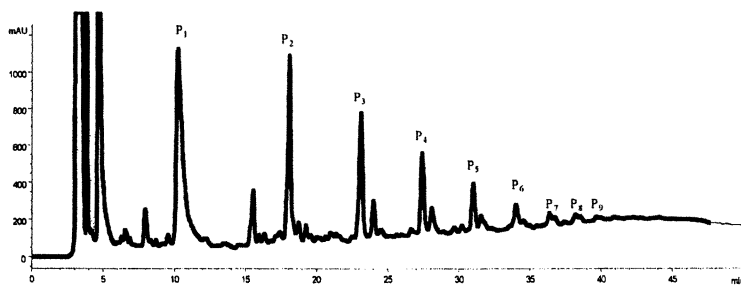


Figure 2. UV trace at 280nm for the separation of procyanidins in chocolate (8).

### Potential Cardiovascular Health Benefits of Flavonoids

The biological activity and potential health benefits of flavonoids from many food sources is attracting a great deal of interest in the research community. Early epidemiological research has shown promising results, although strong associations have been inconsistent (12,13). Positive associations were found between flavonoid intake and the incidence of death from coronary heart disease (14,15). In addition, preliminary findings suggest that procyanidins may have the following positive biological functions: act as a potent antioxidant and free radical scavenger (16,17), reduce lipid peroxidation (3,18,19), spare vitamin E (20), exhibit antibacterial and plaque inhibiting properties (21), be related to longevity (22), act as an antidepressant (23), modulate immune function (2), possess anti-tumor activity (24), and exhibit numerous other cardioprotective effects (25,26).

Several biomarkers and/or mechanisms, other than elevated blood cholesterol levels, have been identified as potential risk factors for cardiovascular disease (27), e.g., LDL oxidation, excessive platelet aggregation, altered endothelial function, and compromised immune function. Thus, it is interesting to note that *in vitro* studies have

shown that polyphenols may modulate oxidant defenses, platelet aggregation, hypertension, and immune function (28,26,29,2).

The possible health benefits of polyphenols present in chocolate and cocoa have been investigated in preliminary experiments focusing on the antioxidant properties of these compounds (3,4). Phenolic extracts of cocoa powder, baker's chocolate and dark chocolate were evaluated for their ability to inhibit LDL oxidation *in vitro* and were found to inhibit oxidation similar to that of other phenol-containing products (3). Kondo *et al.* demonstrated that LDL collected from subjects 2 hours after consumption of 35 g of defatted cocoa was more resistant to oxidation *in vitro* than were LDL obtained from the same subjects prior to ingestion of the cocoa (4).

The research objectives of the present study were to better understand the antioxidant potential of procyanidins present in certain chocolates and cocoas and to determine their ability to inhibit LDL oxidation. The antioxidant capacity of a cocoa powder was measured using the ORAC assay and purified individual procyanidins, from the same cocoa, were further tested for their ability to inhibit copper-catalyzed LDL oxidation *in vitro*.

## Materials and Methods

### Samples

Cocoapro™ cocoa was provided by Mars, Inc. (Elizabethtown, NJ). Brazilian cocoa beans were provided by Almirante Cacau (Mars, Incorporated), Brazil.

### Sample Preparation and Procyanidin Extraction

Samples were prepared for the ORAC assay by extracting with 20 mL in a ratio of 50:49.5:0.5 of acetone, water and acetic acid, respectively. The sample was shaken at a speed of 400 rpm for 60 minutes at room temperature, then centrifuged at 13000 x g for 15 minutes at 4° C. The resulting supernatant was diluted with phosphate buffer and used for the ORAC assay.

Procyanidin fractions used in the LDL oxidation assay were purified according to Hammerstone *et al.* (8). In short, fresh cocoa beans were frozen with liquid nitrogen and ground in a high-speed laboratory mill to a particle size of approximately 90 µm. Lipids were removed with multiple hexane extractions. The lipid free solids were air-dried and a fraction containing procyanidins was obtained by extracting with 70% by volume acetone in water. The suspension was centrifuged for 10 minutes at 1500 x g; the acetone layer was then passed through a funnel with glass wool. To remove residual lipids, the aqueous acetone was re-extracted with hexane. The hexane layer was discarded and the aqueous acetone was rotary evaporated under partial vacuum at 40°C to a final volume of approximately 200 mL and freeze dried. Individual

procyanidin fractions were collected using normal-phase HPLC as described by Hammerstone *et al.* (8).

### Antioxidant Capacity Using the automated ORAC assay

A COBAS FARA II spectrofluorometric centrifugal analyzer (Roche Diagnostic System Inc., Branchburg, NJ; emission filter = 565 nm) was used for the automated ORAC assay. The procedure reported by Cao and co-workers (30) was modified for the COBAS FARA II. Four mM of the peroxy radical generator, 2,2'-azobis (2-amidinopropane) dihydrochloride (AAPH) was mixed with 16.7 nM of R-phycoerythrin (Sigma, St. Louis, MO), which was included as the target of free radical attack. The final assay mixture was a total volume of 0.4 mL. The standard control was 1.0  $\mu\text{M/L}$  of Trolox. The analyzer recorded the fluorescence of R-PE every 2 minutes after the addition of AAPH. Measurements were based on the difference between initial and the two-minute interval readings. Calculations included the difference of areas under the R-PE decay curves, the blank, and a sample. The final results are expressed as  $\mu\text{M}$  of Trolox equivalent (TE)/g.

### Inhibition of LDL Oxidation

The effects of five purified cocoa procyanidins and two procyanidin mixtures (monomer through tetramer and pentamer through decamer) on copper induced LDL oxidation were assessed by measuring the formation of hexanal using headspace gas chromatography. Blood was collected from healthy male volunteers into vacutainer tubes containing ethylene diaminetetraacetate (EDTA). The blood was centrifuged at 15° C and 1500 rpm for 30 minutes. The plasma LDL were further separated by sequential density ultracentrifugation and dialyzed overnight with deoxygenated phosphate buffer (pH 7.4) at 4° C to remove the EDTA. Using a Sigma Protein Lowry kit (St. Louis, MO), the protein concentration in the LDL was quantified and diluted with phosphate buffer to a final concentration of 1 mg LDL/mL.

The individual procyanidins and procyanidin mixtures were tested at concentrations of 1  $\mu\text{M}$  and 5  $\mu\text{M}$ . The controls included LDL and copper sulfate. The test compounds, at their respective concentrations, were added to 250  $\mu\text{L}$  of LDL in a 6 mL headspace vial and gently mixed. Copper sulfate was added for a final concentration of 80  $\mu\text{M}$ . The vial was vortexed, and incubated in a 37° C shaking water bath for 2 hours. The headspace vials were then injected into a gas chromatograph equipped with a static headspace sampler to measure hexanal production. A hexanal standard was run to determine the retention time. All samples were run in quadruplicate and the experiment was repeated three times.

## Results

### ORAC Results

The mean ORAC value  $\pm$  the SEM for *Cocoapro*<sup>TM</sup> cocoa, based on analyses of four samples, was  $974.5 \pm 48.7 \mu\text{M TE/g}$ . The result for cocoa was compared with previously reported values for green tea, blueberry, garlic and strawberry in Figure 3.

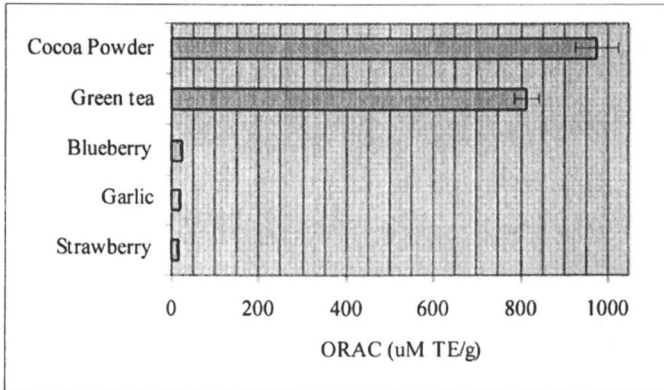


Figure 3. ORAC results for *Cocoapro*<sup>TM</sup> cocoa and compared with previously reported values for strawberry (33), blueberry (32), garlic and green tea (31).

### Inhibition of LDL Oxidation

All procyanidins at both concentrations inhibited copper-catalyzed LDL oxidation to some degree. At the  $5 \mu\text{M}$  concentration, each of the pure compounds, monomer through hexamer, inhibited oxidation by approximately 96% (Figure 4).

The large variation seen with the extract of mixtures of the procyanidins suggest some instability with these samples. At  $1 \mu\text{M}$  concentrations, oxidation was inhibited to varying degrees based on the size of the procyanidin oligomer, with the larger oligomers being most potent (Figure 5).

## Discussion

These results clearly demonstrate the ability of cocoa powder, and of select procyanidins present in certain chocolates and cocoas, to act as potent antioxidants *in*

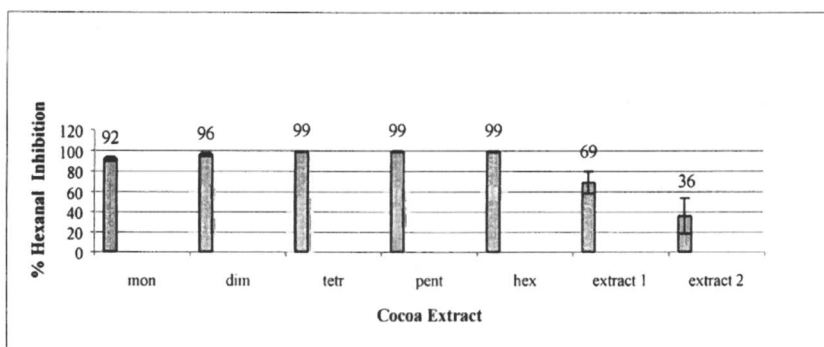


Figure 4. Inhibition of Copper-catalyzed LDL oxidation by cocoa extracts at a concentration of 5  $\mu$ M.

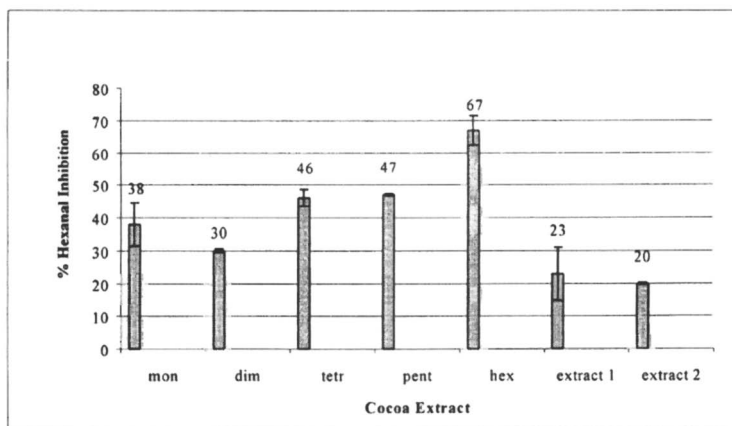


Figure 5. Inhibition of Copper-catalyzed LDL oxidation at a concentration of 1  $\mu$ M.

*in vitro*. Interestingly, the ORAC assay demonstrated that the cocoa powder tested had antioxidant activity equivalent to, or greater than, many fruits and vegetables (31,32,33). Most importantly, however, these experiments suggest that individual procyanidin fractions present in chocolate and cocoa may have significantly different antioxidant activities based on structural differences. Thus, it is reasonable to suggest that individual procyanidin fractions will have different biological activities *in vivo*.

By virtue of their structure, procyanidins have the potential to be potent antioxidants. Polyphenols can act as antioxidants by several mechanisms, including donation of hydrogens, quenching singlet oxygen, radical scavenging, and chelating redox active metals (34). Three main structural criteria have been established for the antioxidant potential of flavonoids: 1) *o*-dihydroxy structure in the B ring, 2) 2,3 double bond with a 4 oxo functional group in the C ring, and 3) 3 and 5-OH groups with the 4-oxo functional group in the A and C rings (34). As an example, quercetin has all three characteristics, while epicatechin satisfies only the first. Thus, according to this definition, quercetin should be a more potent antioxidant than a catechin compound. This theoretical conclusion, however, does not always agree with experimental results. In a recent study using linoleic acid oxidation, an erythrocyte ghost system, and microsomal oxidation, epicatechin was found to have slightly stronger antioxidative potential than quercetin. The only exception was in microsomal oxidation where the two had similar activity (35). Thus, caution must be used when interpreting the antioxidant potential of a given compound, or mixture of compounds, because results may vary depending on the assay used and, more importantly, the biological system in which the flavonoid may be present.

The results from this study suggest that, in the context of LDL oxidation, the larger procyanidin oligomers present in chocolate and cocoa may have a greater antioxidant potential than either the monomer or smaller oligomers (e.g., dimer and trimer). A possible explanation for this observation may be the higher number of hydroxyl groups present on the higher oligomers in contrast to either the monomer epicatechin or the smaller oligomers. It is tempting to suggest that the substantial antioxidant capacity demonstrated by the cocoa powder in the ORAC assay was driven by the oligomeric procyanidins present in the powder. However, it should be noted that the mechanisms to generate free radicals in the ORAC and copper induced LDL oxidation assays are different. Thus, additional testing of the individual procyanidin fractions in the ORAC assay are required to determine if the oligomeric fractions display relative activities similar to the results obtained for the copper induced LDL oxidation assay.

Understanding the bioavailability of oligomeric procyanidins is required to understand their potential cardiovascular benefit when consumed in the diet. While it has been shown that certain monomeric flavonoids are absorbed from tea and cocoa (36,37), similar data on the oligomeric fractions present in these products are lacking. Data on the metabolism of these phytochemicals is also lacking.

Finally, well controlled clinical studies are needed to determine if procyanidins in chocolate and cocoa offer true benefit to cardiovascular health. In these studies it will

be important to control for the presence of other nutritionally important factors present in chocolate and cocoa, such as magnesium and copper, which are important for cardiovascular health (38,39).

## Conclusion

This study demonstrates that certain cocoa powders have significant antioxidant potential, and that procyanidins present in certain chocolates and cocoa exhibit *in vitro* antioxidant activity that may be beneficial for cardiovascular health. Moreover, it was demonstrated that higher oligomeric cocoa procyanidin fractions have greater antioxidant potential than smaller oligomers as measured by the inhibition of copper induced LDL oxidation assay. Additional research is needed to understand the bioactivities cocoa procyanidins with respect to platelet activity, cyclooxygenase activity, and other biological processes critical to cardiovascular health.

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## Chapter 20

# An Overview of Coffee Roasting

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The coffee beverage as presently consumed is an aqueous extract of the roasted and ground seed of the fruit of the coffee plant. It is consumed for its pleasant flavor and aroma and its stimulatory properties due to caffeine. The flavor of roasted coffee is quite complex and this chapter will discuss in general the chemical and flavor changes which occur when the bean is roasted and nearly 800 aroma compounds are generated.

### Historical

It is not known exactly where and when coffee was first cultivated, but some authorities believe that it was grown initially in Arabia near the Red Sea about *ad* 675. Coffee cultivation was rare until the 15th and 16th centuries, when extensive planting of the tree occurred in the Yemen region of Arabia. The consumption of coffee increased in Europe during the 17th century, prompting the Dutch to cultivate it in their colonies. In England, the coffee house became the center of intellectual debate and free speech which transferred to the colonies of North America and played an important role in revolutionary thinking that eventually led to founding of the United States. In 1714 the French succeeded in bringing a live cutting of a coffee tree to the island of Martinique in the West Indies. This single plant was the genesis of the great coffee plantations of Latin America. The name coffee probably derives from the Turkish word, *kahveh*, (from the Arabian word *gahwah*), signifying an infusion of a roasted bean (1).

### Horticultural Aspects

The coffee plant is a relatively small tree, native to Africa, belonging in the family Rubiaceae and genus *Coffea*. The shrub or small tree, 4.6 to 6 m (15 to 20 ft) high at maturity, bears shiny green, ovate leaves that persist for three to five

years and white, fragrant flowers that bloom for only a few days. During the six or seven months after appearance of the flower, the fruit develops and changes from light green to red and, ultimately, when fully ripe and ready for picking, to deep crimson. The mature fruit of the coffee tree resembles a cherry. These grow in clusters attached to the limb by very short stems, and usually contain two seeds, surrounded by a sweet pulp. The fruit on a tree does not all ripen at the same time.

In some regions, such as Colombia, only the ripe fruit is picked and in other regions, such as Brazil, all the fruit is picked at one time resulting in the presence of predominantly ripe, plus some underripe and overripe fruit. Each fruit typically contains two light green or yellowish seeds called coffee beans (1). The coffee tree produces its first full crop when it is about 5 years old. Thereafter it produces consistently for 15 or 20 years. Some trees yield 0.9 to 1.3 kg (2 to 3 lb) of marketable beans annually, but 0.45 kg (1 lb) is considered an average annual yield.

The majority of the 80 varieties known are wild and have demonstrated adaptation to specific elevations, altitudes, soil types, and rainfall. Cultivation is carried out in tropical or semitropical areas around the world ranging between the Tropic of Cancer and Capricorn. The soil in which coffee is grown must be rich, moist, and absorbent enough to accept water readily, but sufficiently loose to allow rapid drainage of excess water. Although coffee trees are damaged easily by frost, they are cultivated in cooler regions. Two species are of commercial importance: *Coffea arabica* Linn. (commonly known as arabica coffee) and *Coffea canephora* Pierre ex Froehner (commonly known as robusta coffee) (2).

The arabica is believed to have originated in Northern Africa (Ethiopia) and is generally grown at higher altitudes. The *Coffea arabica* variety *typica* and variety *bourbon* are the common varieties of arabica coffee under cultivation today (3). The Americas, where *C. arabica* is grown, produce approximately two-thirds of the world's supply and arabica coffee is considered of higher quality than robusta.

The robusta is believed to have originated in the rain forests of Central Africa. They are more robust, and will grow at lower altitudes, tolerate higher temperatures and greater rainfall and are generally more resistant to diseases and pests. They are primarily grown in Uganda, Ivory Coast, Indonesia and regions of Brazil (e.g. Conillon).

These two species differ somewhat in chemical composition as shown in Table 1. Arabica typically contains more lipids, trigonelline, and sucrose while robusta contains more chlorogenic acids and caffeine (1). Because the arabica varieties have a more desirable flavor, they are grown more widely (constituting about 80% of the world trade) and command a higher price. The robustas because of their lower cost are used as fillers in roast and ground blends and they add a characteristic earthy phenolic note to dark roasted espresso coffees. Robustas are also used in the production of instant (soluble) coffees because they produce greater solids in commercial percolation equipment.

**Table 1. Green and Roasted Coffee Composition**

<i>Component</i>	Typical average content (% dry basis)			
	<i>Arabica</i>		<i>Robusta</i>	
	<i>Pre-Roast</i>	<i>Post-Roast</i>	<i>Pre-Roast</i>	<i>Post-Roast</i>
Alkaloids (caffeine )	1.2	1.3	2.2	2.4
Trigonelline	1.0	0.4	0.7	0.2
Ash	4.2	4.5	4.4	4.7
<b>Acids</b>				
total chlorogenic	6.5	2.5	10.0	3.8
quinic	0.4	0.8	0.4	1.0
aliphatic	1.0	1.6	1.0	1.6
Sucrose	8	0.0	4	0.0
Reducing sugars	0.1	0.3	0.4	0.3
<b>Polysaccharides</b>				
mannan	22		22	
arabinogalactan	15		18	
cellulose	7		8	
pectins	2.0	2.0	2.0	2.0
Total	46.0	35	50.0	39
Lignin	3.0	3.0	3.0	3.0
<b>Proteinaceous</b>				
protein	11.0	7.5	11.0	7.5
free amino acids	0.5	0.0	0.8	0.0
Lipids	16	17.0	10	11.0
Caramelized/condensation	na	25.5	na	25.5
Total (dry basis)	100.0	100.0	100.0	100.0
Moisture	12.5 %	3-5%	11%	3-5%

adapted from reference 5

## Green Bean Harvesting, Processing & Transport

Two methods of harvesting are used. One is based on selective picking; the other involves shaking the tree and stripping the fruit. After that, the outer portion of the fruit is removed by a wet or dry process.

### Wet Method

The wet method is employed in areas such as Colombia and Central America where water is plentiful and the product is called washed beans. In the wet process the cherries are depulped mechanically, soaked in water and fermented in

large tanks to remove remaining pulp, washed again, and finally dried in the open or in heated, rotating cylinders. These beans are usually more uniform in appearance and of higher flavor quality.

### **Dry Method**

The dry method is generally practiced in areas such as regions of Brazil and Africa where water is scarce during harvesting. In the dry process, the beans are dried either in the sun or in mechanical dryers. When the coffee cherries are dry, they are transferred to hulling machines which remove the skin, pulp, parchment shell, and silver skin in a single operation leaving the desired bean (1).

In either case the final product, called green coffee, is sorted by hand or machine to remove defective beans (especially "black beans") and extraneous material and is graded according to size. The green beans may be transported in bags or loose in containers on ships to the roaster. It is critical that moisture level be held below 12% or mold growth may occur which is accompanied by severe off-flavor development. Storage should be at temperatures below 26°C to prevent flavor deterioration.

## **Coffee Roasting**

Heat is required for the conversion of green coffee to roasted coffee. The heat transfer can be achieved via convective, conductive or radiative mechanisms or combinations thereof depending upon the design of the coffee roaster. Commercial roasters heat coffee beans with hot combustion gases in either horizontal rotating cylinders (drums) that provide a tumbling action to prevent uneven heating or in fluidized bed systems. These can be of batch or continuous design having a capacity of greater than 2000lb/hr. Temperatures for roasting range from about 193°C (about 380°F) for a light roast, through about 205°C (about 400°F) for a medium roast, to about 218°C (about 425°F) for a dark roast. The major variables determining degree of roast are time and temperature; either temperature is kept constant and roasting time varied, or vice versa. Roasting time can range between about 0.75 min to about 25 min although 1.5 min to about 6 min are typical ranges for modern commercial roasting equipment. Short time roasting processes produce greater bean expansion, and this discovery has been applied to "high yield" coffee products due to greater extraction of solids and flavor upon brewing. Fast roasts can also be achieved in older batch roasters by decreasing the initial charge weight of green coffee in the roaster.

Smoke generated during roasting consists of a mixture water, carbon dioxide, volatile organic compounds and fine particulates. These organic compounds are considered pollutants and are usually destroyed in after-burners or catalytic converters located at the exhaust end of commercial roasters. When the desired degree of roast is achieved, the coffee roasting process is ended by "quenching" the

hot beans with either water or a cooling gas such as air. The quenched beans are conveyed to storage bins for equilibration of moisture and temperature as well as for degassing to occur prior to grinding and packing (1).

## Roasting Chemistry Overview

Green coffee has a moisture content of about 10-12%. During the early phase of roasting, while the coffee dries, the temperature remains close to 100°C. When relatively dry, the temperature rises and the reactions change from endothermic to exothermic. At a temperature generally exceeding 190°C, the coffee beans begin to emit crackling or popping sounds and physically expand or puff to about twice their initial volume (3). Non-volatile precursors are converted to water, carbon dioxide and organic flavor and aroma compounds through reactions such as Maillard, chemical dehydration and oxidative reactions. At the desired degree of roast or flavor development, the exothermic reactions are quenched as previously described.

The freshly roasted beans retain a significant amount of gas which is mostly carbon dioxide (up to 2% by wt.) and organic flavor compounds under pressure (3). Some of this gas is released upon standing during the outgassing phase in the storage bins. Additional gas is released during grinding (grinder gas). Grinder gas, which contains many pleasant aromatic compounds, including sulfur compounds, carbonyls, and short chain acids is often captured by cryogenic condensation and trapped in a suitable carrier (e.g. coffee oil) and utilized to aromatize instant coffees (4).

The percent dry mass loss (on a dry basis green coffee) in roasted coffee correlates well with what is termed the “degree of roast” which embodies both the development of roast color and the development of flavor. The degree of roast plays an important role in determining the overall flavor character of brewed coffee (3).

### Color Development Versus Dry Mass Loss

- |             |       |
|-------------|-------|
| • light     | 1-5%  |
| • medium    | 5-8%  |
| • dark      | 8-12% |
| • very dark | >12%  |

### *Measuring Degree of Roast*

In large scale commercial roasting the “degree of roast” is generally monitored by roast color. The color is typically measured by grinding the coffee to a powder. The material is sieved and pressed into a disc and measured by color reflectance meters such as Photovolt, Gardiner, Agtron and Hunterlab (3). In some commercial batch roasters weight loss or final bean temperature is an indicator of degree of roast. In smaller batch roasters, the experienced operator will utilize his eyes, ears and nose to determine when the desired degree of roast is achieved.

## Flavor Management

Preferred flavor profiles are obtained by blending the varieties before or after roasting. Better quality beans may stand alone or several bean types may be blended to achieve a wider array of flavor notes. The flavor character of Colombian and washed Central American coffees is generally described as mild, winy-acid, and aromatic; Brazilian coffees are described as heavy body, moderately acid, and aromatic; and African robusta coffees vary from heavy bodied to neutral, slightly acidic, and slightly aromatic. Better quality coffee blends may contain higher percentages of better quality arabicas such as Colombian, Central American, or Kenyan coffees (1). Coffee roasters will also select the degree of roast based on the optimal flavor they wish to achieve for a given bean variety. Generally, robustas in the United States are roasted to lighter roast colors and arabicas to darker roast colors.

## General Effects of Roasting on Components

Green coffee has only a low green-bean like aroma; the desirable aromas are developed upon roasting. Many complex physical and chemical changes occur during roasting including the obvious change in color from green to brown. In the first stage of roasting, loss of free water occurs. In the second stage complex chemical dehydration, fragmentation, recombination and polymerization reactions occur. Many of these are associated with the Maillard reaction and lead to the formation of lower molecular weight compounds such as carbon dioxide, free water, and those associated with flavor and aroma. In addition, higher molecular weight colored materials, some soluble and some insoluble in water, are generated which are called melanoidins.

Certain of the reactions are exothermic and produce a rapid rise in temperature, accompanied by a sudden expansion or puffing of the beans, with a volume increase of 50-100%. The loss of carbon dioxide, other volatile substances, as well as the water produced by chemical dehydration during the latter stages account for most of the 2-5% dry weight roasting loss. Most of the polymeric carbohydrate, lipid, caffeine and inorganic salts survive the roasting process. Table 1 presents a comparison of the composition of coffee before and after roasting.

The major compositional changes which occur during roasting are:

1. Decrease of protein and amino acids
2. Loss of chlorogenic acid
3. Loss of sucrose
4. Decrease of arabinogalactan and reducing sugars
5. Decrease of trigonelline

## 6. Formation of melanoidins

### 7. Loss of water

The following section will review the chemical changes which occur during the roasting of green coffee. For an expanded discussion on this topic see Spiller (6).

## Proteins and Amino Acids

The protein level of green coffee is between 10 and 13% (dwb). The roasting of coffee denatures and insolubilizes much of the protein and causes a concomitant small measurable loss of protein (5). All arginine is destroyed; substantial amounts of cysteine, lysine, methionine, serine, and threonine are also lost (7). These amino acids are the polar ones which possess free -OH, -SH, and -NH<sub>2</sub> groups and generate distinct and interesting aromas upon reaction with sugars (8).

In addition, there is complete loss of the 0.5% free amino acids upon roasting. These species are extremely important as aroma precursors, both for reaction with carbohydrates to generate flavors, aromas and melanoidins as well as to undergo the Strecker degradation.

## Chlorogenic Acids

The chlorogenic acids are a group of phenolic acids esterified to quinic acid. This class of acidic compounds accounts for up to 10% of the weight of green coffee. One of the most abundant chlorogenic acids is 3-caffeoyl quinic acid which represents 4 to 5% of the total green coffee weight. Other mono- and dicaffeoyl quinic acids have been identified in coffee with substitution at the 3-, 4-, and 5- position of quinic acid. The phenolic acid fraction of coffee is composed of caffeoyl-, p-coumaroyl- and feruloyl- acids. Para coumaric acid is reported to occur in robusta but not in arabica coffee.

The per cent composition of green coffee (dry basis) is reported to be:

	<u>Caffeoylquinic</u>	<u>Dicaffeoylquinic</u>	<u>Feruloylquinic</u>
Arabica	6.0	0.92	0.35
Robusta	7.25	1.83	1.06

adapted from reference 5

All the chlorogenic acids are susceptible to decomposition during roasting; for example, a medium roast destroys about 60% of the chlorogenic acid. The aromatic portion of the ester is converted to various phenolic compounds. Caffeic acid yields catechol, 4-ethyl catechol, 4-vinyl catechol and 3,4-dihydroxy cinnamic aldehyde upon heating. Ferulic acid readily decarboxylates upon heating to produce vinyl guaiacol as well as guaiacol. Vinyl guaiacol is a major component of roasted coffee.

One undesirable characteristic of dark roasted robusta coffees is their tarry, phenolic character. It has been proposed (1) that the higher load of chlorogenic

acids in green coffee is responsible for these notes upon roasting. The polyphenolic compounds generated upon roasting are highly reactive and enter into the production of brown polymeric materials found in roasted coffee.

Quinic acid is also formed from chlorogenic acid breakdown. Further thermal degradation of quinic acid during roasting produces phenol, catechol, hydroquinone, and pyrogallol. Quinic and caffeic acids are believed to be the precursors of the diphenols found in coffee aroma.

Quinic acid contributes to coffee sourness. Heating of quinic acid generates the inner lactone "quinide" which is not sour. As roasting continues, the level of both quinic acid and quinide increase proportionally. Quinide in coffee brew slowly hydrolyzes to quinic acid upon standing at elevated temperature (e.g. 80°C) which helps explain why the sourness of brewed coffees increase upon standing (9).

Various decarboxylation reactions of chlorogenic acids add to the load of carbon dioxide generated during roasting.

## Acids

The acidity of brewed coffee is important to its taste and is associated with the best high grown arabicas. Lowest brew pH is achieved at about 11% (as is) roasting loss while maximum perception of acidity is found at about a 15% (as is) roasting loss. Certain volatile aliphatic acids such as acetic and formic are generated upon the pyrolysis of carbohydrates. Other acids such as citric and malic, which are native to green coffee, decrease upon roasting (10). Phosphoric acid is believed to play an important role in perceived acidity.

## Carbohydrates

About one half of the weight of a green coffee bean is soluble and insoluble carbohydrate. The soluble carbohydrate is mainly sucrose with smaller amounts of reducing sugars: i.e. fructose and glucose. The sucrose content of good quality arabicas, such as Colombians, is typically about 8% which is about twice as high as generally found in robustas.

The insoluble carbohydrate in the coffee bean is mainly polysaccharide and consists of three polymers: mannan, arabinogalactan and cellulose (11).

### *Mannan*

Mannan, a  $\beta$ -1,4 linked linear polymer of mannose (an epimer of glucose), is a low molecular weight polymer of about 3200-6400 daltons. The mannan content of robustas and arabicas is very similar, typically about 22% on a green bean weight basis.

### *Arabinogalactan*

The arabinogalactan, consists of a backbone of  $\beta$ -1,3 linked galactose units with frequent side chains of between 2 and 4 sugars units which are typically arabinose



and galactose. It is a high molecular weight polymer ranging up to 200,000 daltons and often links other cell wall components together. The arabinogalactan level is some what higher in robustas compared to arabicas, typically 16-18% versus 14-16% respectively.

### *Cellulose*

Cellulose, a  $\beta$ -1,4 linked linear polymer of glucose, is also of high molecular weight and occurs in fibrillar form to provide the structural frame for the cell walls. The cellulose content of robustas and arabicas ranges from 7-9% of the bean weight (11).

Almost all of the simple sugars (sucrose, hexoses, pentoses) are lost during roasting, producing water, carbon dioxide, color, aroma and flavor. Some of the arabinose is also lost, probably from the side chains of the arabinogalactan, during roasting. The cellulose and mannan are more stable to roasting .

### **Trigonelline**

Fifty to 80% of this green coffee compound is degraded during coffee roasting. It readily generates pyridine, picolines, nicotinic acid, and the methyl ester of nicotinic acid. In model system studies (12), thermolysis of trigonelline was shown to generate pyridines, pyrroles, and bicyclic compounds. The decarboxylation of trigonelline is another source of carbon dioxide generated during coffee roasting. Arabica coffee contains more trigonelline than does robusta.

### **Melanoidins**

Melanoidins are yellow to dark brown heterogeneous polymeric products that are end products of the roasting process. It is speculated that the melanoidins are condensation products of Maillard reactions, sugar caramelization products, proteins, polymeric carbohydrates and the degradation products of the chlorogenic acids. The literature indicates that the melanoidin level of brewed coffee is 15% (13) and roasted coffee about 23% (10) on a dry weight basis. Both of these values were obtained by difference, since little is known about their true chemical composition or structure. It is reported that melanoidins are negatively charged high molecular polydispersed substances containing both aliphatic and aromatic molecules. Peptides, amino acids, polymeric and monomeric carbohydrates and phenolic compounds have been identified in melanoidins and their similarity to the humic acids has been noted (14). Melanoidins are believed to contribute to the bitterness of brewed coffee (15).

### **Caffeine**

Caffeine is the component which is responsible for the mild stimulatory effect of coffee on the central nervous system. It has been shown to promote feelings of

well being and increased ability to perform mental tasks efficiently. Caffeine survives the roasting process; however some may be lost by sublimation. The level in green robusta coffee is twice that of arabica. It has a bitter threshold of approximately 200ppm (200mg/kg) in water, which is approximately the level of caffeine in brewed coffee. Other purine alkaloids such as theobromine and theophylline are also present in green coffee at low levels and survive the roasting process. Again, these are at higher levels in robusta coffees.

### **Lipids**

Green coffee beans contain between 7 to 17 % lipid material. Green coffee oil is composed of triglycerides (70-80%), free fatty acids (0.5-2.0%), diterpene esters (15-19%), triterpenes and sterols (1.4-3.2%). Coffee wax (~0.25%) is located on the surface of the bean and contains C20 to C26 acids present as amides of 5-hydroxytryptamine (adapted from Viani, ref 10).

The fatty acid profile of coffee lipids is rather similar to that of an edible vegetable oil. Ninety five percent of the saponifiable fraction is composed of linoleic, palmitic, oleic and stearic acids. The thermal breakdown of these fatty acids and triglycerides is similar to that of any vegetable oil and would generate hydrolysis, autoxidation, pyrolysis and polymerization products.

Hydrolysis of the triglycerides yields free fatty acids. Short chain fatty acids contribute a rancid, goaty character while higher fatty acids are soapy. Autoxidation of the higher unsaturated acids (primarily C18) produces unsaturated aldehydes such as the 2,4-alkadienals and 2-enals.

The diterpene esters are primarily cafestol and kahweol palmitate and linoleate. Partial hydrolysis of these esters occurs during roasting; the sterols survive.

### **Coffee Aroma**

Most of the delightful aromatic character of coffee is the result of roasting. However some of the native green bean aroma survives and contributes to the roasted coffee aroma.

### **Green Beans**

Unroasted coffee beans have only a faint green-bean like aroma. The volatiles of green coffee have been studied (16) and approximately 228 compounds have been reported. The classes and number of compounds are presented in Table 2.

The majority of the odor active compounds of green coffee possess green and fatty notes.

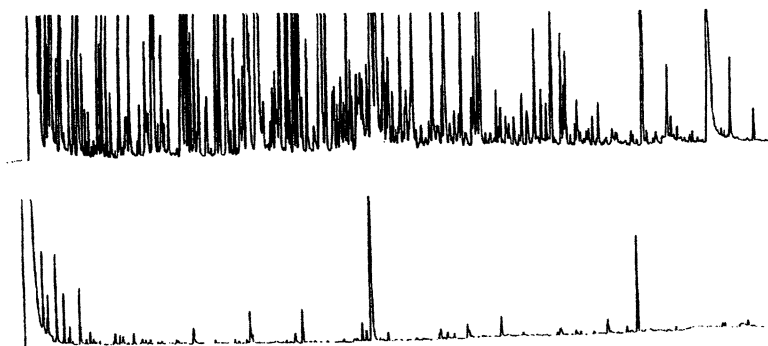
**Table 2. Aromatic Components of Green and Roasted Coffee**

<u>Chemical Class</u>	<u>Green Coffee</u>	<u>Roasted Coffee</u>
Hydrocarbons	41	74
Alcohols	24	20
Aldehydes	32	30
Ketones	21	73
Acids	3	25
Esters	26	31
Lactones	4	3
Phenols (and ethers)	10	48
Furans	17	127
Thiophenes		26
Pyrroles		71
Oxazoles		35
Thiazoles		27
Nitrogen compounds	38	208
Pyridines		19
Pyrazines		86
Amines and misc. nitrogen compounds		32
Sulfur compounds	7	47
Miscellaneous	5	17
<i>Total</i>	<i>228</i>	<i>791</i>

SOURCE: Reproduced from Ref 15, Copyright 1995 American Chemical Society

### Roasted Beans

Upon roasting a myriad of chemical reactions occur, generating more than 800 identified compounds. Figure 1 compares the gas chromatographic patterns of green and roasted coffees. The dramatic increase in aromatic compounds is apparent. The level at which these compounds occur in roast coffee ranges from the ppm ( $1 \times 10^{-6}$ ) to ppt ( $1 \times 10^{-12}$ ). Table 2 lists the numbers and classes of volatile compounds identified in coffee aroma (1). Of particular interest are the heterocyclic compounds which are present since many of these are critical flavor notes. At least 394 heterocyclic compounds have been identified, the majority of which are generated during roasting. Some of the most important compounds to coffee aroma are the sulfur containing furans and the pyrazines.



*Figure 1. GC comparison of roasted (upper) and green (lower) coffee volatiles. (Reproduced from reference 15, Copyright 1995 American Chemical Society.)*

The pioneering research on coffee aroma composition was performed in 1926 by Reichstein and Staudinger (17). They identified 29 components including alkyl pyrazines, alpha-diketones and furfuryl mercaptan that are important to coffee aroma. The effort involved in their research cannot be over emphasized, since it was done before the days of GC/MS and these researchers used classical identification techniques including crystallization of derivatives and distillation. In the ensuing years, various research groups have added to the knowledge base of coffee aroma until today there are about 800 compounds identified.

More recently researchers have emphasized less the identification and more the contribution of the components to the aromas well as the origin of these compounds.

### **Coffee Impact Aroma Compounds**

This very important topic will be covered the following chapter.

### **Origins of Coffee Aroma Compounds**

An excellent summary of the routes of coffee aroma formation of the important compounds upon roasting has been recently published (18); a number of primary routes were described.

#### **1. Compounds present in green coffee before roasting:**

These are the compounds originally present in green coffee and include such important compounds as the alkoxyalkylpyrazines, linalool and some lipid degradation products as well as the biologically derived aldehydes, acids, esters etc.

#### **2. Caramelization products:**

Non-volatile sugars (pentoses, hexoses, disaccharides) are degraded by heat to produce products with caramel, sweet, and burnt type aromas and dark colors. Examples of the aromatics include Furaneol® (2,5-dimethyl-4-hydroxy-3-(2H)-furanone), maltol, cyclotene, other enolone structures, and dicarbonyl compounds such as diacetyl.

### 3. Cyclic enolones:

Two compounds of organoleptic importance are sotolone (4,5-dimethyl-3-hydroxy-2-(5H)-furanone) and abhexone (5-ethyl-4-methyl-3-hydroxy-2(5H)-furanone) (19). These can arise from the reaction of pyruvate and  $\alpha$ -ketobutyric acid; the latter is a degradation product of threonine.

### 4. Lipid degradation products:

Lipid oxidation of unsaturated fatty acids produces hexanal, nonenal, other enals and dienals. 2-Nonenal plays a prominent role in coffee aroma; it possesses a very low threshold of 0.08ppb and has been patented as the woody note of coffee (20).

### 5. Degradation of phenolic acids:

Non-volatile phenolic acids such as ferulic acid are present in relatively high concentrations in green coffee. Thermal degradation produces phenolic compounds such as vinyl guaiacol and guaiacol as discussed in the section on chlorogenic acid.

### 6. Maillard reaction products:

Many of the compounds in coffee originate, directly or indirectly, from the Maillard Reaction which is one of the most important routes to aromas in heated foods. In the Maillard reaction the carbonyl group of a reducing sugar reacts with the amino group of an amino acid. The condensation products are labile and degrade to a number of aromatic and colored products. Vernin (21) has reported that pyridines, pyrazines, oxazoles, thiazoles, pyrroles, and imidazoles arise from this pathway. Substituted furans result from the interaction of sugars and amino acids. Excellent reviews of the Maillard Reaction exist (22-25).

### 7. Strecker degradation of amino acids (via Maillard Reaction described above):

An alpha amino acid is deaminated and decarboxylated in the presence of a dicarbonyl to yield an aldehyde of one less carbon.

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## Chapter 21

# Sensory Studies on the Key Odorants of Roasted Coffee

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Sensory experiments reviewed in the paper indicated that the following odorants are essential for the flavor of roasted coffee: 2-furfurylthiol, acetaldehyde, propanal, methylpropanal, 2- and 3-methylbutanal, 2-ethyl-3,5-dimethylpyrazine, 2-ethenyl-3,5-dimethylpyrazine, 2,3-diethyl-5-methylpyrazine and 4-vinylguaiacol. Model experiments revealed that 2-furfurylthiol, the outstanding odorant in the class of sulfur compounds, was formed during roasting by reactions of cysteine with arabinose. The latter was released from polysaccharides.

In modern flavor analysis the volatiles contributing to the aroma are differentiated from those which do not by Charm analysis (1), aroma extract dilution analysis (AEDA) (2), aroma extract concentration analysis (AECA) (3) as well as gas chromatography/olfactometry of headspace samples (GCOH) (4, 5). As recently reviewed (6) 28 odorants of the more than 800 volatiles have been identified in roasted coffee by AEDA, AECA and GCOH. In these experiments the volatiles are separated by gas chromatography (GC) and then the odor impact is evaluated. Interactions of the odorants, which in most cases are characterized by inhibition and suppression (7), are abolished. Therefore, the question of which compound among the potent odorants actually contributed to the flavor remains open.

To answer this question a synthetic blend of the odorants (aroma model) duplicating the odor profile of roasted coffee was prepared on the basis of quantitative data. Changes in the overall flavor of the aroma model were evaluated by a sensory panel after omission of one or more odorants. The present paper summarizes the results of this study and gives an insight into the formation of a compound which was evaluated by the sensory experiments as key odorant.

## Key Odorants of Ground Roasted Coffee

The concentrations of 28 odorants screened in previous studies (6) were quantified by stable isotope dilution assays (8, 9) in medium roasted Arabica coffee blends originating from Colombia (Col), Brazil, El Salvador and Kenya (9). The data obtained varied at the most by a factor of three which was found for 2,3-butanedione and 3-mercapto-3-methylbutylformate (9).

The differences in the concentrations of potent odorants were most likely the cause for the different flavor profiles of the provenances. Nevertheless, there was no doubt that each of the four provenances smelled like roasted coffee suggesting that each of them contained the odorants causing this unique flavor in sufficient concentrations. We selected a sample of the provenance Col for an evaluation of these odorants.

To imitate the flavor of Col a suitable base for the preparation of the aroma model was chosen. For this purpose sensory experiments with different materials were carried out (10) using the odorants in the concentrations found in Col. Only 2-ethenyl-3-ethyl-5-methylpyrazine was replaced in the models by a corresponding higher amount of 2-ethyl-3,5-dimethylpyrazine (8). This change was permissible as the two pyrazines agree in the odor quality and odor threshold (11). The experiments indicated that the flavor profile of the model containing the odorants dissolved in a mixture of sunflower oil and water (1:20, v/v) was closest to the original coffee aroma (10). In particular, the very characteristic roasty/sulfurous note of coffee was as intense as in the real coffee sample (Table I).

In the aroma model for Col, one or more components were omitted and then 10 assessors compared each reduced model with two complete models in triangle tests which were repeated (10). The results (Table II) indicate that 2-furfurylthiol (23) was the most important odorant of ground roasted coffee. Its absence in experiment (exp.) 15 changed so clearly the odor of the model that 15 of 20 answers were correct. The assessors reported that, in particular, the intensity of the sulfurous/roasty note was lowered. In the experiments in which one odorant was omitted in addition to 23 only the flavor change caused by the lack of vinylguaiacol (19) was recognized by a significant but smaller number of assessors than in the exp. with 23. For  $\beta$ -damascenone (16), guaiacol (17), 2-methyl-3-furanthiol (21), 3-mercapto-3-methylbutylformate (25) and 3-methyl-2-butenthio (26) the number of panellists who were able to find the aroma model in which these odorants were lacking were too small to reach the confidence limit for significance (exp. 9, 12, 16, 17 and 18 in Table II). The other experiments listed in Table II indicated that the absence of some groups of odorants impaired significantly the overall flavor of the aroma model for ground roasted Col, e.g. acetaldehyde (1), propanal (2), methylpropanal (3), 2- and 3-methylbutanal (4, 5) in exp. 1 or the furanones in exp. 8.



**Table I. Flavor Profile of the Coffee Sample Col and the Corresponding Aroma Model Col M**

Attribute	Col	Col M <sup>a</sup>
	Intensity <sup>b</sup>	
Sweetish/caramel-like	1.0 (0.0)	1.4 (0.9)
Earthy	1.6 (0.5)	1.3 (0.5)
Roasty/sulfurous	2.3 (0.5)	2.1 (0.2)
Smoky	1.7 (0.5)	1.4 (0.9)
Similarity <sup>c</sup>		2.3 (0.3)

<sup>a</sup> The odorants were dissolved in sunflower oil-water (1:20, v/v).

<sup>b</sup> The intensity of the attributes was scored on a scale of 0 (absent) to 3 (strong). The results obtained by 10 panellists were averaged; the standard deviations are given in parentheses.

<sup>c</sup> Similarity rating scale: 0 (no similarity) to 3 (identical with the coffee sample).

Source: Reproduced from (10).

Exp. 4 and 5 indicate that the overall flavor of the model was significantly changed by the absence of the alkyl pyrazines **8**, **9** and **10**. Methoxy pyrazine **11** which in contrast to exp. 4 was present in exp. 5 did not affect the flavor of the model. This result is of interest as pyrazine **11** was the most odor-active constituent of raw coffee (Table III) and was stable during roasting (unpublished results). Therefore, we assume that roasting of the raw material does not only produce the pleasant aroma which is characteristic for coffee but, in addition, generates odorants which masks the peasy odor note caused by pyrazine **11**. Only, when the concentrations of **11** and other methoxy pyrazines are too high in the raw beans, then a peasy off-flavour might break through in the roasted coffee as observed by Becker et al. (12).

Table II indicates that the sulfur compounds **21**, **22** and **24-27** did not belong to the key odorants of Col as the flavor difference caused by their absence in exp. 14 was not recognized by a significant number of assessors. However, one or the other sulfur compound might exceed the confidence limit for significance in those provenances in which it occurs at higher concentrations than in Col. Candidates might be 2-methyl-3-furanthiol (**21**), 3-mercapto-3-methylbutylformate (**25**) and 3-methyl-2-butenthio (**26**) which were 52 %, 102 % and 110 %, respectively, higher in Arabica blends from Brazil (**21**), Kenya (**25**) and El Salvador (**26**) than in Col (9).

### Formation of 2-Furfurylthiol

The important role established here for 2-furfurylthiol (**23**) confirmed the assumption of Reichstein and Staudinger (13) that **23** is a key component of the flavor of ground roasted coffee. Therefore, we were interested to identify its precursor in raw coffee and to get an insight into its formation.

**Table II. Flavor of the Model for Col as Affected by the Absence of Compounds**

<i>Exp. no.</i>	<i>compound(s) omitted</i>	<i>Number<sup>a</sup></i>
1	Acetaldehyde (1), propanal (2), methylpropanal (3), 2- and 3-methylbutanal (4, 5)	17*
2	Acetaldehyde (1), propanal (2)	12*
3	2,3-Butanedione (6), 2,3-pentanedione (7)	6
4	2-Ethyl-3,5-dimethylpyrazine (8), 2-ethenyl-3,5-dimethylpyrazine (9), 2,3-diethyl-5-methylpyrazine (10), 3-isobutyl-2-methoxypyrazine (11)	13*
5	Pyrazines 8, 9 and 10 as in exp. 4	12*
6	4-Hydroxy-2,5-dimethyl-3(2H)-furanone (12), 2-ethyl-4-hydroxy-5-methyl-3(2H)-furanone (13)	8
7	3-Hydroxy-4,5-dimethyl-2(5H)-furanone (14), 5-ethyl-3-hydroxy-4-methyl-2(5H)-furanone (15)	9
8	Furanones 12, 13, 14 and 15	11*
9	$\beta$ -Damascenone (16)	6
10	Diones 6, 7, furanones 12 to 15, $\beta$ -damascenone (16)	17*
11	Guaiacol (17), 4-ethylguaiacol (18), 4-vinylguaiacol (19), vanillin (20)	13*
12	Guaiacol (17)	10
13	4-Vinylguaiacol (19)	11*
14	2-Methyl-3-furanthiol (21), dimethyl trisulfide (22), methional (24), 3-mercapto-3-methylbutylformate (25), 3-methyl-2-buten-1-thiol (26), methanethiol (27)	10
15	2-Furfurylthiol (23)	15
16	2-Methyl-3-furanthiol (21)	10
17	3-Mercapto-3-methylbutylformate (25)	10
18	3-Methyl-2-buten-1-thiol (26)	10

<sup>a</sup> In triangle tests the number of assessors who found out by sniffing the sample what was different in the overall flavor. The number of correct answers (maximum 20) was summed up, and the significance of the result ( $p < 0.05$ ) was evaluated.

\* Significant result.

Source: Reproduced from (10).

**Table III. Most Odor-Active Volatiles of Raw Coffee**

<i>Compound</i>	<i>Concentration (<math>\mu\text{g}/\text{kg}</math>)</i>	<i>OAV<sup>a</sup></i>
3-Isobutyl-2-methoxypyrazine (11) <sup>b</sup>	97	490
2-Methoxy-3,5-dimethylpyrazine	0.5	83
Ethyl 3-methylbutyrate	22	37
3-Isopropyl-2-methoxypyrazine	2.3	23

<sup>a</sup> OAV, odor activity value: Ratio of the concentration to the odor threshold determined in cellulose.

<sup>b</sup> Number refers to Table II. Source: Unpublished results.

**Table IV. Formation of 2-Furfurylthiol During Roasting of Ground Raw Arabica Coffee<sup>a</sup>**

No.	Roast conditions	2-Furfurylthiol ( $\mu\text{g}/\text{kg}$ )
1	Air	120
2	Argon (0.1 MPa)	870
3	Argon (1.9 MPa)	820
4	Addition of cysteine (100 mg)/argon (0.1 MPa)	9220

<sup>a</sup> The material (5 g) was heated in an autoclave for 2 h at 200°C.

Source: Unpublished results.

A comparison of exp. 1 and 2 in Table IV indicates that the amount of **23** increased 7 fold when air was replaced by argon in the autoclave used for the roast process. An increase of the pressure by a factor of 19 did not affect the production of **23** in exp. 3. In the following experiments, due to these results, roasting was performed after replacement of air by 0.1 MPa argon. Shortage of the sulfur source had limited the production of **23** in exp. 2 and 3. Addition of cysteine in exp. 4 enhanced the amount of **23** strongly.

The reaction of furfural, a well-known dehydration product of pentoses (**14**), with hydrogen sulfide yielded **23** (**15**). In raw coffee small amounts of free ribose and a larger pool of polymeric arabinose as well as cysteine were proposed as precursors of **23** on the basis of model experiments (**16**, **17**). Increasing amounts of **23** were formed when ribose, 3-deoxypentose and furfural were individually heated in the presence of hydrogen sulfide (**18**). These results led to a hypothetical reaction route which explains the generation of **23**. In aqueous media **23** resulted in a smaller yield also from the breakdown of hexoses in the presence of cysteine (**18**). However, under dry-heating conditions, e.g. in the roast process, the pentose ribose compared to glucose was the most effective precursor of **23** (**18**).

Raw coffee was fractionated and after hydrolysis the monosaccharide composition of each fraction was gas chromatographically determined to evaluate the monosaccharides which may act as precursors of **23**. In agreement with the literature (**19**) arabinose was the only pentose of which considerable amounts occur in raw coffee (Table V). Galactose, mannose and glucose, of which the latter most likely originated from saccharose, were found in the hexose fraction given as sum in Table V. In addition, the concentration of free cysteine was determined in some fractions. A comparison of exp. 1 and 2 in Table V shows that the yield of **23** was lowered when the raw material was defatted before roasting. The defatted raw coffee was then separated into the water-soluble and the insoluble fraction. Roasting of the former fraction (exp. 3) yielded 7 times more **23** than the latter one (exp. 4). The main reason for this decrease in the generation of **23** in expt. 4 might be the separation of cysteine, which was completely extracted with water from the major portion of arabinose which remained in the water-insoluble fraction (Table V). This suggestion was confirmed in exp. 5, as the amount of **23** increased strongly when the water-insoluble fraction was roasted in the presence of added cysteine.

**Table V. Formation of 2-Furfurylthiol (FFT) During Roasting of Raw Coffee and Its Fractions**

No.	Sample/fraction (yield)	Amount (g/kg)			FFT <sup>c</sup> ( $\mu\text{g/kg}$ )
		Cys <sup>a</sup>	Arab <sup>b</sup>	Hex <sup>b</sup>	
1	Ground raw coffee (100 %)	n.d.	n.d.	n.d.	689
2	No. 1, defatted (88 %)	n.d.	40	319	477
3	Water-solubles of no. 2 (29 %)	0.6	11	174	81
4	Water-insolubles of no. 2 (59 %)	<0.01	61	401	12
5	Water-insolubles of no. 2 plus cysteine <sup>d</sup>				581
6	Permeate ( $M < 10^3$ ) of no. 3 (21 %)	0.41	<2	169	0
7	Retentate ( $M > 10^3$ ) of no. 3 (8 %)	0.46	25	138	121

<sup>a</sup> Cysteine (Cys) was quantified after derivatization with vinylpyridine.

<sup>b</sup> Arabinose (Arab) and hexoses (Hex, sum of galactose, glucose and mannose) after hydrolysis.

<sup>c</sup> FFT formed during roasting in an autoclave at 200°C for 1 h.

<sup>d</sup> Cysteine (0.1 g) was mixed with the freeze-dried water-insolubles (5 g) and then roasted.

n.d., not determined.

Source: Unpublished results.

The importance of arabinose as precursor of **23** was shown after separation of the water-soluble fraction by ultrafiltration since roasting of the permeate (exp. 6), in contrast to the retentate (exp. 7), yielded no thiol **23** due to the lack of arabinose. The finding that only the pentose, but not the hexoses occurring in raw coffee, is active as precursor of **23** is in agreement with results of model experiments (18) discussed above.

The major portion of arabinose occurring in raw coffee has been identified as building block of polysaccharides (20). To study bound arabinose as precursor of **23**, an arabinogalactan was isolated from raw coffee (21) and then roasted in the presence of an excess of cysteine. Exp. 1 in Table VI indicates bound arabinose as precursor of **23**. However, the yield of **23** increased, when a mixture of monosaccharides which was equal to the composition of the arabinogalactan was roasted (exp. 2) and **23** peaked when only arabinose was treated (exp. 3).

Polysaccharides from the larch tree and from oat spelts were roasted in exp. 4 and 5. The amounts of these materials were adjusted to the arabinose content of the arabinogalactan from raw coffee in exp. 1. The arabinogalactan from larch tree, containing only arabinose as pentose, produced nearly as much **23** in exp. 4 as the arabinogalactan isolated from coffee (exp. 1). This indicated that the higher peptide content of the latter, which was reflected by the higher N-content (Table VI), had not affected the formation of **23** in exp. 1. The xylan from oat spelts provided only double as much of **23** (exp. 5) as the arabinogalactans, although it contained xylose in addition to arabinose in a molar ratio of 10 to 1. This result is corroborated by experiments indicating that xylose was less active as precursor of **23** than arabinose (data not shown).

**Table VI. Formation of 2-Furfurylthiol During Roasting of Purified Polysaccharides, Monosaccharides and Cysteine**

No.	Polysaccharide (source)/ monosaccharide	Composition (mmol) <sup>a</sup>				2-Furfurylthiol <sup>b</sup> (µg)	
		Arabinose	Xylose	Hexoses	N-Content	I	II
1	Arabinogalactan (coffee)	0.49	n.d.	1.36	1.02	0.37	9.6
2	Mixture of monosaccharides <sup>c</sup>	0.49	--	1.36	--	n.d.	26.8
3	Arabinose	0.49	--	--	--	n.d.	48.5
4	Arabinogalactan (larch tree)	0.49	n.d.	1.96	0.02	0.01	8.9
5	Xylan (oat spelts)	0.49	4.90	0.07	0.05	0.08	18.7

<sup>a</sup> Composition of the model system.

<sup>b</sup> Roasting (200°C, 60 min) was performed without (I) and with (II) an excess of cysteine.

n.d.: not determined.

Source: Unpublished results.

## Conclusions

The results indicate that the characteristic odor notes in the flavor profile of medium-roasted Arabica coffee can be imitated by mixing the potent odorants identified previously by a combination of sensorial with instrumental analytical methods. 2-Furfurylthiol, 4-vinylguaiacol, several alkyl pyrazines, furanones, acetaldehyde, propanal and the „malty“ smelling Strecker aldehydes mainly cause the unique flavor of roasted coffee and, in addition, mask the peasy odor of 3-isobutyl-2-methoxypyrazine which originates from raw coffee. 2-Furfurylthiol is undoubtedly confirmed as the outstanding odorant in the class of sulfur compounds. During roasting it is formed by reactions of cysteine with arabinose which is released from the polysaccharides occurring in raw coffee.

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## Chapter 22

# Formation of Sulfur-Containing Volatiles under Coffee Roasting Conditions

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Experiments support a new hypothesis for the formation of key aroma components in roasted coffee. Furfuryl mercaptan and related compounds are readily formed by reactions of Maillard-derived furfuryl alcohols and amides of S-containing amino acids at acid pH. Model reactions suggested that soluble coffee proteins may be the precursors of flavor significant sulfur volatiles found in roasted coffee.

### Introduction

Volatile sulfur compounds, especially furan derivatives like 2-furfurylthiol, i.e., furfuryl mercaptan (**3a**) have long been associated with coffee flavor chemistry. Furfuryl mercaptan is unique among the sulfur volatiles of roasted coffee since its vapor alone, in high dilution, can convey the unmistakable pleasant scent of roasted coffee. In the first detailed, quantitative analysis of roasted coffee aroma, Silwar *et al.* separated the steam volatile components into six fractions by partition chromatography on silica gel. Of these, a single fraction exhibited "typical, pleasant roast coffee aroma" and was shown to contain eleven (2-furyl)methanethio derivatives including **3a** in addition to six 3-furylthio compounds (1). More recently the organoleptic significance of **3a** and 2-methyl-3-furanthiol in coffee products was confirmed by GC-olfactometry techniques (2). The purpose of our research was to gain a better understanding into the formation of **3a** and related (2-furyl)methanethio compounds during coffee roasting.

### Aroma Formation Hypothesis

Extensive model studies have shown that **3a** and similar compounds are readily formed by heating cysteine or methionine with sugars and/or furfural and its homologs (3) and it has been suggested that reactions of this kind are important for

coffee aroma formation. Model system studies generally indicate Strecker degradations of sulfur-containing amino acids and subsequent reactions of hydrogen sulfide or methanethiol with sugar-derived intermediates such as furfurals yield furfuryl mercaptan. However, the existing model system studies are open to some criticism. Current model systems do not produce true coffee aroma nor do they explain why similar foodstuffs, e.g., sesame seeds which contain flavor precursors similar to those found in coffee do not smell like coffee upon roasting (4). Also, most current model systems require cysteine as a reactant and there seems to be some question as to whether free cysteine actually exists in unroasted coffee beans (5). In view of these questions we began to consider non-Strecker flavor-forming reactions. We and others (6) propose an alternative model for coffee aroma formation in which soluble raw bean protein serves as the limiting source of sulfur for the formation of key aroma compounds. Bean protein provides constant, biogenetically determined numbers and locations of sulfhydryl and methylthio residues which conceivably can offer more control over aroma formation compared with reactions of free amino acids. Moreover, reducing sugars in coffee glycoprotein may be uniquely positioned to interact with sulfur-containing amino acid residues during roasting. We hypothesize that reactive intermediates formed during caramelization (or Maillard reaction) of ambient sugars, i.e., glucose, fructose or rhamnose will interact with pendant sulfur functional groups on bean proteins to produce coffee aroma.

## Experimental Results and Discussion

### General Procedures

To test the new aroma formation hypothesis we allowed furfuryl alcohol (**1a**) or 5-methylfurfuryl alcohol (**1b**) to react with various sulfur-containing amino acid derivatives in pH 4.0 aqueous acetate buffer solution at 100°C to simulate protein reactions in the initial stage of coffee bean roasting. Following the reactions, the mixtures were made basic by adding sodium hydroxide solution and subjected to atmospheric steam distillation extraction (SDE) with water/methylene chloride to isolate volatile products. Details have been reported elsewhere (7). After isolation, the SDE-volatile products were identified and quantified by sniffport GC and by GC/mass spectrometry techniques. Yields of individual volatile products are reported as GC area percentages of total volatiles area minus solvent peaks (% TV), Table I. Compounds **1a** and **1b** were selected as reactants because they both are prevalent in roasted coffee and because of their predicted reactivity in acidic media (8). We suspect that **1a** is being formed during roasting by autoxidation/decarboxylation of the prevalent hexose degradation product, 5-hydroxymethylfurfural. Loss of the aldehyde carbon as carbon dioxide is also



Table I. Formation of Sulfur-containing Volatiles

<i>Amino acid or Derivative</i>	<i>Alcohol</i>	<i>Reaction Time (h)</i>	<i>Sulfur Volatile</i>	<i>% TV</i>	<i>Structure Proof</i>
Cysteine	1a	2.0	3a	t	a
S-Methylcysteine	1a	16.0	3b	t	c
Homocysteine	1a	2.0	3a	ND	
same @ 0.5 M	1a	21.5	3a	t	c
Methionine	1a	2.5	3b	0.05	a
Ethionine	1a	3.0	3c	6.5	a
N-Acetylcysteine	1a	2.0	3a	7.9	a
same	1b	3.0	3d	11	b
same	4	2.0	6a	30	b
N-Acetylmethionine	1a	2.5	3b	1.4	a
same	1b	2.5	3e	0.03	b
same	4	2.0	6b	0.92	b
Glutathione	1a	2.0	3a	2.7	c
same	1a	15.3	3a	18	a
Glycylmethionine	1a	5.0	3b	ND	

NOTES: Equimolar amounts of reactants initially at 0.25 M in 0.1 M pH 4.0 acetate buffer refluxed under nitrogen atmosphere for (h) hours; TV = area % of total FID volatiles; t=trace, ND=none detected; structure proof: (a) GC/MS comparison with authentic substance, (b) MS compares well with literature spectrum (no standard available), [c] retention time and sniffport aroma only.

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consistent with previously reported C-13 labelling experiments (9) in which unlabelled **1a** was obtained as a product in Maillard reactions of aldehyde-labelled glucose. Reactions of amino acids and derivatives are shown in Figure 1.

### Reactions of Free Amino Acids

At 100°C, **1a** and cysteine generated only traces of **3a** whose odor dominated the smell of the reaction mixture. Significantly, cysteine plus furfural produced even less **3a** (detectable only by sniffport/GC) and a reaction odor dominated by hydrogen sulfide. Reactions of homocysteine and **1a** led only to traces of **3a** at extended reaction times. For free amino acids, the low yields of **3a** may have due to poor nucleophilicity of the sulfur atom at pH 4 where cysteine and homocysteine exist mainly in their zwitterionic and monoprotonated forms. S-alkyl amino acids were more effective for producing S-containing furan derivatives. Methionine plus **1a** produced methyl furfuryl sulfide (**3b**) at 0.046% TV, a significant enhancement in S-volatile formation compared to unmethylated homocysteine. Similarly, ethionine plus **1a** generated ethyl furfuryl sulfide (**3c**) as 6.5% TV.

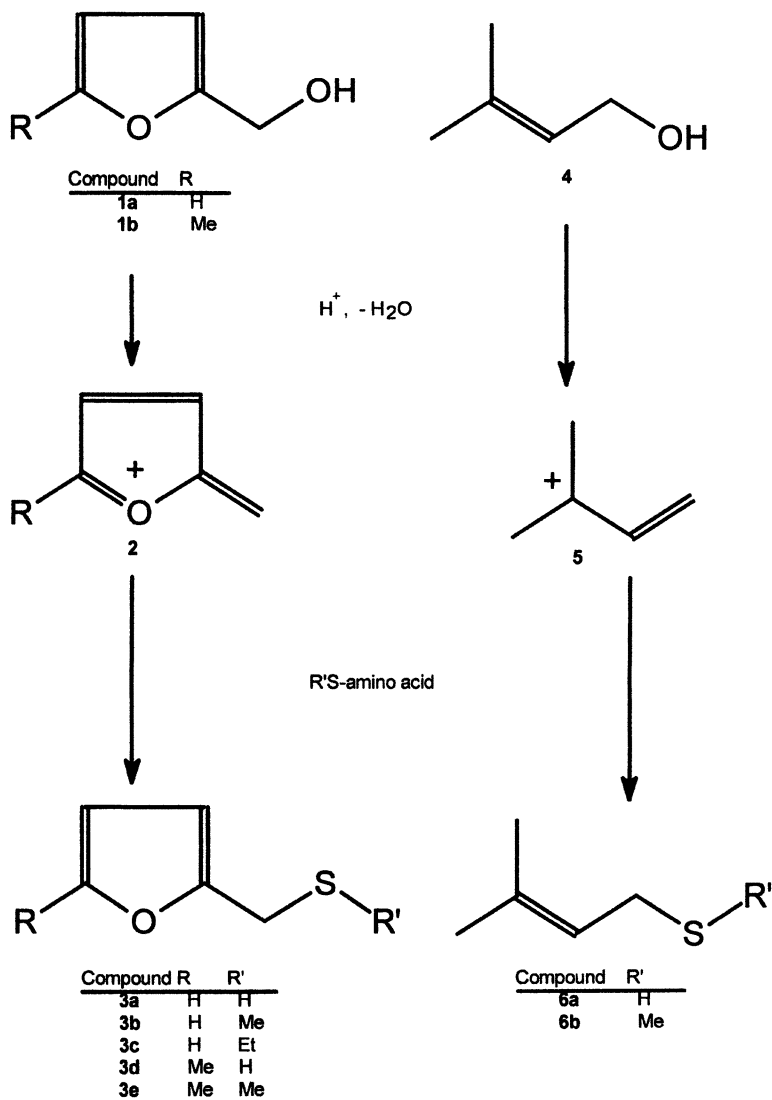


Figure 1. Formation of Sulfur-containing Volatiles

## Reactions of N-Acetyl Amino Acids and Peptides

Reactions of N-acetyl amino acids were investigated as simple models to simulate peptide/protein reactions. It was predicted that N-acetyl amino acids should be more prone to electrophilic alkylation at sulfur since as amides they are uncharged molecules in pH 4 solution. In fact, N-acetylcysteine (AcCys) and **1a** produced more **3a** (7.9% TV) compared to free cysteine. Also, a similar reaction with 5-methylfurfuryl alcohol (**1b**) afforded 5-methylfurfuryl mercaptan (**3d**) as 11% TV. In addition, N-acetylmethionine (AcMet) with **1a** and **1b** produced **3b** (1.4% TV) and 5-methylfurfuryl methyl sulfide (**3e**) (0.03% TV) respectively. As expected, the yield of **3b** was higher with AcMet when compared with free methionine, but still strangely low compared with the AcCys results. At this stage more sophisticated experiments are needed to evaluate the meaning of reaction yields in terms of steric and electronic effects and/or complications due to unspecified side reactions. The cysteine tripeptide glutathione reacted with **1a** to produce more **3a** (2.7% TV) than free cysteine suggesting that protein-bound cysteine could function as a direct precursor of **3a** during food processing. The methionine dipeptide, glycyl-methionine, failed to generate **3b** from **1a** apparently due to its possessing a pK<sub>1</sub> greater than the 2.28 reported for free methionine. A higher pK<sub>1</sub> will lead to more net positive charge on the peptide at pH 4 and therefore reduced reactivity (nucleophilicity) at sulfur.

## Reactions With 3-Methyl-2-butene-1-ol (**4**)

The above results with furfuryl alcohols suggested that other unsaturated alcohols present in coffee might also function as precursors of important sulfur volatiles. In particular, 3-methyl-2-butene-1-ol (prenyl alcohol) (**4**) was recently described as a reasonable precursor of 3-methyl-2-butene-1-thiol (prenyl mercaptan) (**6a**) (10). In our system, **4** reacted with AcCys to form **6a**, a compound known to exist in roasted coffee volatiles, in surprisingly high relative yield (30% TV). The reason for such a high yield is best explained by less by-product formation compared with furfuryl alcohol reactions. Compound **4** also reacted with AcMet to generate the homolog, methyl prenyl sulfide (**6b**, 0.92% TV). Methyl prenyl sulfide has not yet been reported in coffee, but it has been observed as a component of hop oil volatiles (11).

## Reaction Mechanism

Formation of sulfur-containing volatiles is explained by an acid-catalyzed

alkylation of amino acid sulfur atoms, followed by hydrolysis (7), Figure 1. Products formed during acid-catalyzed polymerization of furfuryl alcohol have been rationalized by invoking furfuryl cations similar to **2** (8). In a similar fashion, unsaturated alcohols like **4** can, via dehydration, proceed via cationic intermediates with structure **5**. In general, unsaturated alcohols may form electrophilic species in acidic solution which in turn can react with ambient nucleophilic sites, i.e., amino acid sulfur atoms. Initial products of sulfur alkylation will be sulfonium salts which finally may undergo hydrolysis to form the observed sulfur volatiles **3** and **6**.

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## Chapter 23

# Characterization of the Roasting Temperature and Time-Dependent Physicochemical and Sensory Evaluation of Various Coffee Beans

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Roasting temperature and time-dependent changes in bean density (puffing), color, moisture and soluble solids content (percolation) of Colombian, Brazil and Madagascar coffee beans were studied under normal and quick roasting conditions. The greater bean expansion effects were observed the shorter the roasting time, and the shorter roasting time resulted from higher heating and starting temperature. Roast color ranges are 37.0-40.0, 42.5-45.0 and 46.0-49.0 in Colombian, Brazil and Madagascar, respectively. From the PCA test analysis, better flavor and better mouth-feel were obtained with shorter roasting time in all three coffee beans. Overall quality of roast beans improved with higher temperature and shorter roast time in terms of aromatic impact, cup strength, intensity of taste, and quality of extract.

Roasting time and temperature of green coffee beans determines color, flavor and taste. Coffee beans may be roasted by normal or quick roasting processes. It takes 13-15 minutes for normal roasting and 2-3 minutes for quick roasting. Quick roasting method has the advantage of short duration of roasting. Roasting methods can be classified into two procedures. One of these is Shop Roasting (use Shop roaster), which is a hot air impingement method, and the other one is the Probat Roasting, which is an indirect heating method.

### Purpose

This study is aimed to determine difference between normal and quick roasting when varying roasting temperature and to determine the physicochemical and sensory properties of normal and quick roasted coffees. In addition, this study compares qualities of coffee beans by Shop Roasting and Probat Roasting. Results of this study

will provide information on the overall equipment and manufacturing process for roasting beans to produce optimum product.

### Materials and Preparation

Colombian, Brazil and Madagascar green coffee beans were used as samples in this study (1-3). For the sample preparation, Shop Roasting and Probat Roasting were used for each sample of green coffee bean. For example Shop Roasting of Colombian beans had 4 roasting times. That is, process A takes 1-2 minutes, process B takes 2-3 minutes, process C takes 3-7 minutes and process D takes 8-14 minutes. The preparation of samples using Probat Roasting method was carried out manually according to the degree of roast desired and took 12-16 minutes. The heating parameters are shown in Table 1.

**Table I. Treatment of Green Coffee Bean.**

<i>Type of Roasting</i>	<i>Kind of coffee bean</i>		<i>Heating Temp. (°C)</i>	<i>Starting Temp. (°C)</i>	<i>Final Temp. (°C)</i>	
Shop Roasting	Colombia	A	300	270	250~244	
		B	285	245	246~240	
		C	260	225	232~226	
		D	245	220	216~215	
	Brazil	A		300	270	250~244
		B		285	245	246~240
		C		260	225	232~226
		D		245	220	215
	Madagascar	A		300	270	252~246
		B		285	245	250~244
		C		260	225	236~230
		D		245	220	216
Probat Roasting	Colombia	#1				
	Brazil	#2				
	Madagascar	#3				

### Analytical Measurements

Analytical measurements consisted of roasting time, roasted bean density, roast color, roast moisture and soluble solids. Flavor components in each sample using

peak area of gas chromatogram measured by internal standard was determined (4-8). For the sensory evaluation, the strength and quality of each sample by well-trained panels was measured and preference for flavor and taste was examined (9-13).

## Results

Table II shows roasting time for each green bean. With each of the green coffee beans: Colombian, Brazil, and Madagascar, the higher the heating temperature and start temperature, the shorter the roasting time.

**Table II. Roasting Time for Each Green Coffee Bean**

<i>Types</i>	<i>Final Temp. (°C)</i>	<i>Colombia (min)</i>	<i>Brazil (min)</i>	<i>Madagascar (min)</i>
A	250	1.55	1.52	1.58
	248	1.53	1.50	1.54
	246	1.48	1.47	1.51
	244	1.44	1.43	1.45
B	246	2.56	2.48	2.54
	244	2.34	2.38	2.36
	242	2.26	2.22	2.26
	240	2.21	2.15	2.20
C	232	6.52	6.39	6.04
	230	4.44	5.40	5.00
	228	4.11	4.30	4.21
	226	3.49	3.46	4.59
D	216	12.30	14.30	14.00
	216	11.10	12.30	12.00
	215	9.40	10.30	10.30
	215	8.12	8.30	
Probat Roasting	#1	14.20	14.10	15.30
	#2	13.48	13.24	15.05
	#3	13.25	12.44	14.50

Figure 1 shows the results of roast bean density, namely expansion of each green coffee bean. The shorter the roasting time, the higher the expansion and the greater the expansion, the higher the soluble solids yield.

Figures 2 and 3 show the results of the roast bean moisture and roast color of each green coffee bean, respectively. Figure 4 shows the results of soluble solid of each green coffee bean. For example, maximum value of Shop Roasting is 1.580 and maximum value of Probat Roasting is 1.179. Thus there is a significant difference between these roasting processes.

Figure 5 shows the summary of the results of the physical analysis. This figure is summarized in terms of similar roast color for several conditions and compares the roasting bean density, roasting bean moisture and soluble solid of the samples. A, B, C and D are the samples used with Shop Roasting which is method of hot air impingement and E is Probat roasting which is indirect heating.

Table III shows the Principle Component Analysis (PCA) of Colombia beans. With Colombian, floral, sharp and sour odors were detected to be stronger, but tarry odor was detected to be one step lighter than Brazil and Madagascar beans.

**Table III. The PCA Results of Colombia Beans**

	<i>Probat Roasting</i>	<i>Quick Roasting</i>			
		<i>A</i>	<i>B</i>	<i>C</i>	<i>D</i>
Degree of Roast	R-SL-C	R-SL-C	R-SL-C	R-SL-C	R-SL-C
Aromatic Impact	LOW~MOD	2 steps strong	2 steps strong	1 step strong	same*
Aromatic Character					
Floral	1	1~2	1~2	1~2	1
Sharp	X~1	1~2	1~2	1	X~1
Tarry	1	X~1	X~1	X~1	X~1
Cup Strength	LT~MED	2 steps strong	2 steps strong	1 step strong	same*
Basic Taste Intensity					
Sour	1~2	1~2	1~2	1~2	1~2
Bitter	1~2	1~2	1~2	1~2	1~2
Astringent	1	1~2	1~2	1	1

\* : same as Brazil and Madagascar beans.

Table IV shows the PCA results of Brazil coffee. Earthy odor was detected to be one step stronger and tarry odor was detected to be one step lighter. Table V shows PCA results of Madagascar. With Madagascar, cereal odor was detected to be one step stronger and tarry odor was detected to be one step lighter. It was observed that



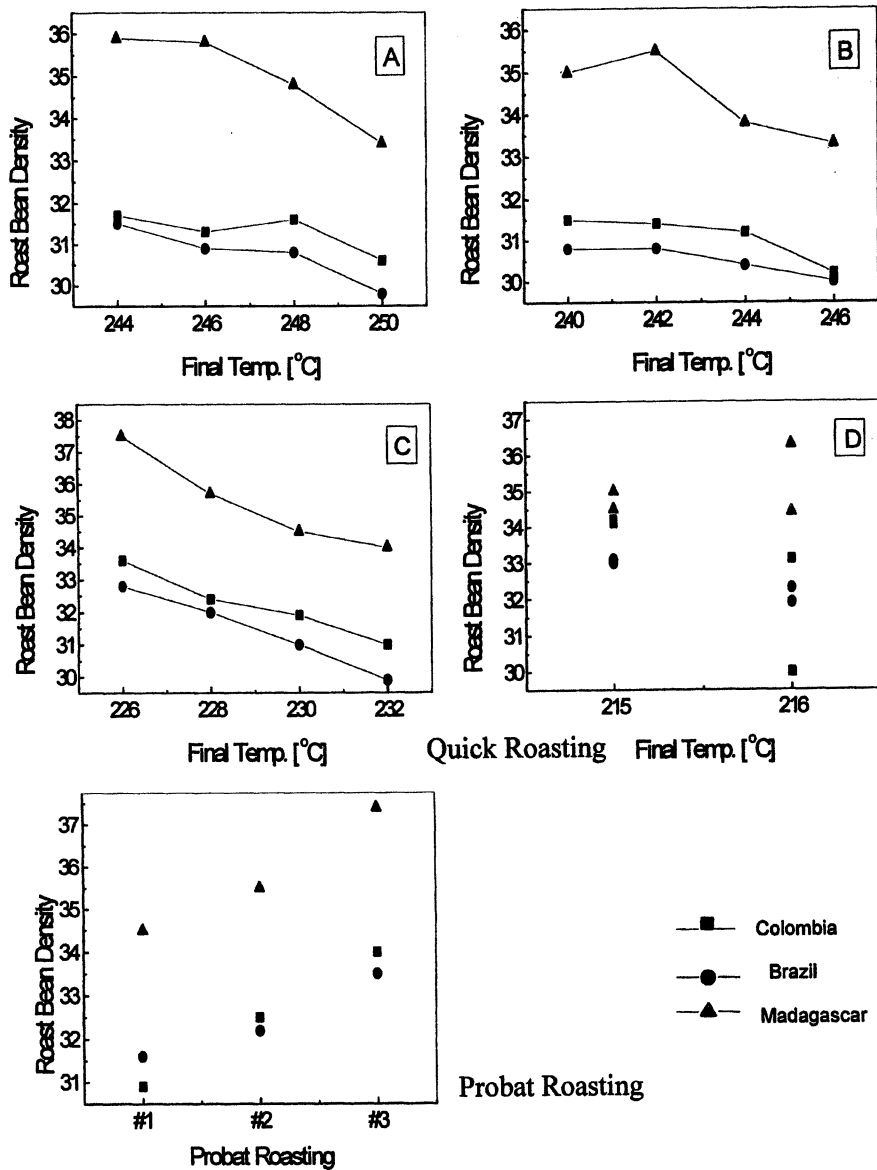


Figure 1. Roast bean density of each coffee bean sample.

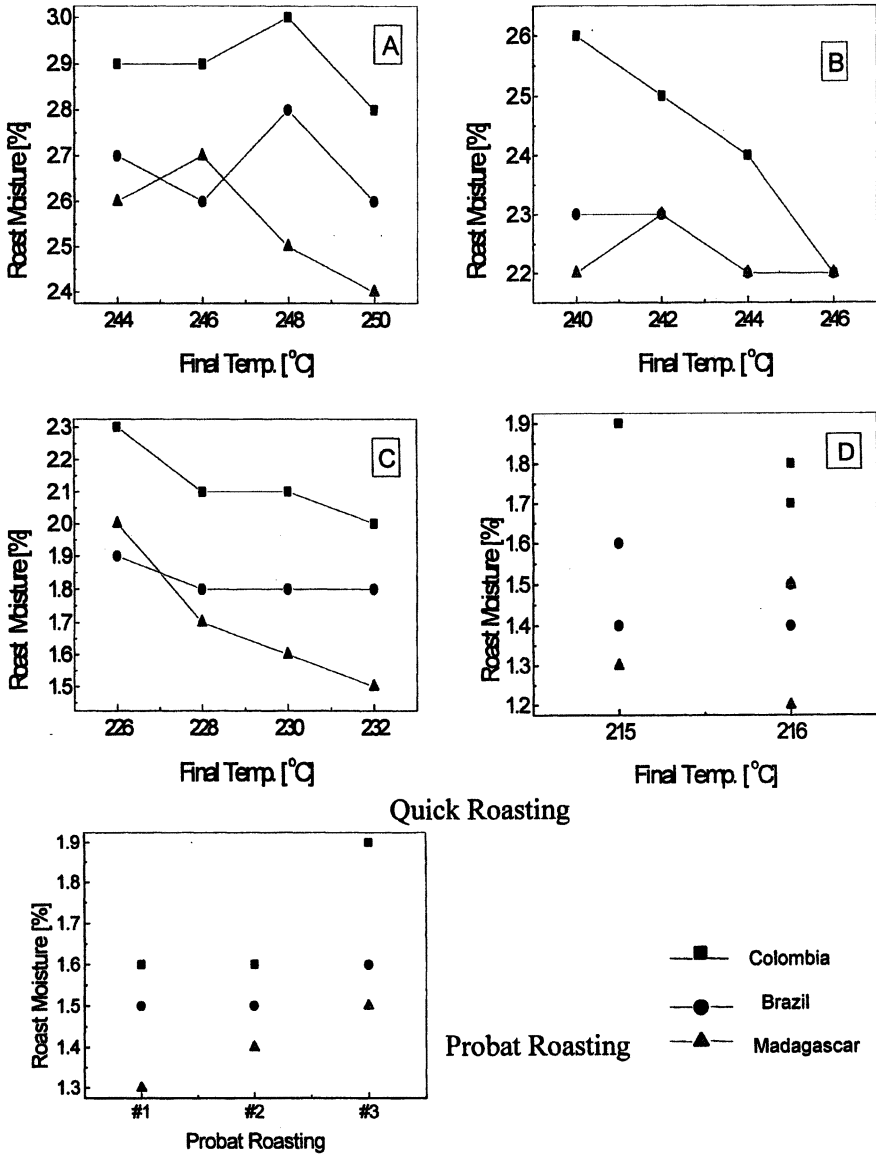


Figure 2. Roast bean moisture content of each coffee bean sample.

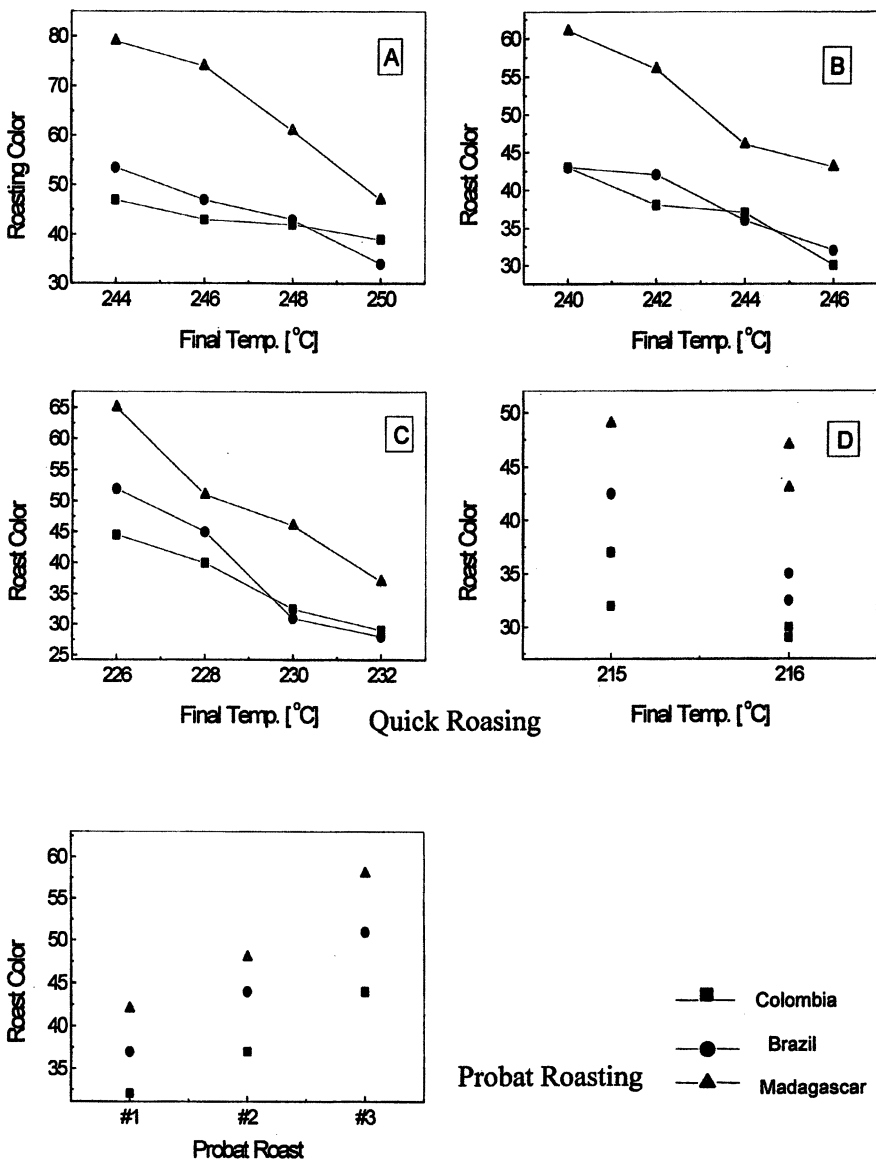


Figure 3. Roast color of each coffee bean sample.

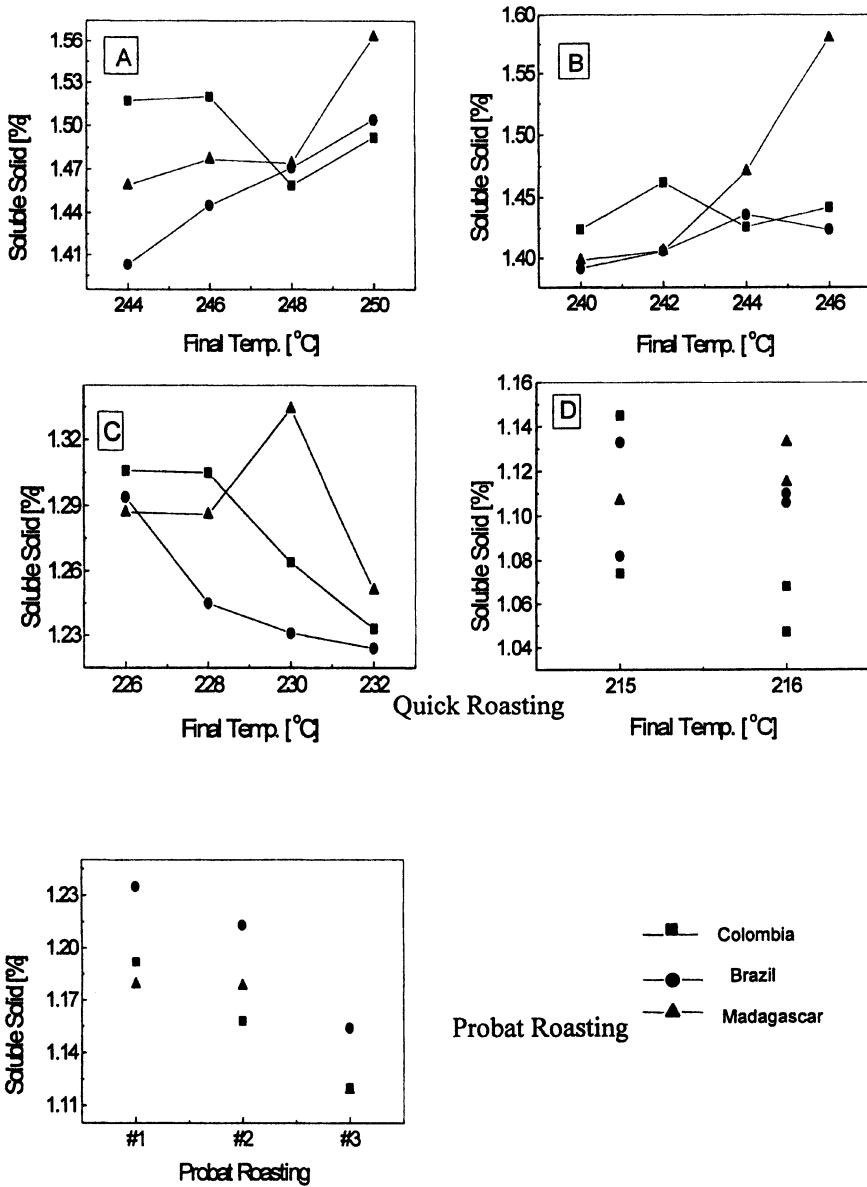


Figure 4. Soluble solid of each coffee bean sample.

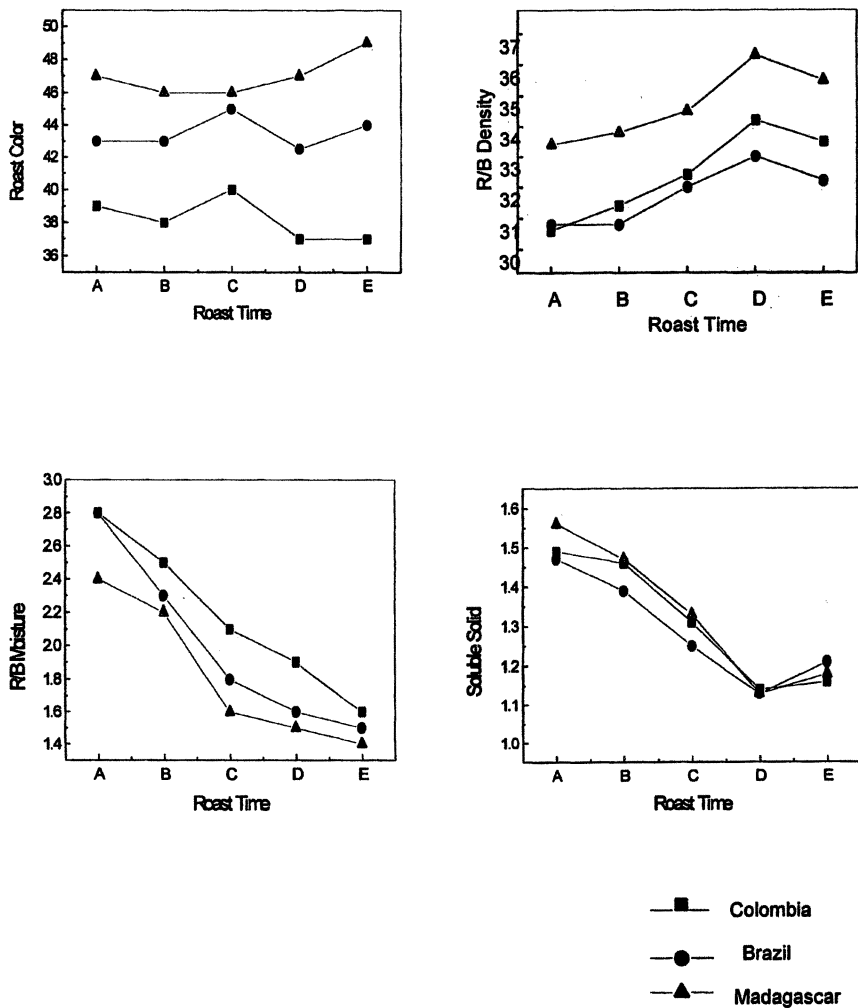


Figure 5. Summary of physical analysis of each coffee bean sample.

with Colombian, Brazil and Madagascar, the shorter the roasting time, the stronger the overall flavor and aroma.

**Table IV. The PCA Results of Brazil Beans**

	<i>Probat Roasting</i>	<i>Quick Roasting</i>			
		<i>A</i>	<i>B</i>	<i>C</i>	<i>D</i>
Degree of Roast	R-SL-C	1 step strong	1 step strong	same*	same*
Aromatic Impact	moderate	2 steps strong	2 steps strong	1 step strong	same*
Aromatic Character					
Earthy	1	1~2	1~2	1~2	1
Sharp		1	X~1		
Tarry	1	X~1	X~1	1	1
Cup Strength	medium	1 step strong	1 step strong	same*	same*
Basic Taste Intensity					
Sour	1	1~2	1~2	1	1
Bitter	1~2	1~2	1~2	1~2	1~2
Astringent	1	1~2	1~2	1~2	1

\*: same as Brazil and Madagascar beans.

**Table V. The PCA Results of Madagascar Beans**

	<i>Probat Roasting</i>	<i>Quick Roasting</i>			
		<i>A</i>	<i>B</i>	<i>C</i>	<i>D</i>
Degree of Roast	R-SL-C	1 step strong	1 step strong	same*	1 step light
Aromatic Impact	moderate	2 steps strong	2 steps strong	1 steps strong	same*
Aromatic Character					
Cereal	1~2	1~2	1~2	1~2	1~2
Tarry	1~2	1	1	1~2	1~2
Cup Strength	medium	2 steps strong	2 steps strong	1 step strong	same*
Basic Taste Intensity					
Sour	1	1	1	1	1
Bitter	1~2	1~2	1~2	1~2	1~2
Astringent	1	1~2	1~2	1~2	1

\* : same as Brazil and Madagascar beans.

Table VI. Analysis of Flavor Components of Colombian Beans by GC

Aromatic Components	Shop Roasting				Probat Roasting
	A	B	C	D	
Light aroma	686	558	544	402	542
Medium aroma	145	127	139	130	182
Heavy aroma	600	439	409	221	266
Total aroma	1431	1124	1092	753	990
Acetaldehyde	33	36	22	11	20
Acetoin	138	116	125	92	132
Furan	17	10	16	14	14
Isobutyric acid	30	30	28	20	27
Diacetyl	48	35	32	14	23
2,3-Pentanedione	-	-	39	17	28
Methyl ethyl ketone	27	19	27	22	28
2-Methylfuran	66	51	53	60	79
Isovaleraldehyde	42	39	37	25	37
2-Methylbutanal	37	37	38	30	39
Pyrazine	43	25	60	83	110
Furfural	108	69	62	18	29
Furfuryl alcohol	152	144	65	42	59
Methylpyrazine	28	28	25	17	16
Dimethylpyrazine	-	29	27	18	18
Trimethylpyrazine	-	20	22	16	16
pH	5.214	5.156	5.279	5.662	5.491
TA	1.227	1.171	1.025	0.559	0.74

**Table VII. Analysis of Flavor Components of Brazil Beans by GC**

<i>Aromatic components</i>	<i>Shop Roasting</i>				<i>Probat Roasting</i>
	<i>A</i>	<i>B</i>	<i>C</i>	<i>D</i>	
Light aroma	564	638	285	409	498
Medium aroma	170	200	110	187	228
Heavy aroma	479	451	212	231	384
Total aroma	1213	1289	607	827	1110
Acetaldehyde	19	31	8	17	16
Acetoin	127	151	77	95	118
Furan	10	15	6	10	12
Isobutyric acid	30	39	17	22	26
Diacetyl	40	45	16	14	23
2,3-Pentanedione	39	49	18	20	28
Methyl ethyl ketone	22	28	15	23	25
2-Methylfuran	50	50	34	46	63
Isovaleraldehyde	37	47	19	28	29
2-Methylbutanal	37	48	23	37	36
Pyrazine	63	88	64	120	141
Furfural	59	62	20	15	25
Furfuryl alcohol	138	78	50	55	122
Methylpyrazine	35	39	17	22	24
Dimethylpyrazine	34	36	17	22	25
Trimethylpyrazine	25	24	20	16	22
pH	5.343	5.377	5.589	5.811	5.702
TA	0.965	0.922	0.656	0.442	0.531



Table VIII. Analysis of Flavor Components of Madagascar Beans by GC

<i>Aromatic components</i>	<i>Shop Roasting</i>				<i>Probat Roasting</i>
	<i>A</i>	<i>B</i>	<i>C</i>	<i>D</i>	
Light aroma	599	738	609	608	551
Medium aroma	125	197	191	174	187
Heavy aroma	302	423	337	304	328
Total aroma	1026	1358	1137	1086	1066
Acetaldehyde	39	35	26	24	24
Acetoin	138	163	145	132	135
Furan	15	20	19	18	19
Isobutyric acid	56	61	33	48	29
Diacetyl	34	41	27	25	23
2,3-Pentanedione	25	31	19	20	14
Methyl ethyl ketone	26	32	36	30	33
2-Methylfuran	57	73	77	76	80
Isovaleraldehyde	57	68	40	52	33
2-Methylbutanal	61	74	48	65	44
Pyrazine	50	91	65	64	81
Furfural	37	44	27	22	21
Furfuryl alcohol	72	119	64	77	101
Methylpyrazine	28	36	32	29	23
Dimethylpyrazine	27	37	36	31	26
Trimethylpyrazine	19	26	26	21	24
pH	5.508	5.56	5.826	6.147	5.834
TA	0.62	0.504	0.317	0.092	0.325

Tables VI, VII and VIII show the flavor component results of each green coffee bean by GC analysis. With all samples, Shop Roasting which takes about 3 minutes of roasting gave better flavor extracts by percolation than Probat Roasting which takes 13-15 minutes and is the current manufacturing method. With Colombian beans, longer roasting time results in less total aroma. Probat Roasting is currently used commercially and shows more excellent results than D of Shop Roasting. With Brazil, Probat Roasting method shows a greater amount of total aroma and diketones, namely diacetyl and 2,3-pentanedione, and more 2-methylfuran than C and D. Probat Roasting method shows similar results to C and D. However, overall, Probat Roasting method is considered to produce inferior flavor quality to A and B.

With Madagascar coffee, Probat Roasting method yielded lower amount of total aroma than B of Shop Roasting and similar level of total aroma under other roasting conditions. Additionally, it gave a lower diketone level and a higher level of 2-methylfuran than those with the Shop Roasting method. Overall it gave inferior quality compared to Shop Roasting method.

## Conclusions

Overall, the higher the roasting temperature and the shorter the roasting time, the better the quality of roasted bean. Quick roasting method gave better expansion, therefore, greater yield of soluble solid extracts and higher cup strength than with Probat roasting. The shorter the roasting time, the higher the aromatic impact, cup strength, intensity of taste and the richer aroma. Quick roasting methods gave higher aromatic flavor and one step less tarry odor than the Probat roast. The shorter the roasting time, the better were extracts by percolation based on chromatographic results.

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## Chapter 24

### Degradation of Furfuryl Mercaptan in Fenton-Type Model Systems

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The stability of furfuryl mercaptan (Fur-SH) was studied in aqueous solutions in the presence of reagents for the *Fenton* reaction. The impact of hydrogen peroxide, iron, ascorbic acid, and EDTA was studied by incubating them in various combinations with Fur-SH for 1 h at 37°C. About 80% of Fur-SH was lost in the presence of hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) and the ferrous iron generating system Fe(II) and ascorbic acid. Volatile components formed were mainly dimers of Fur-SH, i.e. Fur-S<sub>n</sub>-Fur with n = 1-3, difurfuryl disulfide being the major compound. In the model systems containing Fe(II) and H<sub>2</sub>O<sub>2</sub>, the volatiles generated account for about 1/3 of the total Fur-SH lost. Electron paramagnetic resonance spectroscopy in the presence of the spin traps POBN, DMPO, and DEPMPO indicated the formation of hydroxyl and carbon-centered radicals arising from H<sub>2</sub>O<sub>2</sub> and Fur-SH, respectively.

Furfuryl mercaptan (Fur-SH) has been suggested to be a key odorant of coffee (1). Its sensory relevance, evidenced by various groups (2, 3), is due to the roasty, coffee-like aroma note and low odor threshold of 0.01 ng/L air (4). The concentration of Fur-SH in roast and ground coffee was determined to be 1-2 mg/kg (5). However, only 20-40 µg/kg of Fur-SH were detected in coffee brews (50 g/L), which correspond to about 1/3 of the total amount (6). This might be explained by the low extractability of Fur-SH during preparation of the coffee beverage or high sensitivity of Fur-SH to oxidative processes. Formation of hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) in liquid coffee has been reported (7). In the presence of transition metals, H<sub>2</sub>O<sub>2</sub> produces hydroxyl radicals (·OH) via the *Fenton* reaction (8) as shown below:



Caffeine has been shown to be an effective radical scavenger, trapping OH radicals and forming 8-oxocaffeine (9). Yet, OH radicals may also attack aromatic thiols, which may then lead to a distortion of the coffee aroma. The objective of this work was to study the stability of Fur-SH in *Fenton*-type model systems. We were aiming at (i) quantifying the losses of Fur-SH caused by *Fenton* reagents, (ii) identifying the corresponding volatile degradation products, and (iii) characterizing short-lived radical species.

## Experimental Procedures

### Materials

Furfuryl mercaptan (Fur-SH), benzyl mercaptan (Ben-SH), difurfuryl monosulfide (DFMS), difurfuryl disulfide (DFDS) and ascorbic acid were from Aldrich (Buchs, Switzerland).  $\alpha$ -(4-Pyridyl-1-oxide)-*N*-*t*-butylnitron (POBN), 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) were from Sigma (Dorset, England, UK). Ethylenediaminetetraacetic acid (EDTA, disodium salt), hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), ferric chloride (FeCl<sub>3</sub> · 6 H<sub>2</sub>O), and sodium dodecyl sulfate (SDS) were from Merck (Darmstadt, Germany) and 5-(diethoxyphosphoryl),5-methyl-1-pyrroline-*N*-oxide (DEPMPO) was from Calbiochem-Novabiochem (Beeston, Nottinghamshire, UK).

### Sample Preparation

The aqueous solutions were prepared fresh before use, i.e. ascorbic acid (20 mM), EDTA (25 mM), H<sub>2</sub>O<sub>2</sub> (1.5 %), FeCl<sub>3</sub> · 6 H<sub>2</sub>O (10 mM), K<sub>2</sub>HPO<sub>4</sub> (20 mM, pH 5.5), and Fur-SH (3.3 mM, dissolved in aqueous SDS, 3.3 mg/mL). The reaction was initiated by adding Fur-SH to mixtures as indicated in Table I. Samples were incubated (1 h, 37°C) and adding ethanol (0.1 mL) terminated the reaction. EPR measurements were made on solutions to which a spin trap was added immediately prior to the Fur-SH. Chemical analyses were performed after adjusting the pH to 3.5 and extracting neutral compounds with diethyl ether (1 mL). The organic phase was centrifuged (5 min, 3500 rpm) and analyzed by GC.

**Table I. Experimental Design to Study the Effect of *Fenton* Reagents on the Loss of Furfuryl Mercaptan (Fur-SH) in Aqueous Model Systems<sup>a</sup>**

Reagents	#1	#2	#3	#4	#5	#6	#7	#8
Fur-SH	100	100	100	100	100	100	100	100
FeCl <sub>3</sub>	10	-	10	10	10	-	10	-
H <sub>2</sub> O <sub>2</sub>	10	10	-	10	10	-	-	-
EDTA	10	10	10	-	10	-	-	-
Ascorbic acid	50	50	50	50	-	50	50	-
Phosphate buffer	800	800	800	800	800	800	800	800
Water	20	30	30	30	70	50	40	100

<sup>a</sup> The values correspond to volumes expressed in  $\mu$ L. The total volume of each sample was 1 mL. In all samples, the initial concentration of Fur-SH was 3.3  $\mu$ mol.

## Capillary Gas Chromatography

A Hewlett Packard gas chromatograph (HP-5890) equipped with a HP-7673A autosampler and cold on-column injection was used. Samples were analyzed on an OV-1701 fused silica capillary column (30 m x 0.25 mm, film thickness 0.25  $\mu\text{m}$ ). The column pressure was 80 kPa using helium as carrier gas. The effluent was split 1:1 to a flame ionization detector (FID) and a flame photometric detector (FPD). The GC oven was temperature programmed (4).

## Quantification

Benzyl mercaptan (Ben-SH) was used as internal standard (0.5 mL of 49.4 mg/100 mL diethyl ether) added to the samples after quenching with ethanol. The pH was adjusted to 3.5 and the clean-up was performed as described above. FID detection was used for quantification. Response factors were determined using mixtures of known amounts of Ben-SH and the compounds to be quantified (e.g. 1.75 for Fur-SH).

## Gas Chromatography-Mass Spectrometry (GC-MS)

Electron impact (EI) and positive chemical ionization (PCI) mass spectra were obtained on a Finnigan MAT 8430 mass spectrometer at 70 eV and 150 eV, respectively. Ammonia was used as reagent gas for PCI. Volatile compounds were sampled via a cold on-column injector (HP-5890 GC) using the conditions described above. Relative abundances of the ions are given in %.

## Electron Paramagnetic Resonance (EPR) Spectroscopy

This was made at room temperature at X-band frequencies ( $\sim 9.5$  GHz) using a Bruker ESP300E computer-controlled spectrometer incorporating an ER4103TM cylindrical microwave cavity. All spectra were collected in 1024 data points using a modulation frequency of 100 KHz. Most spectra were recorded as 1<sup>st</sup> derivatives of the microwave absorption and displayed as functions of absorption versus magnetic field at a constant microwave frequency (except for one spectrum with POBN for which a 2<sup>nd</sup> derivative spectrum is shown).

# Results and Discussion

## Loss of Fur-SH in *Fenton*-type Model Systems

The degradation of Fur-SH and concomitant formation of reaction products was investigated on a series of eight *Fenton*-type model systems at pH 5.5 (Table II). Sample #1 (full *Fenton* model) contained all of the reagents for a *Fenton* reaction. EDTA was used to prevent precipitation of Fe(III) and ascorbic acid reduced Fe(III) to Fe(II), which initiated decomposition of  $\text{H}_2\text{O}_2$  to the hydroxyl radical ( $\cdot\text{OH}$ ). The reference (sample #8) was a 3.3  $\mu\text{mol}$  Fur-SH solution.

The concentration of Fur-SH found after reaction was strongly dependent on the composition of the model system (Table II). The most significant loss of Fur-SH, i.e. 80%, was observed in sample #1 containing the typical *Fenton* reagents  $H_2O_2$  and Fe(II). The presence of EDTA was not essential under the experimental condition chosen, as about 70% of Fur-SH were lost in sample #4, which contained no EDTA. Similarly, the absence of ascorbic acid did not prevent degradation of Fur-SH, where a 65% loss was observed in sample #5. This might be explained by the ability of Fur-SH or its degradation products to reduce Fe(III) to Fe(II).

**Table II. Effect of *Fenton* Reagents on the Loss of Furfuryl Mercaptan (Fur-SH) in Aqueous Model Systems<sup>a</sup>**

Reagents	Concentration of Fur-SH ( $\mu\text{mol}$ )	Loss of Fur-SH (%)
# 1: Full <i>Fenton</i> system	0.7	80
# 2: No Fe(III)	1.8	45
# 3: No $H_2O_2$	2.3	30
# 4: No EDTA	1.0	70
# 5: No ascorbic acid	1.1	65
# 8: Reference sample	3.3	<3

<sup>a</sup> Mean values of duplicates obtained by quantification using Ben-SH as internal standard added to the sample after quenching with ethanol.

In contrast, omitting the typical *Fenton* reagents  $H_2O_2$  or Fe(II) from the model systems significantly reduced the losses of Fur-SH. In the absence of  $H_2O_2$  losses of Fur-SH were reduced to about 30% or less (samples #3), and only traces of degradation products were found in samples #6 and #7 (data not shown). This demonstrates the crucial role of  $H_2O_2$ , in which reductive cleavage of the O-O bond can be catalyzed by ferrous iron or ferrous iron complexes which generate powerful oxidants such as OH or, as recently proposed (10), metal-based oxidizing species such as peroxy  $Fe^{II}(\text{OOH})$  and iron(IV)-oxo complexes. The OH radicals that are generated can react with compounds such as thiols and initiate formation of volatile and non-volatile degradation products.

Sample #2, in which no iron was added, surprisingly showed about 45% loss of Fur-SH. This relatively high loss of Fur-SH is probably due to a combination of traces of transition metal impurities and the effects of light on the stability of the  $H_2O_2$ .

### Formation of Volatile Degradation Products of Fur-SH

Loss of Fur-SH is accompanied by the formation of volatile degradation products, which were particularly pronounced in samples #1, #4, and #5. As shown in Figure 1, difurfuryl disulfide (DFDS) was the major degradation product identified. Furfural, difurfuryl monosulfide (DFMS), and difurfuryl trisulfide (DFTS) were further characteristic volatile degradation products.

DFTS has not been reported as a constituent of coffee aroma, but DFMS and DFDS are known volatiles in roasted coffee (11, 12). However, there is no information available about their sensory relevance in coffee aroma. The aroma notes of DFMS and DFDS can be described as burnt, sulfury, roasty, and rubbery, but they lack the characteristic coffee-type aroma of Fur-SH. Taking into account their low odor thresholds, e.g. 0.001 ng/L air for DFDS (13), it is likely that the loss of Fur-SH and concomitant formation of various difurfuryl sulfides can generate an imbalance in coffee aroma during storage or under conditions of oxidative stress.

Several volatile compounds were detected showing the fragment at  $m/z$  81 that is characteristic for the furfuryl moiety (Figure 1). Four of these signals correspond to Fur-SH, DFMS, DFDS, and DFTS, but some remain unidentified. Their structures should, however, be closely related to Fur-SH, as the main fragment was found to be at  $m/z$  81. Experiments to identify them, particularly  $U_1$ ,  $U_2$ , and  $U_3$ , are in progress and will be reported elsewhere.

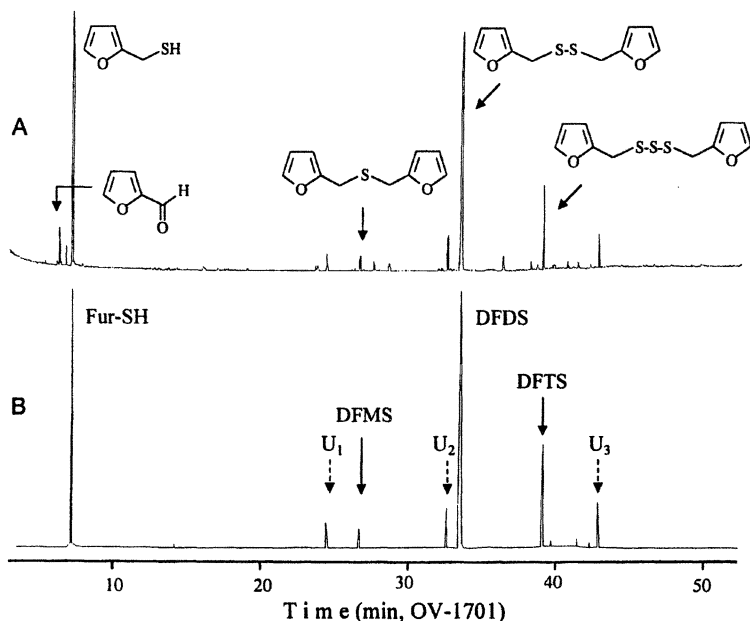


Figure 1. GC-MS identification of volatile compounds detected in sample #1. The upper trace (A) represents the total ion current (TIC) while (B) is an extract of TIC showing only the trace of  $m/z$  81. Furfural (RI= 970), Fur-SH: furfuryl mercaptan (RI= 1000),  $U_1$ : (RI= 1530), DFMS: difurfuryl monosulfide (RI= 1605),  $U_2$ : (RI= 1825), DFDS: difurfuryl disulfide (RI= 1860), DFTS: difurfuryl trisulfide (RI= 2125),  $U_3$ : (RI= 2380).

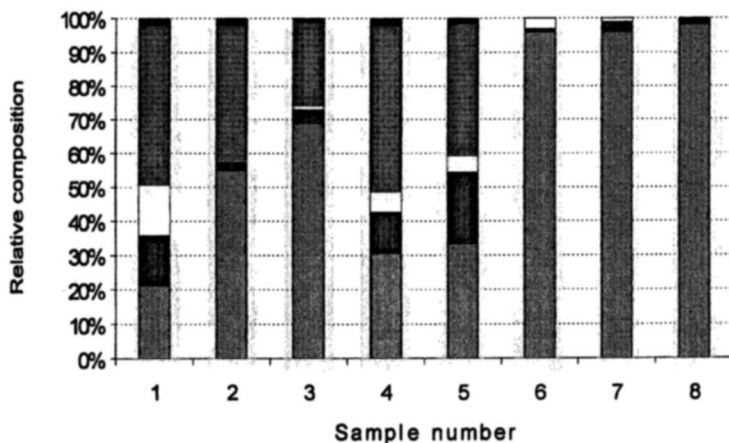


Figure 2. Relative composition of samples #1 to #8. The concentration of Fur-SH (bottom) and DFDS (black) was quantified using response factors. The concentration of DFMS, DFTS, and unknown compounds (white) was estimated using the response factor of 1.7. The difference of volatiles to total Fur-SH equivalents is defined as 'non-volatiles' (upper part in bar diagram).

#### Non-volatile Degradation Products of Fur-SH

In addition to volatile reaction products, degradation of Fur-SH in *Fenton*-type reactions can generate non-volatile compounds. The amount of non-volatiles was estimated by mass-balance between reactants and products (Figure 2). Volatiles found in samples #1, #4, and #5 account only for about 50-60% of the initial Fur-SH amount, with DFDS as the major volatile decomposition product. Consequently, an appreciable fraction of the Fur-SH degradation products is in the form of non-volatile materials.

These non-volatile compounds can not be analyzed by gas chromatography. They might be ionic low molecular weight compounds or polymeric materials. In a study of ascorbate and transition-metal mediated oxidation of methanethiol, Chin and Lindsay (14) proposed methanesulfenic acid ( $\text{CH}_3\text{SOH}$ ) as an intermediate in the formation of dimethyl disulfide and dimethyl trisulfide. However, no evidence was found for furfurylsulfenic acid in this study using GC methods, although this might be due to low volatility and high reactivity of such species, as shown for methanesulfenic acid (15). HPLC-MS is currently being used in an attempt to verify the occurrence of urfurylsulfenic acid in sample #1 and to study decomposition products, including high molecular weight components.

#### Short-lived Free Radical Species Investigated by EPR Spectroscopy of Spin-trapped Adducts

The reaction products from sample #1, as determined by GC, suggest that both C- and S-centered radicals are involved as intermediates in the reaction pathway. In order to attempt to obtain direct experimental evidence for their presence, we have



embarked on a series of experiments using EPR spectroscopy that provides information on chemical species with unpaired electrons (i.e. free radicals and paramagnetic metal ions). Many free radicals, however, have limited chemical stability and as a consequence are difficult to study. In this work we have used a chemical method, in which unstable free radicals react with a chemical (often called a "spin trap") to generate a new stable radical, which can then be characterized by EPR spectroscopy.

Several examples of "spin traps" are nitrones, which after reaction with a free radical produce nitroxides (Figure 3), stable free radicals that give EPR spectra with characteristic 3-peak hyperfine structure from interaction of the unpaired electron with the  $^{14}\text{N}$  ( $I=1$ ) nucleus. POBN, DMPO, and DEPMPO are water-soluble spin traps used in the present study. Each has some advantages and disadvantages compared to the others. POBN forms adducts with free radicals that are stable and easy to study, but there are little differences in the EPR spectral parameters for C-centered and  $\cdot\text{S}$ -centered radical adducts. DMPO provides better discrimination between parent radicals than POBN, but some adducts have limited stability. DEPMPO is reported as overcoming the problems of discrimination and stability mentioned above, but it is relatively new and there is only a very limited library of parameters for different types of radical adduct. With all spin traps, however, there may be appreciable differences in their affinities for different types of free radical.

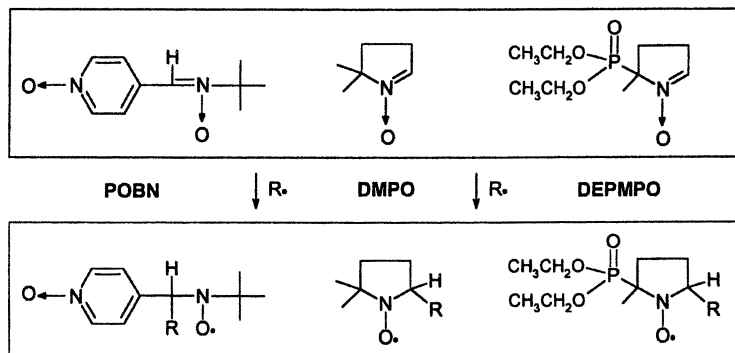
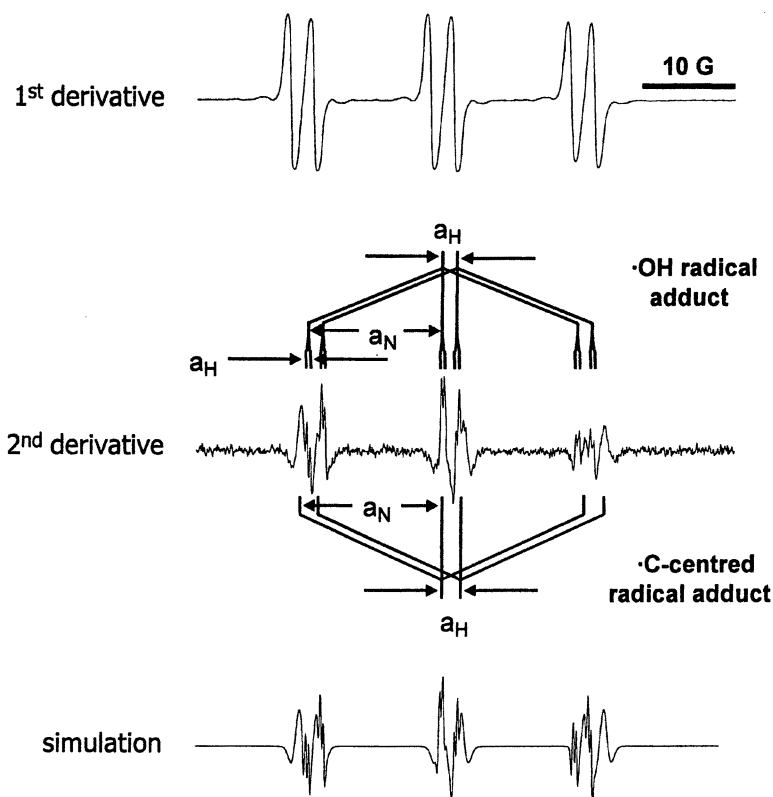


Figure 3. Chemical structures of the spin-traps used in this study to characterize radical species generated in the Fenton model systems with Fur-SH and the corresponding adducts of a free radical  $\cdot\text{R}$ .

### POBN

The 1<sup>st</sup> derivative spectrum of model solution #1 with POBN shows a 6-peak hyperfine structure (Figure 4), which arises from interaction of the unpaired electron with  $^{14}\text{N}$  ( $I=1$ ) and the  $^1\text{H}$  ( $I=1/2$ ) on the C atom adjacent to the nitroxide group. The weak satellite peaks, seen when the intensity-axis is magnified, arise from  $^{13}\text{C}$  ( $I=1/2$ , natural abundance 1.108%) in the *t*-butyl group and the carbon atom adjacent to the nitroxide. No hyperfine structure is seen from  $^{17}\text{O}$  ( $I=5/2$ ), because of its low abundance (0.037%). The 2<sup>nd</sup>



*Figure 4. Trapping OH and C-centered radicals with  $\alpha$ -(4-pyridyl-1-oxide)-*N*-*t*-butylnitrone (POBN). The 1<sup>st</sup> and 2<sup>nd</sup> derivative EPR spectra obtained with model solution #1 and POBN are shown as well as a simulation of the experimental 2<sup>nd</sup> derivative spectrum using known coupling constants.*

derivative spectrum, however, shows clearly the presence of more than one component. The accompanying “stick diagrams” show the positions of the peaks from the OH and typical C-centered radical adducts and the bottom trace a simulation using known coupling constants.

Although the spin trap POBN clearly showed an EPR spectrum consistent with a superposition of trapped OH and C-centered radical adducts (Figure 4), the chemical nature of the C-centered radical cannot be determined from the current data. Also, POBN does not provide good discrimination between C-, O-, and S-centered radicals, so it is not possible to use these spectra to substantiate the presence of  $\cdot$ S-centered radicals (16).

#### DMPO

The spin trap DMPO gives good discrimination between O- and S-centered adducts, provided they are trapped. The experimental spectrum (Figure 5) obtained with model solution #1 shows a  $\cdot$ C-centered radical

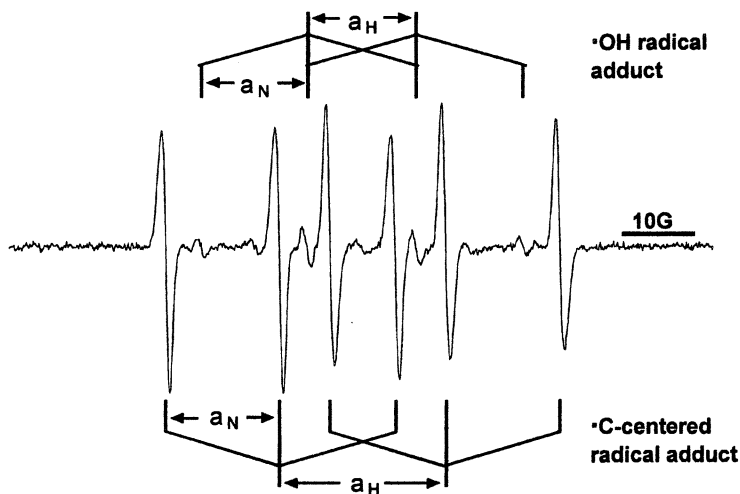


Figure 5. Trapping C-centered radical adducts and OH adducts with DMPO in model system #1. DMPO provides good discrimination between ·OH and C-centered radical adducts as shown in the "stick diagram".

adduct as the major component and the OH adduct as the minor component (16, 17). S-centered radical adducts were absent from the EPR spectrum, which means that either the adducts were not formed (radicals are not trapped by DMPO) or S-centered radicals were not present in the sample.

#### DEPMPO

This spin trap is closely related to DMPO (Figure 3), but produces more stable adducts. In addition to hyperfine structure from  $^{14}\text{N}$  and the  $^1\text{H}$  on the carbon atom adjacent to the nitroxide group, DEPMPO has a further doublet splitting from the  $^{31}\text{P}$  ( $I = 1/2$ ) atom (Figure 6). Weak hyperfine structure from  $^{13}\text{C}$  ( $I = 1/2$ , natural abundance 1.108%) can also be resolved, but has been little studied, and cannot therefore be used at present for adduct characterisation.

The main adduct seen with DEPMPO corresponds to a ·C-centered radical and only minor amounts of the ·OH adduct (not seen in Figure 6) were found in the present experiments (18, 19). Figure 6 shows additional satellite peaks from unidentified species, but no peaks corresponding to ·S-centered radical adducts.

### Conclusions

This work provides the first evidence for fast degradation of the coffee flavour compound Fur-SH under *Fenton* conditions leading to volatile and non-volatile compounds. Since the main reagents of the *Fenton* model system are also present in coffee beverages, analogous reactions may take place in liquid coffee. Therefore, it is likely that loss of Fur-SH and other aroma-relevant thiols and concomitant formation of various difurfuryl sulfides and related degradation products can lead to a distortion of coffee aroma during storage or under conditions of oxidative stress.

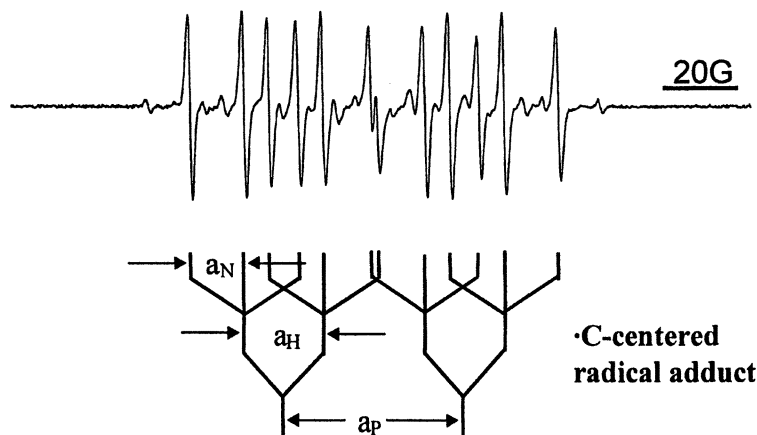


Figure 6. Trapping ·C-centered radical adducts with DEPMPO in model sample #1.

### Acknowledgments

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## Chapter 25

### Diterpenes in Coffee

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In recent years, the diterpenes in the lipid fraction of coffee have become of considerable interest: 16-O-methylcafestol as an indicator for Robusta coffee, the diterpenes cafestol and kahweol as components with different physiological effects. In comparison to green coffee, a large number of additional diterpenes were obtained in roasted coffee samples. Aside from diterpene derivatives formed by the loss of a water molecule, with cafestol and kahweol two further degradation products were elucidated. Many of these compounds have been detected and quantified in differently prepared coffee beverages as well.

The two most important coffee species, *Coffea arabica* and *Coffea canephora*, var. *robusta*, contain between 7 and 17% fat in the coffee beans. The lipid content of Arabica coffee averages some 15%, whilst Robusta coffee contains much less, namely around 10% lipids.

The main components in coffee oil are triglycerides, followed closely by diterpenes with a share of 20%. These compounds are important not only because of their amount, but also because of their physiological effects, which is also the reason for the increasing interest in them in recent years. The structural formulae of four diterpenes are assembled in Figure 1.

Until now, the physiological activity of only cafestol and kahweol has been researched and in several studies reported that, through the enjoyment of coffee, the serum cholesterol level can increase. Initially, triglycerides were said to be responsible for this effect. Since then, however, it has been established that it was the diterpenes which influence the serum cholesterol level (1,2,3).

In addition, a substantial number of scientific publications exist where positive effects of diterpenes are introduced. It was shown that cafestol stimulates the glutathion-S-transferase activity, through which the decomposition of xenobiotica is accelerated (4). Other authors reported that the diterpenes cafestol and kahweol protect against B1-induced genotoxicity (5,6).

Up until today, only a few of these compounds have been examined in detail. To what extent additive, reversible or even synergistic effects show up, can only

be judged when the entirety of the diterpene compounds found in coffee is included in this study. Hence, the following shall be an overview of the current state of knowledge with regard to diterpene compounds in coffee, where, above all, the results of this research team, gathered from 1985 to the present, will be presented.

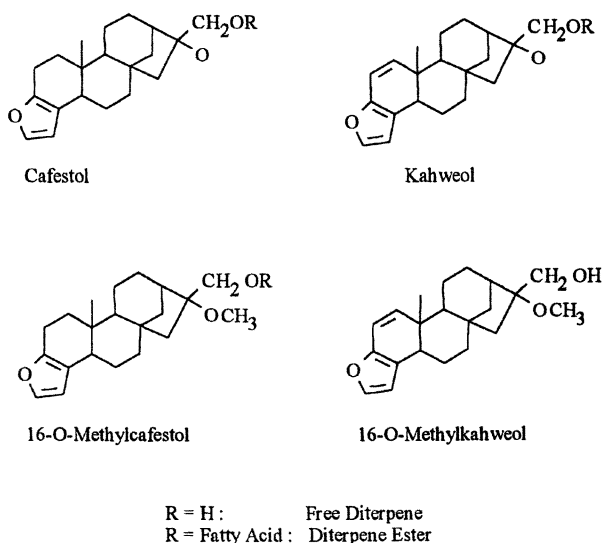


Figure 1. Structural formulae of the diterpenes.

### Diterpenes in the Lipid Fraction of Green Robusta and Arabica Coffees

It has been well-known since the 1930s that coffee contains diterpenes (7). Working groups under Wettstein (8), Chakravorty (9), Djerassi (10), and Haworth (11) worked for several years to identify the structure of two of the coffee diterpenes, namely kahweol and cafestol. Through later work by Kaufmann and Hamsagar (12,13) and also by Folstar (14,15), it could be shown that both diterpenes are predominantly esterified with different fatty acids and exist freely only in small amounts.

In order to analyze the total amount, it was necessary to extract the fat from the coffee bean, to saponify and then to determine the diterpenes in the unsaponifiable matter by means of GC or faster by HPLC (16,17) (Figure 2).

Several green Arabica and Robusta coffees were investigated (Figure 3). In Arabica coffee beans, the diterpenes cafestol and kahweol were found, whereas Robusta coffee beans contain cafestol, small amounts of kahweol and, additionally, another diterpene -- the 16-O-methylcafestol (16-OMC) (18,19). The new diterpene was present only in the Robusta coffee; therefore, it is the

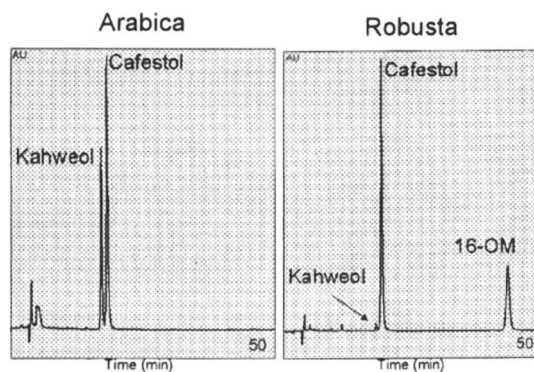


Figure 2. HPLC-chromatograms of green Arabica and Robusta coffees. Conditions: column 250x4mm, Nucleosil 120-3 C<sub>18</sub>, eluent: acetonitrile/water (50:50), detection: UV 220 nm.

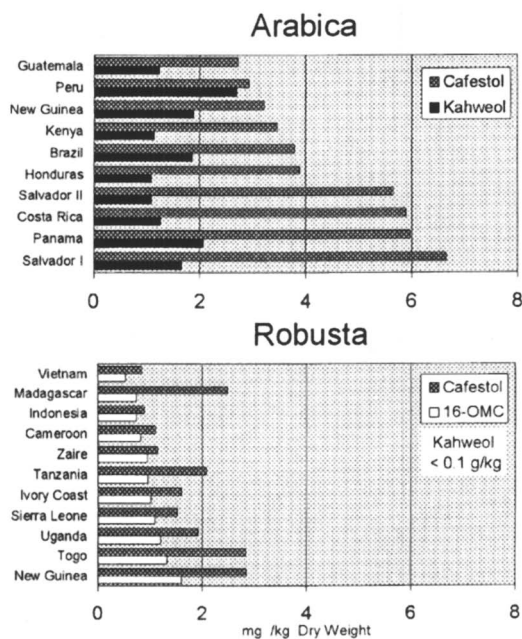


Figure 3. Contents of diterpenes in the unsaponifiable matter of different green Arabica and Robusta coffees.



indicator substance used to detect parts of Robusta in Arabica coffee mixtures. A validated method of determination used in Germany has just been published as the DIN 10779 of the German Institute for Normalization. This method makes it possible to identify very small parts of Robusta -- parts under 2 percent -- in mixtures of Arabica coffees.

Recently, a further diterpene was found and tentatively introduced as a 16-O-methyl derivative of kahweol. It should be present exclusively in *coffea stenophylla*, a coffee species which is not being used commercially (20). However, this derivative has now also been found in many Robusta coffee samples and, using different spectroscopic methods, it was clearly identified as 16-O-methylkahweol (21).

### Free Diterpenes

In their free form, the diterpenes cafestol, kahweol and 16-OMC occur only as minor components in coffee oil (22,23). Quantifying them requires an effective separation from the major compounds which interfere with the analysis. By means of gel permeation chromatography, it is possible to simultaneously analyze and quantify the small amounts of these compounds by subsequent HPLC.

Several coffees were analyzed. In the Arabica coffees, both free cafestol and free kahweol were determined (Figure 4). In addition to cafestol and 16-OMC, traces of kahweol were detected in some of the Robusta coffees. Only traces of free kahweol, if at all, had been expected, because the contents of total kahweol achieved after saponification were very small, with amounts between 10 mg/kg to 60 mg/kg.

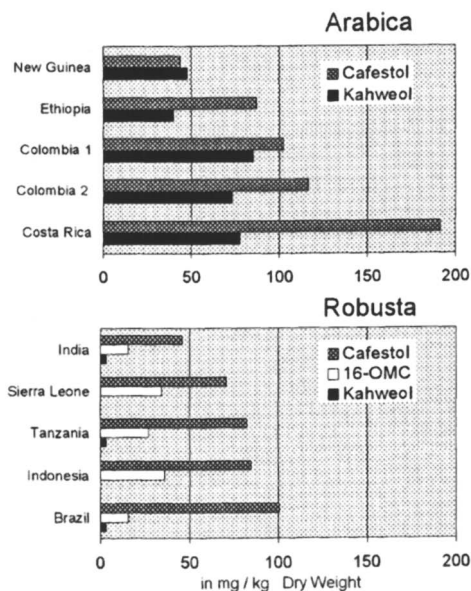


Figure 4. Contents of free diterpenes in green Arabica and Robusta coffees.

When the content of the free diterpene was contrasted with the total content of the diterpene determined after saponification, a similar result for each examined diterpene was obtained. In Arabicas, the proportions ranged from 0.7 to 2.5%, in Robustas the proportions of the free diterpenes were slightly higher with 1.1 to 3.5%.

### Diterpene Fatty Acid Esters

Until now only a few diterpene esters have been reported. In order to identify the individual diterpene esters, it was necessary to separate the other compounds of coffee oil such as sterol esters, sterols, phosphatides, free fatty acids, free diterpenes and mainly the triglycerides. Using the same gel chromatographic system, the diterpene esters were separated with success. However, they were still present in the same fraction together with sterol esters, which were removed by using solid phase extraction on silica cartridges. Finally for Arabicas, one fraction was achieved containing the cafestol and kahweol esters; for Robustas a second fraction was achieved which included the 16-OMC esters. The fractions were analyzed by HPLC, and as exemplarily shown in Figure 5 for a Robusta coffee sample, it was possible to determine the individual cafestol esters.

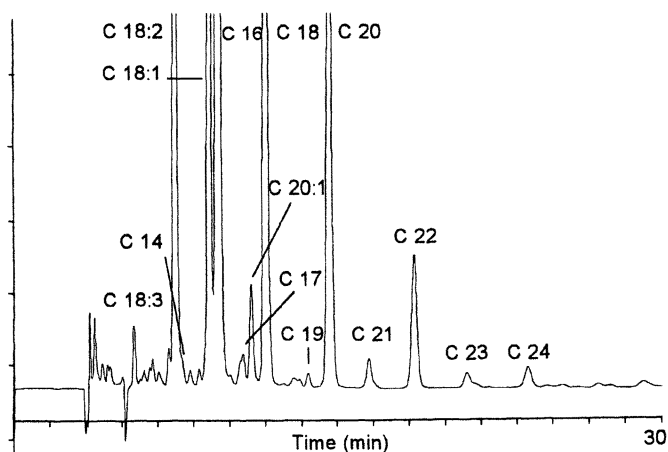


Figure 5. HPLC-chromatogram of cafestol fatty acid esters. Conditions: column 250x4mm, Nucleosil 120-3  $C_{18}$ , eluent: acetonitrile/iso-propanol (60:40), detection: UV 220 nm.

Esters with fatty acids such as  $C_{14}$ ,  $C_{16}$ ,  $C_{18}$ ,  $C_{18:1}$ ,  $C_{18:2}$ ,  $C_{18:3}$ ,  $C_{20}$ ,  $C_{22}$ ,  $C_{24}$  were identified, as well as esters with the fatty acid  $C_{20:1}$  and some odd-numbered fatty acids such as  $C_{17}$ ,  $C_{19}$ ,  $C_{21}$  and  $C_{23}$ .

The individual diterpene esters were irregularly present in the coffee oil. The odd-numbered fatty acid esters were minor components, whereas the diterpenes esterified with palmitic, linoleic, oleic, stearic, arachidic and behenic

acid existed in larger amounts. The focus was therefore placed on these six diterpene esters, which make up a sum of nearly 98% of the respective diterpenes (24,25,26).

The total content of these six quantitatively significant cafestol esters in different Arabica coffees was analyzed. The established content fell between 9.4 and 21.2 g/kg dry weight, corresponding to 5.2 to 11.8 g/kg cafestol. In different Robusta coffees, it was determined to be between 2.2 and 7.6 g/kg dry weight, corresponding to 1.2 to 4.2 g/kg cafestol, thus, notably less than in the Arabica coffees.

## Diterpenes in the Lipid Fraction of Roasted Robusta and Arabica Coffees

During the roasting process, temperatures in the middle of the coffee bean reach up to 230 °C. Through this process, a large number of compounds are changed. Therefore, the question presents itself, to what extent the diterpenes are also affected.

### Free Diterpenes

In order to investigate the free diterpenes, one type of green Arabica and one of green Robusta coffee were roasted at different temperatures for three minutes each, and then analyzed. The results are shown in Figure 6. In this figure, the amounts of the free diterpenes are referred to in kilogram of lipid, in contrast to the previous data, where they were referred to in kilogram of dry matter. This modification makes sense because the dry matter of coffee highly increases during roasting, whereas the total lipid content remains stable. The results illustrate that increasing roasting temperature diminishes the contents of the free diterpenes kahweol, cafestol, and 16-OMC. Up to 80% of the initial amounts, which were analyzed in the green coffees, were lost.

During roasting, two additional peaks become clear in the HPLC-chromatogram of Arabica coffee. It is a question of decomposition products from cafestol and kahweol which could be identified as dehydrocafestol and dehydrokahweol (Figure 7). Both compounds increased with raising roasting temperatures (27,28,29).

### Diterpene Fatty Acid Esters

The stable behaviour of diterpene fatty acid esters during roasting is quite different. Examinations of the 16-OMC esters have shown that these are clearly stable during roasting and in spite of different roasting temperatures, the proportional distribution for the diterpene esters is almost the same (30).

On the contrary, the contents of the diterpene esters in cafestol and kahweol decrease depending on the roasting temperature. An increase in roasting temperature leads to a decrease in the total cafestol ester content, but the distribution of the esters remains nearly the same. Subsequently, all of the esters decompose in a similar manner.

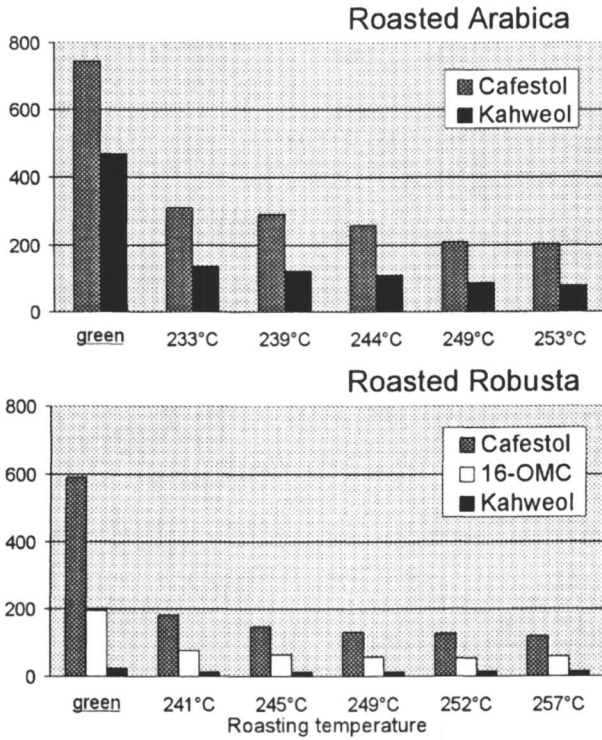


Figure 6. Contents of free diterpenes in roasted Arabica and Robusta coffees.

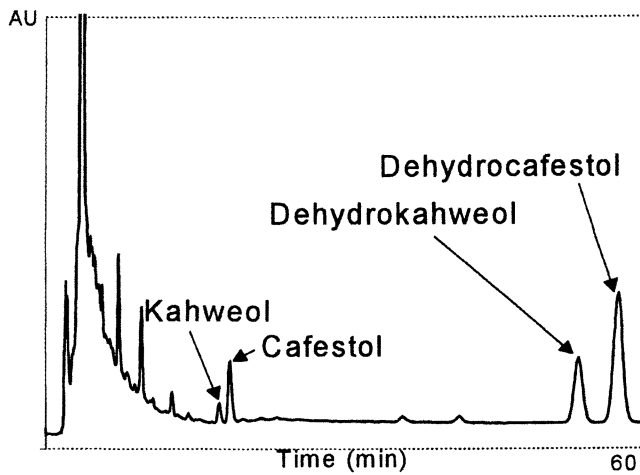


Figure 7. HPLC-chromatogram of free diterpenes in roasted Arabica coffee.

### Formation of Dehydrocafestol and Dehydrokahweol Fatty Acid Esters

As reported earlier, the free diterpenes cafestol and kahweol were dehydrated to their dehydro compounds during the roasting process. Up to the present, it has been unknown whether only the free diterpenes or the diterpene fatty acid esters, too, could be decomposed to the respective dehydro derivatives. In model experiments with cafestol palmitate and cafestol linoleate it has been proved that cafestol fatty acid esters can be dehydrated as well. Dehydrocafestol palmitate and dehydrocafestol linoleate were identified in roasted coffee, too. From this point, it is possible to assume that further fatty acid esters from both dehydrocafestol and dehydrokahweol may be identified (31) (Figure 8).

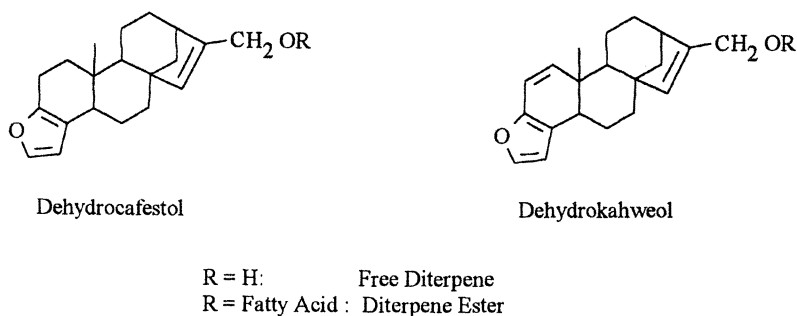


Figure 8. Structural formulae of decomposed diterpenes.

### Cafestal - A Degradation Product of Cafestol

Aside from the described compounds, a further component has been discovered in the unsaponifiable matter of commercial roasted coffee (32). The structure elucidation was carried out by means of NMR, FTIR, and mass spectrometry and checked by chemical conversion. Following the designation of cafestol, cafestal is proposed as the common name.

In order to study the roasting behaviour of cafestal, one Arabica and one Robusta coffee sample were roasted at two different temperatures (Figure 9). As expected, Cafestal was not found in the green coffees. In the roasted coffees, the contents of cafestal were determined from 0.25 to 0.95 mg/g coffee oil. By raising the roasting degree, the content of cafestal increased as well. In addition to cafestal, one corresponding compound of kahweol has, in the meantime, also been identified.

## Diterpenes in Coffee Beverages

In 1993, using the example of 16-OMC ester, it could already be shown that when preparing a coffee drink, lipophile diterpene esters flow into the coffee and are even detectable in instant coffee granules (33).

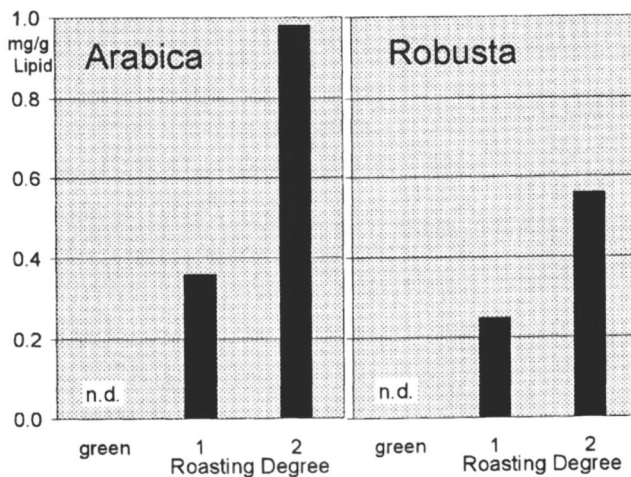


Figure 9. Contents of cafestol in green and roasted coffees.

The amount of diterpenes in the drink is decisively dependent on the method of preparation and is directly connected with the amount of lipids in the drink. With filtered coffee prepared in a common household coffee machine, the amount of lipids is less than 0.2%. On the contrary, when one prepares an espresso, then between 1-2% of the lipids and thereby diterpenes as well, flow from the finely ground espresso coffee into the drink.

When coffee is prepared Scandinavian style, it can even contain up to 22% of the coffee fat. The proportional distribution of diterpenes in the coffee drink is nearly identical to the distribution in the roasted coffee which was used.

In an espresso prepared from Arabica coffee, a total of 1.3 mg cafestol fatty acid esters and 0.5 mg kahweol esters per 50 ml cup were determined, corresponding with approximately 1.5% of cafestol esters and approximately 1.0% of kahweol esters in the roasted ground coffee. These results confirm the findings for the 16-OMC (34). In addition, the decomposition by-products dehydrokahweol, dehydrocafestol and cafestol as well as some esters from dehydrocafestol were identified in the coffee beverage.

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## Chapter 26

# Headspace Analysis of the Coffee Beverage with and without Different Milk Additives

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Milk and vegetable products as an additive for coffee beverages have an effect on the release of aroma substances in the brew through their lipid, protein and carbohydrate components. For the investigation of these effects an external dynamic headspace sampling technique and an external static headspace technique were developed. The release of volatile compounds of the beverages in the oral cavity of human volunteers was measured by oral vapour gas chromatography. With these techniques the most potent odorants of the coffee beverage were determined. Analyses were performed by gas chromatography/olfactometry, FI- and MS-detection. To characterize the odour profiles of the different beverages GC/O analysis were used. All beverages with a milk or vegetable additive showed reduced, but typical odour profiles for each additive.

### Introduction

Up to now, investigations of the coffee flavour have been confined to the analysis of the aroma substances (1, 2, 3). These investigations showed that about 30 volatile compounds were substantially responsible for the coffee flavour. The aim of this study was to investigate the influence of different milk additives and one coffee whitener on the release of flavour impact compounds from coffee beverages. KIM et al. (4) were the first to investigate the effects of milk additives on the coffee flavour; they used the conventional static headspace technique and only instant coffees.

The purposes of adding these products to the coffee beverage are: to develop a desirable colour change, to impart a body to the coffee beverage, to reduce bitter and sour tastes and to reduce astringency of the coffee.

Ingredients of these additives such as lipids, proteins and carbohydrates affect the retention of volatiles (5). Consequently, these aroma interactions affect quality and quantity of the coffee headspace aroma. Direct injection of a headspace sample onto a GC column gives the most accurate composition of flavours. However, working with static headspace (gastight syringe) was not sufficient, because only low amounts of aroma compounds were collected. Therefore new devices had to be developed, which could collect a larger volume of headspace above the coffee beverage. Static headspace as distinct from dynamic headspace measures the concentration of volatiles under equilibrium conditions. In the present study both methods were carried out by GC-FID / olfactometry and GC-MS / olfactometry. Furthermore oral vapour gas chromatography (OVGC) was performed to characterise the human aroma impression.

## Materials and Methods

### Sample Material

For the beverages the two economically important coffee species were used: one Arabica coffee (Columbia) and one Robusta coffee (Indonesia), both with an average roasting degree. Besides this a soluble coffee was used. All samples were supplied by Kraft Jacobs Suchard (Bremen, FRG).

Eight products, purchased from a local market, respectively Kraft Jacobs Suchard (Munich, FRG), were selected as typical coffee additives.

These different types of dairy and vegetable products were also chosen because of their different lipid and protein contents. These components have the greatest influence on the retardation of volatiles. The ingredients of the additives are listed in Table I (6).

**Table I. Ingredients of the additives (in %)**

<i>Additive</i>	<i>Lipid</i>	<i>Carbohydrate</i>	<i>Protein</i>
UHT-Milk	3.5	4.8	3.3
Condensed milk	10	12.5	8.8
Coffee creamer	10	3.1	4.0
Whipping cream	30	3.2	2.5
Coffee whitener (vegetable)	34	55	6

### Sample Preparations

#### *External Dynamic Headspace Sampling (DHS)*

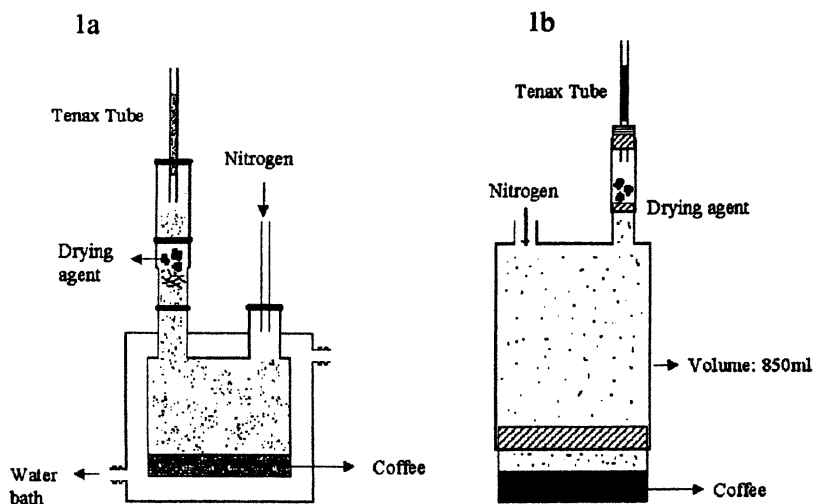
The beans were stored at -17°C and ground directly prior to use in a coffee grinder of the style that is normally used in coffee shops. The brew was prepared in a household coffeemaker with 12g coffee powder and 225g tap water.

Soluble coffee was prepared by pouring boiling water (125g) onto the powder (3.5g). 50g and 125g of soluble coffee brew respectively were placed in the external

dynamic sampling device (Figure 1a), with a headspace volume of 275mL, and an additive was added (10g and 25g respectively). The temperature of the water bath was 40°C. With a flow of 40mL/min for 30 minutes, nitrogen was flushed above the coffee and the volatiles were collected on Tenax TA tubes.

#### *External Static Headspace Sampling (SHS)*

For the external static headspace device 220g of coffee beverage and 45g of additive were used. These liquids were filled in the lower of the two glass vessels (Figure 1b). After an equilibrium time of 15 min at room temperature the lower vessel was replaced by an empty one. Nitrogen was flushed with 100mL/min for 30 minutes and the volatiles were collected on a Tenax TA tube. Standard deviation of 8 replications ranged between 2 and 10% for most of the volatile compounds for both methods.



*Figure 1a and b. External Dynamic Headspace device (1a)  
External Static Headspace device (1b)*

#### *Oral Breath Sampler*

For the OVGC five assessors took 18mL of the freshly brewed beverage in their mouth, the beverage remained in their oral cavity. Their breath was collected using the Oral Breath Sampler (7) as shown in Figure 2. For this they had to place a mouthpiece between their lips in such a way that released volatile compounds were directed onto a Tenax TA tube by a vacuum pump with a constant flow (142mL/min) for 6 minutes. A cold trap (-10°C) was used for freezing out water vapour.

Reproducibility of this method was checked by analysing the same kind of beverage five times with subsequent comparison of the chromatograms.

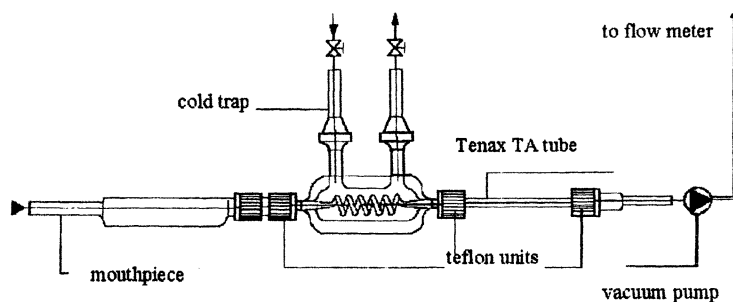


Figure 2. Oral Breath Sampler (J. ROOZEN and A. LEGGER-HUYSMAN, 1994) (Reproduced with permission from reference 7. Copyright 1994.)

## GC-FID, GC-MS and GC-Olfactometry

### DHS / SHS

The volatile compounds were analysed on an HP 5890 GC equipped with an FI-Detector and Sniffing-Port or MS-Detector and Sniffing-Port. The volatiles were desorbed by a thermal desorption device and injected onto a DB-5 capillary column (30m x 0.53mm; 1.5 $\mu$ m film thickness, non-polar). In addition to the DB-5, an OV1701 (60m x 0.32mm; 1.0 $\mu$ m film thickness or 60 x 0.25mm; 0.5 $\mu$ m film thickness, semi-polar) and an FFAP (60m x 0.25mm; 0.5 $\mu$ m film thickness, polar) fused silica capillary column were used.

### Oral breath sampler

The volatile compounds were analysed on a Carlo Erba MEGA 5300 GC equipped with an FI-Detector and/or a sniffing-port. The volatiles were desorbed by a thermal desorption device and injected onto a Supelcowax 10 capillary column (60m x 0.25mm; 0.25 $\mu$ m film thickness).

### Identification of volatiles

The identification of the compounds was achieved by comparison of retention data on DB-5, OV-1701 and FFAP and mass spectral data as well as sensory properties with those of authentic reference substances. Mass spectra were generated at 70eV in the electron impact mode.

## GC/O analysis

The assessors recorded the aroma substances during the sniff runs (Figure 3): When the compound exceeds its threshold, the sniffer records (chart speed: 10cm/min) the event; when the concentration drops below the threshold again, the sniffer also records the event.

Flavour descriptors were generated during preliminary GC / sniffing experiments and clustered to descriptors after group sessions of the panel (five trained assessors).

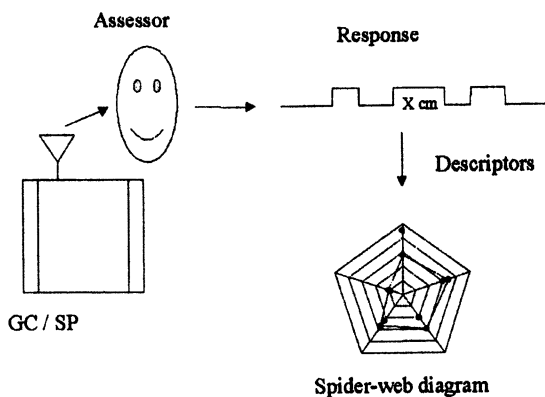


Figure 3. Creation of odour profiles with a GC/O-technique

## Results

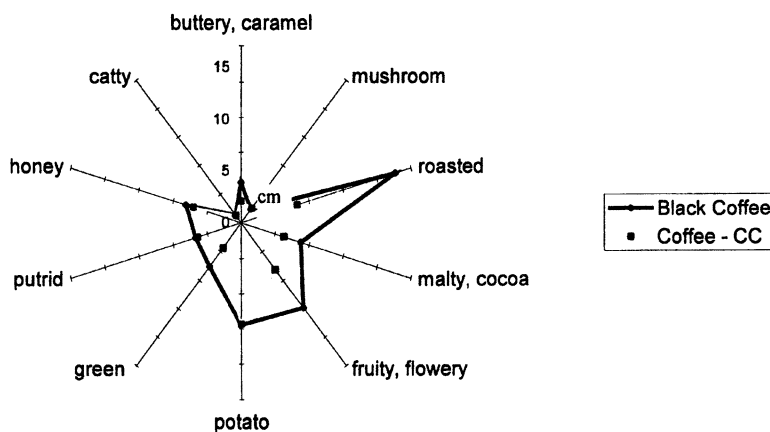
### Comparison between DHS and SHS and the influence of additives on the intensity of coffee volatiles using DHS and SHS analysis

With both methods over 50 potent odorants (Table 2), resulting in a list of ten descriptors, were recognized at the sniffing port. Collecting volatiles with the DHS affected slightly higher amounts of these volatiles. The identification of these volatiles verified most of the contributors of the coffee aroma described in literature (1, 2, 3). The results of the GC/O-analysis showed that both odour profiles were comparable. Thus the influence of non-equilibrium conditions with the DHS was negligible.

In general the additives reduced the intensity of the volatiles (Figure 4), especially for the roasty descriptor. Also the descriptors malty, cocoa, and fruity, flowery showed a significant decrease. The changes in the flavour profile of coffee beverages with an additive can be caused by several effects (8). Interactions (e.g. solution, adsorption, specific binding) of the volatiles with the ingredients of these

**Table 2. Examples of identified potent odorants**

Compound	Retention index on		Aroma quality
	DB-5	OV1701	
methanthiol	<600	<600	putrid
dimethyl sulfide	<600	<600	putrid
2-methylpropanal	<600	616	cocoa
2,3-butanedione	610	665	buttery
3-methylbutanal	653	720	malty
2-methylbutanal	665	726	fruity / malty
2,3-pentanedione	696	755	buttery
2- & 3-methylbutanoic acid	869	980	sweaty
3-methyl-2-buten-1-thiol	881	728	foxy
methional	903	1027	potato-like
2-furfurylthiol	906	1010	roasty / coffee-like
1-octen-3-one	972	1057	mushroom
2, 3, 5-trimethylpyrazine	1002	1072	roasty
3-mercapto-3-methylbutylformate	1027	1218	foxy
phenylacetaldehyde	1055	1171	sweet / honey-like
2-ethyl-3,5-dimethylpyrazine	1062	1151	earthy / roasty
guaiacol	1096	1219	phenolic / burnt
2-isopropyl-3-methoxypyrazine	1144	1144	earthy / roasty
(E)-2-nonenal	1154	1266	cucumber-like
2-isobutyl-3-methoxypyrazine	1186	1237	sweet pepper - burnt

**Figure 4. Influence of Coffee Creamer (CC) on the intensity of coffee volatiles**

### Oral Breath Sampler

Four characteristic impact compounds of the coffee beverage, 2-methylbutanal (2-MB), 3-methylbutanal (3-MB), 2,3-butanedione (2,3-B) and 2,3-pentanedione (2,3-P), were selected for data analysis. These highly volatile compounds dominate the first aroma impression of coffee brews. Furthermore their concentration in the oral cavity enables detection and data evaluation. In agreement with previous investigations (1), the content of 2-MB and 3-MB was higher in Robusta and the content of 2,3-B and 2,3-P was lower in Robusta compared to Arabica. Investigations only with additives showed that except for 2,3-butanedione (whipping cream, coffee whitener) none of the additives had any relevant volatiles which occurred in the black coffee beverage, too. The FID chromatograms of the coffee beverages with additives showed a decrease of the four compounds, caused by the ingredients of these additives. In Figure 5 the combined peak areas of all five panelists shown as percentage related to black coffee.

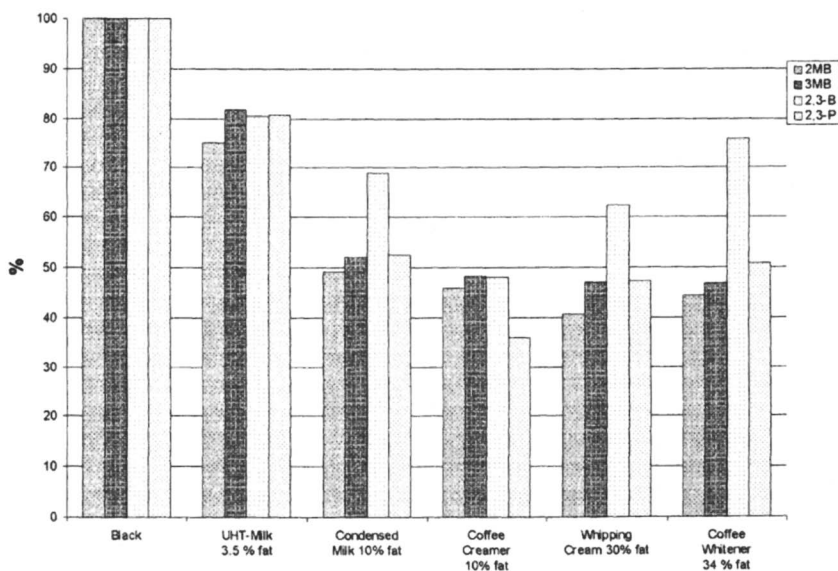


Figure 5. Effect of additives on aroma retention in Robusta coffee beverage: combined peak areas of five panelists for five additives shown as percentage relate to black coffee

## Conclusions

With the SHS and DHS methods the most potent odorants of coffee beverages were determined.

OVGC, carried out with the Oral Breath Sampler, was a valuable method to analyse the release of volatile compounds from the beverages by human volunteers.

The addition of different dairy or vegetable products reduced the amounts of these volatiles in the headspace of the coffee beverage. This effect of retardation caused by components of the additives was typical for each additive.

Further studies will concentrate on structural characteristics that lead to the retention of coffee aroma substances.

## Acknowledgment

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## Chapter 27

# The Chemistry and Technology of Cocoa

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After a short introduction into the history of cocoa and its manufacturing process, the review focuses on the chemistry of cocoa compounds correlated with cocoa quality. I.e., present knowledge on constituents responsible for cocoa bitterness, astringency and flavor is reviewed with special emphasis on quantitative changes occurring during fermentation and roasting. The last chapter discusses fatty acid tryptamides and their use for detecting cocoa mass adulteration.

### Introduction

Besides coffee and tea, cocoa is a third important plant derived food which is preferentially consumed due to its high hedonic value, i.e. its taste and aroma. However, whereas exclusively hot water extracts of coffee powder or tea leaves are consumed, cocoa is eaten as the whole.

Cocoa is manufactured from the so-called cocoa beans which, from the botanical point of view, are the seeds of the cocoa tree *Theobroma cocoa*. The tree was first cultivated in Central America and long before Columbus and Hernando Cortez took the beans over the Europe, Mayas and Aztecs enjoyed the flavor of a drink made of cocoa, water, maize and spices. The drink was called 'Xocolatl' which means „bitter water“ and is the origin of the word chocolate (1). To acknowledge the Aztecs who believed in the divine origin of cocoa, European botanists used the Greek word „theobroma“ which means „Food of the Gods“ as name of the cocoa tree. Also today, due to their unique aroma and taste, cocoa containing foods, in particular chocolate, are liked by consumers all over the world. Excellent and comprehensive reviews have previously been published on the chemical constituents of cocoa and cocoa technology (2-4), and it is quite a challenge to present some new views in such a broad topic. The following review is, therefore, focused on those studies aimed at elucidating compounds which can be proposed as indicators to objectify cocoa quality.

## Cocoa technology

Cocoa trees are cultivated in the humid areas within 15° latitude of the equator. Within 5 to 6 months, the cocoa flowers located on the trunks and main branches develop yellow or violet fruits. These pods are carefully harvested by hand; harvesting time is October to March. As shown in Figure 1, inside the ripe pod, which is about 16 to 20 cm in length and about 7 cm in diameter, twenty to forty seeds are fixed in a mucilaginous pulp.

For manufacturing, the pods are sliced by hand, pulp and beans are withdrawn and this material is then subjected to a fermentation process. The most traditional way is to built up heaps and to cover them with banana leaves (1). More advanced techniques use perforated wooden boxes for curing. From the technological point of view two aims have to be reached by the fermentation process. First, the seeds have to be biologically „dead“, which means that there is no germinating power left and, second, the pulp can easily be removed from the seeds. Depending on the variety, the fermentation time varies from 3 to 7 days.

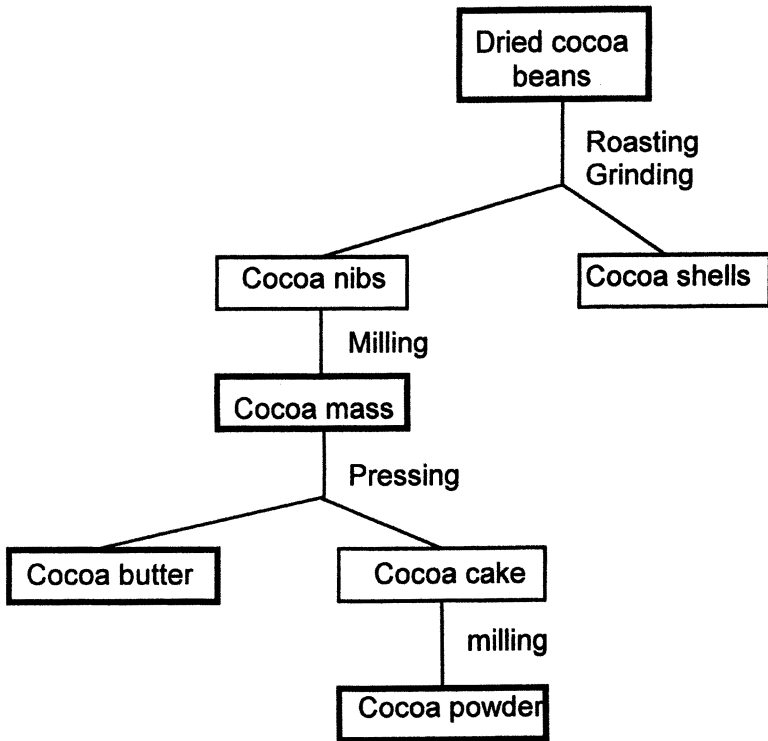
After fermentation, the seeds are usually dried by sun-drying (at about 45°C to 60°C) for about 6 days on trestle tables. Due to an oxidation of phenolic compounds, the beans turn from white to brown in color. In the cocoa manufacturing countries, but more and more also in the producing countries, the dried cocoa beans are then processed as outlined in Scheme 1. First, the dried beans are roasted. The temperatures applied range from 99°C to 121°C for 15 to 70 min. The roasting conditions depend on the further use, e.g., cocoa powder is usually made from higher roasted beans, whilst cocoa butter is produced from slightly roasted beans. The beans are then crushed in the so-called winnowing machines and separated into cocoa nibs and the cocoa shells. The nibs are then milled at elevated temperatures to yield the cocoa mass (or cocoa liquor). By pressing of the mass, two fractions are obtained, namely cocoa butter and the cocoa cake. The latter, by milling, gives the cocoa powder. To increase the intensity of the color and to stabilize cocoa powder suspensions, the Dutchman van Houten in 1829 introduced an alkaline treatment, which may either be performed on the cocoa mass or the cocoa cake.

Two main varieties of cocoa are cultivated named 'Criollo' and 'Forastero'. Due to its higher fruitfulness, the latter covers 90 % of the world cocoa crop. However, due to the distinct aroma formed after roasting, Criollo beans are of higher quality and are mainly used in the manufacturing of darker chocolates.

As shown in Table I, cocoa is becoming increasingly popular, since the world cocoa production has increased by 33 % within the years 1979 to 1994 years. Today, the main cocoa producing countries are the Ivory Coast, Brazil, Indonesia, Ghana and Malaysia. In particular, the more intense cocoa cultivation in Ivory Coast, Indonesia and Malaysia within the last two decades is responsible for the increasing cocoa production.



Figure 1. Cocoa fruit (halved, showing the entire set of cocoa beans)



Scheme 1. Brief outline of cocoa bean processing

**Table I. Comparison of cocoa bean production in 1979 and 1994  
(million tons according to (5))**

Country	Year	
	1979	1994
Ivory coast	<b>0.43</b>	<b>0.81</b>
Brazil	0.33	0.34
Indonesia	<b>0.01</b>	<b>0.28</b>
Ghana	0.27	0.27
Malaysia	<b>0.04</b>	<b>0.23</b>
Nigeria	0.17	0.14
Cameroon	0.12	0.10
Ecuador	0.08	0.08
Colombia	0.04	0.06
Dominican Republic	0.03	0.06
Mexico	0.04	0.04
New Guinea	0.03	0.03
<b>Total</b>	<b>1.63</b>	<b>2.44</b>

## Cocoa chemistry

### Main chemical composition

The predominant chemical class in the cocoa nibs are triacylglycerides (Table II). Interestingly, in contrast to many other plant oils, cocoa fat consists of only three triacylglycerides namely 1,3-dipalmito-2-olein, 1-palmito-3-stearo-2-olein and 1,3-distearo-2-olein in a constant ratio of about 2:4:3. This glyceride composition is the reason for the unique melting behavior of cocoa butter (28°C to 36°C with cooling). Besides proteins and polymeric carbohydrates, such as cellulose, pentosans or starch, the cocoa bean contains comparatively high amounts of mono- and polymeric polyhydroxyphenols (tannins) which are responsible for the brown color developing after fermentation.

It is without doubt that taste and aroma (smell) belong to the most important quality attributes of cocoa and products made thereof. Furthermore, it is generally accepted that both, the fermentation and the roasting procedure have the most significant effect on cocoa chemistry or flavour, respectively. Fermentation is known to decrease bitterness and astringency of the beans and on the other hand to increase acidity, as well as aroma precursors and aroma volatiles. Roasting finally decreases undesired aroma volatiles and leads to the formation of the desired roast aroma compounds.

In the following, changes in cocoa compounds correlated with the quality attributes taste and aroma are described. Correlations were done either by measuring quantitative changes in the compounds themselves responsible

**Table II. Approximate chemical composition (%) of cocoa nibs (according to (6))**

Triacylglycerides (fat)	55.7
Cellulose, Pentosans; Starch	18.0
Proteins	11.9
Polyhydroxyphenols	6.2
Minerals	2.7
Water	2.0
Alkaloids	1.4
Organic acids (Citric acid: 0.6)	1.6
Vitamines, Aroma volatiles etc.	0.5

for the sensory changes, or by using indicator compounds showing a correlation with sensory changes, but having no direct influence on the sensory attributes.

### **Changes in chemical compounds caused by processing**

#### *Taste compounds*

The astringent taste of unfermented cocoa beans is mainly due to phenolic compounds among which the monomeric catechins and leucoanthocyanes predominate. As shown in Figure 2, during fermentation, epicatechin, the main catechin in cocoa beans is decreased to about 10 % of its concentration in the unfermented bean (7). In combination with its taste threshold of 100 µg/kg, these data underline the importance of epicatechin degradation for the decrease in cocoa astringency during curing.

The methylxanthine theobromine, which is present in cocoa beans in concentrations between 1.8 g to 3.8 g per 100 g of dry weight (8) was among the first compounds believed to be responsible for the bitter taste. Because the taste threshold of theobromine is reported to be 10 mg/L in water (9), it is quite evident that the methylxanthine should contribute significantly to the bitter taste of cocoa.

It has been reported in the literature that the fermentation process leads to a decrease in the concentration of theobromine in the cocoa beans. However, recent studies showed (8) that this 'decrease' is caused by a diffusion of theobromine from the cotyledons into the shell during fermentation leading to lower concentrations in the cocoa nibs.

Heating of foods rich in free amino acids may lead to the formation of symmetric di-lactames, the so-called diketopiperazines (DKP). Pickenhagen et al. (10) showed that especially the DKP's formed from hydrophobic amino acids such as cyclo (Phe-Phe) or cyclo (Leu-Phe; structures cf. Figure 3) are extremely bitter. Although both derivatives were not detected in roasted cocoa beans (Table III), the authors (10) showed that, in particular, cyclo (Val-Phe) and cyclo (Ala-Phe), when mixed with theobromine, gave a similar bitter taste when compared to a suspension of cocoa powder in water. Both DKP's were detected as minor compounds in an extract from cocoa powder (Table III).

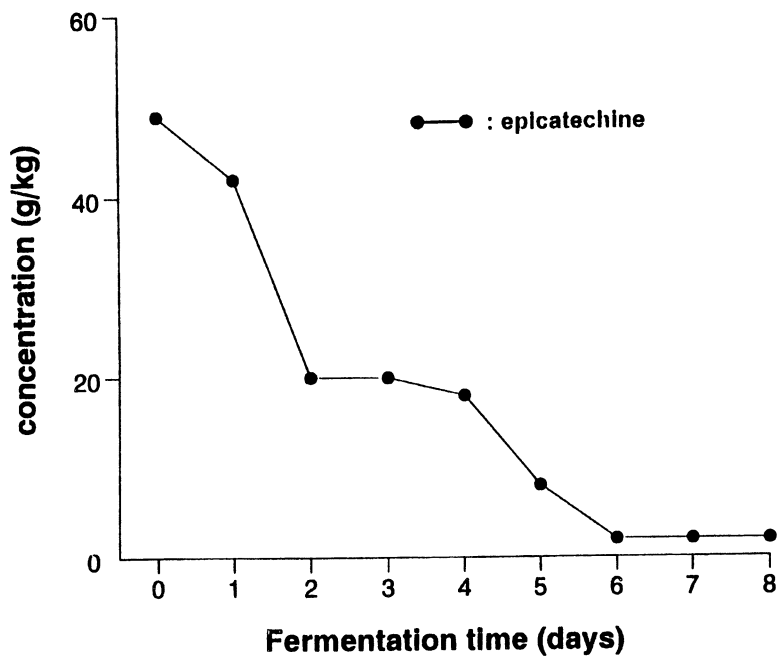
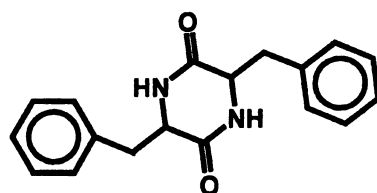
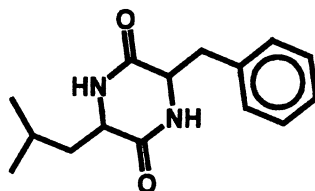


Figure 2. Time course of epicatechine degradation during cocoa fermentation (variety Sanchez; modified according to (7))



Cyclo ( Phe-Phe )



Cyclo ( Leu-Phe )

Figure 3. Structures of bitter tasting diketopiperazines

**Table III. Amounts of diketopiperazines identified in cocoa powder and their contribution to bitter taste (according to (10))**

<i>Diketopiperazine</i>	<i>% Integration</i>	<i>Evaluation of bitterness<sup>a</sup></i>
Cyclo (Ala-Gly)	25.4	±
Cyclo (Ala-Val)	23.7	+
Cyclo (Pro-Leu)	8.0	±
Cyclo (Pro-Gly)	6.9	±
Cyclo (Ala-Pro)	6.1	±
Cyclo (Ala-Phe)	3.0	+++
Cyclo (Val-Phe)	1.6	+++
Cyclo (Gly-Phe)	1.4	+
Cyclo (Pro-Phe)	1.2	n.d.

<sup>a</sup> The taste of a mixture of each diketopiperazine (50 mg) and theobromine (100 mg) dissolved in water (1 L) was compared with that of a suspension of 40 g cocoa powder in 1 L of water.

### Flavor precursors

The fermentation process is believed to be important for the liberation of flavor precursors by enzymic reactions. In particular, changes in proteins and carbohydrates have been extensively studied. This is due to the following reason: The typical aroma of cocoa is mainly formed by chemical reactions occurring during the roasting process. The Maillard reaction between reducing carbohydrates and amino compounds is known to generate caramel-like, roasty or sweet smelling chemicals that are generally believed to contribute to the flavor of thermally processed foods. It is, therefore, quite clear that this reaction was also in the focus of many studies aimed at clarifying cocoa flavor formation.

More than 30 years ago, Rohan and Stewart (11) already showed that during fermentation of different cocoa bean varieties, the soluble nitrogen content was increased by 26 to 59 %. Studies of Reineccius et al. (12), furthermore, indicated that the non-reducing carbohydrate sucrose (1.6 % in the raw beans) is converted into fructose (0.45 %) and glucose (0.22 %) after a three day fermentation. Mohr et al. (13) determined leucine as the predominant free amino acid in fermented, dried cocoa beans followed by alanine and phenylalanine. During roasting of the beans, each amino acid was found to be degraded with phenylalanine showing the most significant decomposition (13) indicating that the Maillard reaction should contribute to cocoa aroma.

As shown in Figure 4, the reaction of free amino acids and, e.g. glucose, leads to the formation of the so-called Amadori-compounds as relatively stable intermediates. Interestingly, such compounds have been identified already in fermented and dried cocoa beans (14). Drying of the beans for 30 min at 98°C slightly increased their concentrations and, in particular, the Amadori-compound of  $\gamma$ -aminobutanoic acid, was enhanced by a factor of

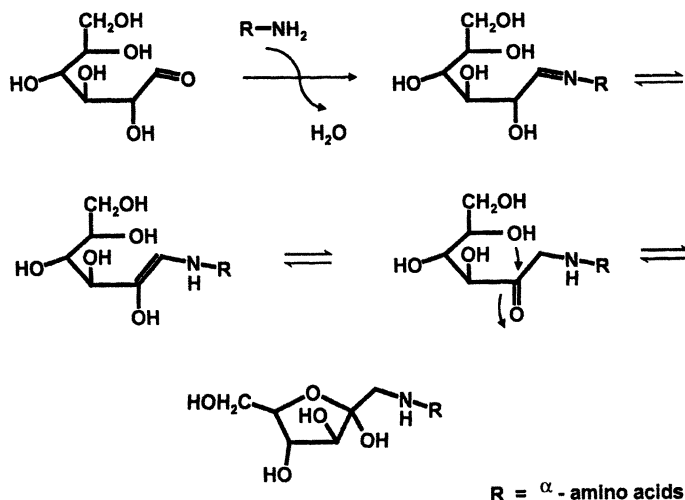


Figure 4. Formation of an Amadori compound from glucose and an amino compound

Table IV. Concentrations of the predominant Amadori compounds in cocoa - influence of drying and roasting

Amadori-compound of	Conc. (mg/kg fat free material)		
	before	after drying (30 min at 98°C)	after roasting (30 min, 130°C)
Leucine	254	229	117
$\gamma$ -Aminobutyric acid	169	667	115
Valine	148	186	51
Alanine	119	314	70
Isoleucine	63	89	36
Threonine	57	66	50

Data modified from (14).

more than four (Table IV). As found for the corresponding amino acids, roasting then led to a drastic decrease of the Amadori compounds.

### Indicator volatiles

Since the first studies by Bainbridge and Davies (15) who identified linalol as an important cocoa volatile, numerous investigations were undertaken to identify other cocoa volatiles and up to now, more than 520



components have been reported to occur in roasted cocoa (16). Nearly one fifth of them are pyrazines (Table V) confirming that Maillard-type reactions play an important role in cocoa flavor development.

In a study aimed at elucidating the influence of cocoa roasting on volatile formation Ziegler (17) showed that among the 95 cocoa volatiles taken into consideration, the six compounds listed in Table VI were most significantly increased during roasting. However, except the caramel-like smelling 4-hydroxy-2,5-dimethyl-3(2H)-furanone, the resting compounds given in Table VI, in the author's opinion, are not likely to contribute to the cocoa flavor, since their odor thresholds are much higher than their concentrations reported in the roasted beans.

**Table V. Volatiles identified in cocoa materials (according to (16))**

<i>Substance class</i>	<i>Number</i>
Pyrazines	94
Esters	69
Acids	62
Hydrocarbons	49
Amines	41
Alcohols	34
Ketones	29
Furans	27
Aldehydes	26
Sulfur compounds	23
Oxazoles	15
Quinoxalines, piperazines and other bases	15
Phenols	9
Acetals	7
Nitriles, amides	7
Epoxides	6
Lactones	6
Ethers	4
Halogens	2
<b>Total:</b>	<b>525</b>

**Table VI. Cocoa volatiles<sup>a</sup> showing a significant increase in their concentration after roasting of cocoa beans (modified from (17))**

<i>Compounds</i>	<i>Conc. range</i>	
	<i>before</i>	<i>after roasting</i>
4-Hydroxy-2,5-dimethyl-3(2H)-furanone (Furaneol)	I	III
2,3-Dihydro-3,5-dihydroxy-6-methyl-4-pyranone	II	IV
2-Methylpyrazine	I	III
2-Acetylpyrrole	I	III
2-Pyrrolcarbaldehyde	I	III
1,2-Benzene diol	I	III

<sup>a</sup> The concentrations of 95 cocoa volatiles were determined. Concentration range I: <0.1 ppm; II: 0.1-0.5 ppm; III: 0.5-2.0 ppm; IV: 2-10 ppm.

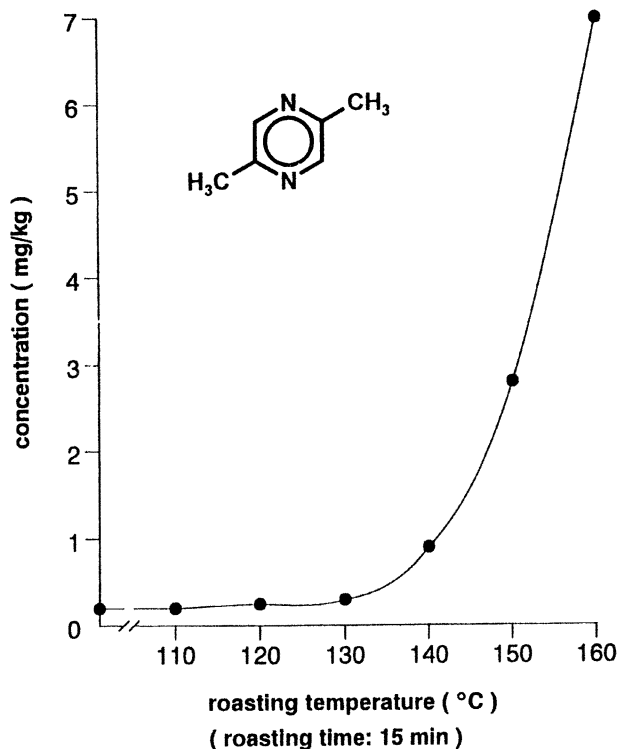


Figure 5. Time course of the formation of 2,5-dimethylpyrazine depending on the roasting temperature (according to (18))

Selected Maillard reaction products may, however, be used as indicators, e.g., to evaluate the roasting degree of cocoa beans. Two odorless volatiles, namely 2,5-dimethylpyrazine (18) and 2,3-dihydro-3,5-dihydroxy-6-methyl pyranone (19) have been proposed, and successfully used for this purpose. As shown in Figure 5, up to a roasting temperature of 135°C, the concentration of 2,5-dimethylpyrazine in the cocoa beans remains below 0.5 mg/kg. Above 140°C there is a steep increase in the pyrazine concentration which was accompanied by a burnt off-odor in the roasted beans.

### Flavor volatiles

Compounds contributing to a food can be distinguished from the bulk of rather odorless volatiles by GC/Olfactometry using dilution to odor threshold

approaches such as CHARM analysis or the Aroma Extract Dilution Analysis (AEDA; cf. review in 20). By application of the AEDA on a cocoa mass prepared from fermented, roasted cocoa beans, 36 odor-active volatiles were detected. The results of the identification experiments in combination with the Flavor Dilution (FD) factors revealed the eight odorants shown in Figure 6 as the most odor-active in cocoa mass (21). The results suggested in particular the sweaty smelling iso-pentanoic acids, the malty smelling 3-methylbutanal and the fruity smelling ethyl 2-methylbutanoic acids as important odorants.

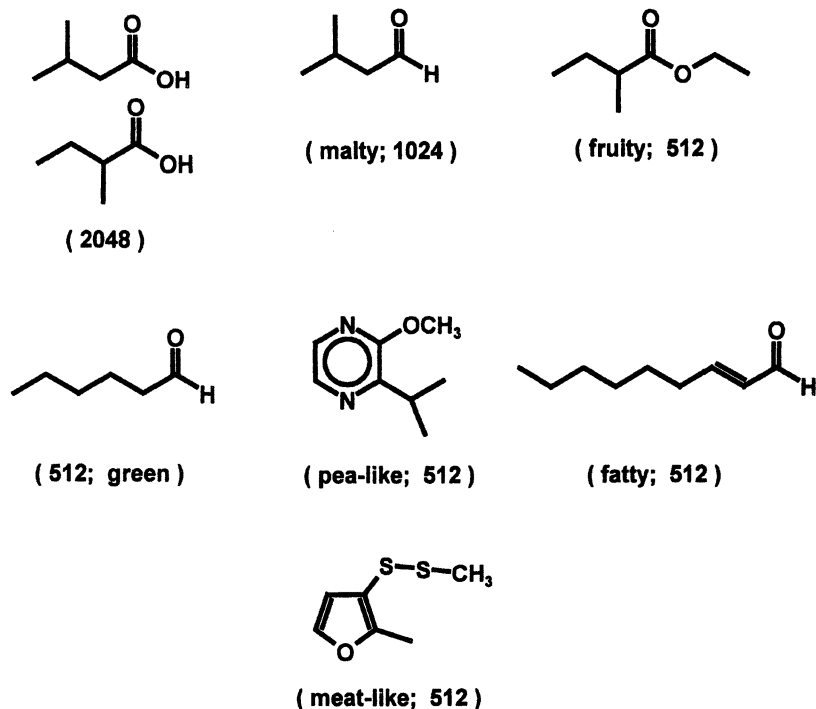


Figure 6. Structures of key odorants identified in cocoa mass (21)

In Tables VII to IX, the 30 odorants identified (21) are listed according to their possible source of formation. The data show that 3-methylbutanal, 2-methyl-3-(methylthio)furan and 2,3-diethyl-5-methylpyridine followed by phenylacetaldehyde are among those odorants most likely formed by Maillard-type reactions (Table VIII). The significant contribution of 3-methylbutanal and 2-phenylacetaldehyde is well in line with the high amounts of the respective precursor amino acids leucine and phenylalanine in cocoa (13). However, also lipid degradation reactions seems to play an important role in cocoa odor, because well-known degradation products of unsaturated fatty acids such as hexanal, (E)-2-octenal and (E)-2-nonenal showed high

FD-factors (Table VIII). Interestingly, also the plant or, alternatively, the microbial metabolism seems to generate further important odorants. E.g., 2-methoxy-3-isopropylpyrazine, known as key odorant in fresh bell pepper, was found with a comparatively high odor activity in the cocoa mass. The fruity, sweet smelling esters ethyl 2-methylbutanoate or ethyl phenylacetate might either stem from the plant metabolisms or may be formed during curing.

Quantitative studies on these key odorants are, however, necessary further steps to elucidate, e.g., the time course of the flavor formation during roasting or the influence of the fermentation on odorant concentrations in the cocoa mass.

**Table VII. Odorants in cocoa mass proposed to be formed by carbohydrate/amino acid reactions (according to (21))**

<i>Odorant</i>	<i>Odor quality</i>	<i>FD-factor<sup>a</sup></i>
3-Methylbutanal	malty	1024
2-Methyl-3-(methylthio)furan	meat-like	512
2,3-Diethyl-5-methylpyrazine	potato-chip like	256
Phenylacetaldehyde	sweet, honey-like	64
Dimethyltrisulfide	sulfurous	32
Trimethylpyrazine	earthy, roasty	32
2-Ethyl-3,6-dimethylpyrazine	potato chip-like	32
2,3-Diethyl-5-methylpyrazine	potato chip-like	32
4,5-Dimethyl-3-hydroxy-2(5H)-furanone (Sotolon)	spicy	32
5-Ethyl-4-methyl-3-hydroxy-2(5H)-furanone (Abhexon)	spicy	32
2,3-Butandione	buttery	16

<sup>a</sup> The Flavour Dilution (FD) factor is a relative measure of the odor contribution of a single odorant in an extract (cf. (21)).

**Table VIII. Odorants in cocoa mass proposed to be formed by lipid degradation (according to (21))**

<i>Odorant</i>	<i>Odor quality</i>	<i>FD-factor</i>
Hexanal	green	512
(E)-2-Octenal	fatty, waxy	512
(E)-2-Nonenal	green, fatty	256
(Z)-4-Heptenal	sweet, bisquit-like	64
Nonanal	soapy	32
(Z)-2-Nonenal	green, tallowy	32
(E)-2-Decenal	fatty, green	32
(E,E)-2,4-Nonadienal	deep-fat fried	32
1-Hexen-3-one	linseed-like	16

**Table IX. Odorants in cocoa mass probably formed by either the plant or the microbial metabolism during fermentation (according to (21))**

<i>Odorant</i>	<i>Odor quality</i>	<i>FD-factor</i>
2- and 3-Methylbutanoic acid	sweaty	2048
Ethyl 2-methylbutanoate	fruity	1024
2-Methoxy-3-isopropylpyrazine	pea-like	512
Ethyl phenylacetate	sweet, waxy	128
$\gamma$ -Decalactone	peach-like	64
Ethyl 2-methylpropionate	fruity	32
2-Phenylethyl acetate	fruity, sweet	32
2-Phenylethanol	sweet, yeast-like	32
$\delta$ -Octenolactone	sweet, coconut-like	32
Ethyl cinnamate	sweet, cinnamon	32

**Table X. Concentrations of behenic acid tryptamide (BAT) and lignoceric acid tryptamide (LAT) in commercial chocolates**

<i>Sample</i>	<i>No.</i>	<i>BAT (<math>\mu\text{g/g}</math> of chocolate)</i>	<i>LAT (<math>\mu\text{g/g}</math> of chocolate)</i>	<i>Concentration of tryptamides based on the fat content (<math>\mu\text{g/g}</math>)<sup>a</sup></i>
Bitter chocolate	1	6.79	13.38	63.0
	2	2.70	5.43	23.1
	4	4.61	9.26	42.9
	5	5.64	10.90	41.4
Milk chocolate	1	3.44	6.69	34.7
	2	4.67	9.43	49.0
White chocolate		5.04	10.38	43.4

<sup>a</sup> The fat content was determined by extraction of the sample with diethyl ether in a Soxhlet apparatus.

### Detection of the shell content in cocoa mass

The German Food Law limits the content of cocoa shells in cocoa butter and cocoa mass to 5 %. Very recently, lignoceric acid tryptamide (LAT) and behenic acid tryptamide (BAT) were identified as the main fatty acid tryptamides occurring in cocoa shells in concentrations between 300 to 390 mg/kg (22), whereas in the cocoa cotyledons their concentrations were much lower. Based on heptadecanoyl-2-(3-indolyl)ethane amide as the internal standard, and using HPLC with fluorescence detection, a very sensitive method was developed for the quantification of tryptamides in cocoa products and chocolate (22). As shown in Table X, significant differences

were found in the tryptamide concentrations of several commercial chocolates indicating different amounts of shells in the cocoa materials used for the chocolate production. However, further authentic samples have to be analyzed before this method can be recommended in the assessment of cocoa mass adulteration.

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## Chapter 28

# Flavor Development of Cocoa during Roasting

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The desired flavor of cocoa results from the compositional changes that occur in the beans principally during roasting. The roasting process of cocoa beans is necessary to develop the final chocolate flavor. The flavor of cocoa is highly complex in character, and unfortunately not yet fully understood, due to the very large number of different substances present. Maillard reaction or non-enzymatic browning is one of the most important and complex reaction involving flavor development during the roasting process. Sugars and amino acids are the components that undergo the most significant changes during roasting and are suited for use as monitors of compositional changes occurring during the process. There are around 500 volatile compounds which have been already identified in cocoa aroma. The roasting process, heating the cocoa beans to 120-140°C during 20-40 minutes in general depending on cocoa bean type, is needed to develop the final flavor desired.

Chocolate manufacturers are very concerned with the sensory properties of cocoa beans when assessed as finished chocolate (1). Flavor in cocoa beans is influenced by several variables including the type of beans used and processing in chocolate factory. Cocoa beans are transformed into chocolate by a combination of many techniques such as fermentation, roasting and conching. The roasting technique is one of the most important factors in determining the organoleptic properties of cocoa beans and eventually chocolate. With chocolate the chemical complexity of flavor development is evident when one realizes the numerous parameters which influence its development (2).

The controlled high-temperature heating step of roasting that magnifies the complex interaction between the flavor precursors and the results in chocolate flavor. Prior to roasting, the beans may taste astringent, bitter,

acidic, musty or nutty. Roasting reduces the acidity as evidenced by the significant decrease in the concentrations of volatile acids, especially acetic acid (3; 4). The roasting process, normally heating the beans to 110-140°C for 20-40 minutes to develop the final flavor desired.

The generation of flavor, the types of reaction and the influencing factors during roasting are very complex fields of study. The development of new techniques have given the chocolate manufacturer a wide choice of methods of carrying out cocoa beans roasting. It is essential for each manufacturer to use one which will produce the appropriate flavor. Cost is also important and considerable differences exist between the capital and running costs of the different procedures (5).

During roasting, one of the most important and complex reactions involving cocoa flavor is called the non-enzymatic browning, Maillard browning or the carbonyl-amine reaction. These reactions involve two major precursors namely the free amino acids and reducing sugars which develop during fermentation (6; 7).

Modern analytical techniques such as capillary gas chromatography and mass spectral data of the volatile aroma fraction have contributed to the understanding of chocolate flavor. There are about 500 compounds which have been already identified in chocolate.

### **The precursors of cocoa flavor**

Work on cocoa aroma started many years ago. The characteristic of chocolate arises as a result of two principal stages in the processing of cocoa bean. The first is called fermentation, which is affected where the cocoa is grown. The second stage is accomplished in the factory, where the fermented beans are roasted. In the absence of either process, no cocoa aroma is observed. Roasted unfermented cocoa beans do not generate cocoa aroma but produce an excessively bitter and astringent taste instead (8).

Investigations into the origin of the characteristic aroma of cocoa beans have essentially followed two different ways. The first sought to concentrate the volatile aroma from fermented and roasted cocoa beans and tried to characterize its components. The second was the indirect approach, which attempted to isolate from unroasted beans the compounds which gave the characteristic aroma when heated, the so called flavor precursors which have developed during the fermentation of cocoa beans.

Free amino acids (leucine, alanine, phenylalanine and tyrosine), peptides and reducing sugars have been shown to be precursors of cocoa flavor and undergo Maillard reactions to produce cocoa flavor during roasting (9; 10).

During fermentation, free amino acids and peptides are formed by proteolytic enzymatic reactions, whereas the reducing sugars, such as, fructose and glucose are the products of invertase hydrolysis of sucrose (8). During the fermentation process, the biochemical process will result in the production of flavor precursors, which later will develop into cocoa-specific flavor and aroma upon roasting.

During fermentation, the total amino acids concentrations showed an almost linear increase with increasing days of fermentation, up to day 4, after which the values remained relatively constant as the fermentation proceeded



to day 6. At the end of the fermentation the concentration of leucine, phenylalanine, tyrosine, valine and isoleucine increased significantly (11). These amino acids accumulated during cocoa fermentation, contribute positively to the formation of cocoa flavor.

In unfermented cocoa beans, sucrose is the only sugar present in significant concentration (more than 90% of the total sugars), fructose and glucose present in trace amounts. During fermentation, the sucrose decreased significantly. In contrast, fructose and glucose concentration increased.

Reducing sugars and amino acids form addition compounds during Maillard reactions, which in turn form glycosylamines or fructosylamines, depending on the initial reducing sugar. One of the key reactions is the rearrangement of these glycosylamines into isomerization products. If glucose is the starting sugar in the reaction, it would be converted to aminated fructose. The intermediate reactions involve the previous compounds as well as sugar and amino acid degradation (Strecker). They are essential for the proper flavor of a cocoa product and involve interactions of numerous compounds.

Odors from mixtures of leucine and glucose, threonine and glucose, and glutamine and glucose, when heated to 100°C, have been described as 'sweet chocolate', 'chocolate' and 'chocolate' respectively. Valine and glucose, upon heating to 180°C, have been described as 'penetrating chocolate'. A change in temperature elicits a change in description, when heated 20°C higher, the leucine-glucose mixture was described as 'moderate breadcrust'.

The isomerization products formed during the initial phase are primarily addition compounds formed from amino acids and sugar. During the intermediate stage they are then dehydrated, fragmented and transaminated, forming complex compounds, depending on temperature and pH. On the acid side, generally hydroxymethylfurfural and other furfural products are formed. If the pH is neutral, the result of the reaction is reductones. In the end a host of compounds, depending on the substrates and the pH, will polymerize and in turn contribute to the final cocoa flavor. Some of the most important compounds are pyrazines, pyrroles, pyridines, imidazoles, thiazoles, and oxazoles.

As important as the carbonyl-amine reactions are, one must realize that it is unlikely that a food system will consist of only amino acids and sugars. Other compounds such as peptides, proteins, vitamins, fats and their oxidation products and other derivatives can enter the reactions and influence the final product. With the many compounds found in chocolate, it is virtually impossible to identify all reactions and pathways needed to produce chocolate flavor (2).

### **Roasting conditions**

The development of cocoa flavor, effective roasting temperatures are necessary depending on the origin of cocoa. The optimum roasting times applied industrially depend mainly on the heat transfer and the temperature gradient in the products. If the maximum temperature and time conditions are exceeded signs of over roasting become noticeable which diminish the

quality of final product. The greatest problem in roasting cocoa beans is controlling a uniform temperature throughout the product.

There are three different roasting procedures to produce cocoa mass from cocoa beans. The first is the roasting of whole cocoa beans which has several disadvantages, among them the size of the beans varying according to the source and the growth season. The degree of roasting which each receives will depend upon its size.

Cocoa beans vary somewhat in size and shape with 1g being a representative weight (12). If the roasting time is optimized for medium-sized cocoa beans, the small beans are inevitably overheated as a result. This has an unfavourable effect on the taste of the cocoa liquor. In addition the large cocoa beans are not roasted sufficiently, which is also detrimental to the final chocolate. The temperature differences between the outer skin and the center of the bean that exist during roasting may be 12°C. This allows the conclusion to be drawn that in the normal course of events over roasting of the outer layer cannot be avoided while the inner parts of the beans may still contain raw components, resulting unequal roasting of the whole bean (13). The advantage of whole beans roasting however is that it loosens the shell making it easier to remove, in order to aid the winnowing of unroasted beans.

The second is Nib roasting which has the advantage to remove the shell before beans roasting, the major problems of fat migration, off-flavors from burning impurities and extra energy use are overcome. In addition the particles being roasted are of a more uniform size, resulting more uniform roasting intensity of beans. The main disadvantage of this type of roasting procedure is that it is necessary to remove the shell from unroasted beans.

The third is the Mass roasting, in this technique the main problems of bean roasting again are overcome, and a uniformity of heat treatment is ensured. The heat input is definitely more efficient during mass roasting in which the homogeneous mass is kept in constant movement instead of the slowly turned solid mass of beans or nibs (14). The beans could be roasted in thin layer, mass roasted in this way under optimum temperature conditions reaches a high flavor intensity without any of the defects arising out of over roasting. This could be explained by a negligibly small temperature gradient, good heat transfer. This procedure, however, requires both the winnowing and the grinding of raw beans. All these methods are currently in use and favoured by different cocoa processors.

The quantity of reducing sugar and amino acids consumed during cocoa bean roasting is variable depending on the type of cocoa beans and time-temperature of the heat treatment. Figure 1 shows the results obtained for roasted cocoa beans (Ivory Coast) during 25 minutes at 110°C to 170°C (15). It is obvious that the quantity of reducing sugars consumed are in linear relation with the roasting temperature. At 140°C the totality of reducing sugars are already consumed in the Maillard reaction, and only 40% of amino acids are still non consumed. This would indicate that the addition of reducing sugars is efficient to improve or to change the flavor of the final product.

It is now possible to control the process of cocoa roasting by adding reducing sugars, amino acids, or a mixture of both, depending on the characteristics of the original cocoa beans. Knowing the exact specification desired, and by testing the raw material, it is possible to control the Maillard

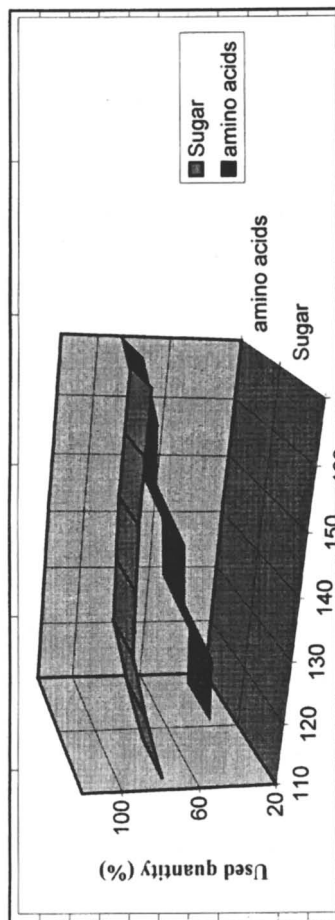


Figure 1. Influence of roasting temperature on the precursors of cocoa flavor

reactions during the roasting of the cocoa beans, so the taste of the final product could be exactly tailored to demand.

Figure 2 shows the process of producing cocoa mass from cocoa beans.

### Aroma compounds formed

The flavor of cocoa has been the subject for intense research during the last three decades. Many researches have been realized to review all volatile components in cocoa beans. There are almost 500 different components (15) divided over 20 different chemical classes.

**Table 1 : Number of identified compounds in cocoa**

<i>Compound</i>	<i>Number</i>
Hydrocarbons	48
Alcohols	28
Aldehydes	21
ketones	27
Esters	61
Lactones	6
Amines	36
Pyrroles	15
Pyridines	15
Pyrazines	95
Sulfurs	9
Thiazoles	9
Thiols	4
Phenols	8
Furans	23
Oxazoles	15
Acetals	6
Ethers	4
Halogens	2
Nitriles	3
Bases	12
Acids	53

The principal components are : pyrazines which represent about 20% of the identified compounds, then the esters 15%, the acids and hydrocarbons 10%, the amines 8%, the alcohols and ketones 6%, the furans and aldehydes 4%.

### Roasting control

Many working parties have studied the possibility to control cocoa beans roasting. The question is very complex, only because cocoa beans contain a

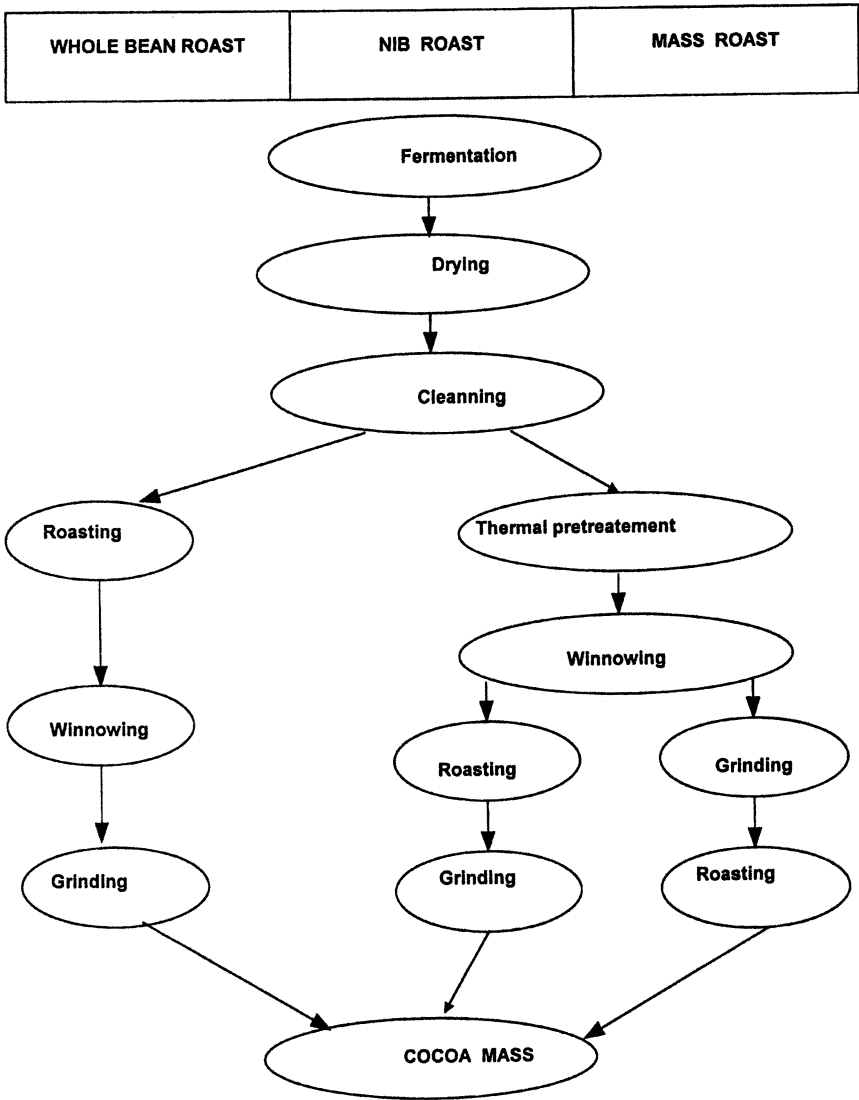


Figure 2 : Process of producing cocoa mass from cocoa beans

large number of substances that have detected which contribute positively to the typical cocoa flavor. For example, the aldehyde has an important role in cocoa bean roasting. The evolution of isopentanal during bean roasting has shown that a concentration of this molecule equal to 60 ppm would give a normal roasting of the beans and a concentration equal to 70 ppm shows an over roasting of the beans (13).

The total quantity of aldehydes could be used as indicator of cocoa roasting (15). Figure 3 shows the evolution of the total aldehyde during cocoa beans roasting. At 120°C the concentration of aldehydes was in its maximum. Their concentration slow down from 130°C. At this temperature the quantity of reducing sugar is practically non existent (Figure 1). That would suggest the importance of reducing sugars to develop aldehydes in cocoa beans during roasting.

The 2,3-dihydro-3-hydroxy maltol was detected in roasted cocoa by HPLC technique (17). It was shown that, the concentration of this compound showed a linear correlation with respect to the extent of roasting and therefore it can be used as an indicator substance for the determination of the degree of cocoa beans roasting.

The pyrazines principal components of cocoa beans flavor, and their ratios could be used as indicator for the degree of cocoa beans roasting. Their concentrations are in a linear correlation with the time-temperature cocoa bean roasting (13; 15; 18). Figure 4 shows the formation of certain methylpyrazines during cocoa beans roasting. The ratio between tetra-/2,5di- and tetra-/tri- decreased at high temperature, over roasting of cocoa beans. Figure 5 shows the same observations when cocoa beans roasted at 150°C at different times.

The recent application of the electronic nose to determine the degree of cocoa beans roasting has shown the possibility to predict the quality of cocoa beans and to monitor the cocoa aroma (19).

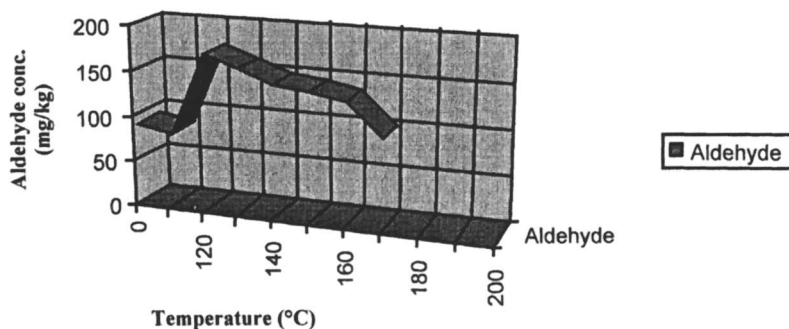


Figure 3 : Influence of roasting temperature on the quantity of total aldehyde

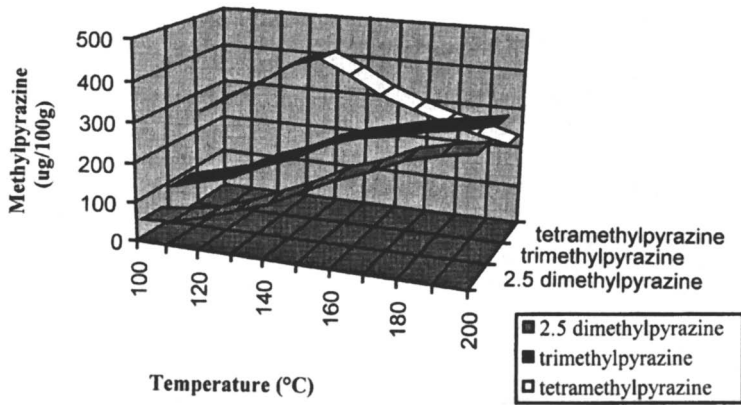


Figure 4 : Formation of certain methylpyrazines in laboratory roasted cocoa beans (Ivory Coast) during 30 min.

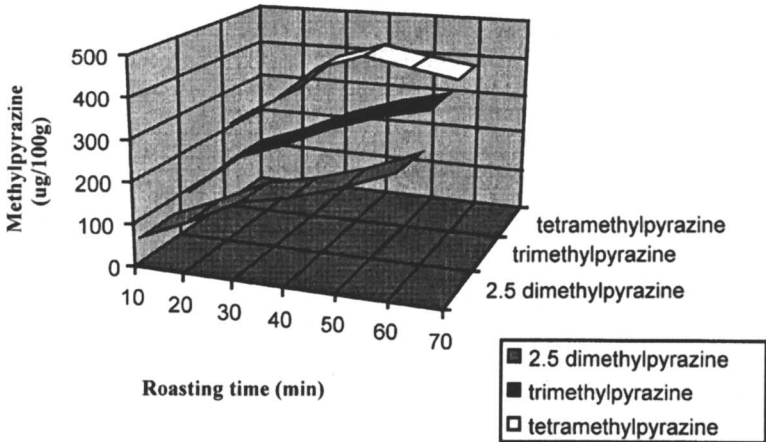


Figure 5 : Formation of certain methylpyrazines in laboratory roasted cocoa beans (Ivory Coast) at 150 $^{\circ}\text{C}$ .

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## Chapter 29

# Chocolate Flavor via the Maillard Reaction

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The characteristic chocolate flavor is a direct result of the interactions of amino acids, sugars, and other flavor precursors. Three samples of cocoa flavor were analyzed. 5-Methyl-2-phenyl-2-hexenal and 4-methyl-2-phenyl-2-pentenal, both of which impart penetrating chocolate-like notes to the overall flavor, are found in the reaction product. Compared to the others, the reaction chocolate flavors have broader varieties and lower production costs.

Chocolate typically derives its characteristic flavor as a result of multiple processes, two of which are very important. The first is the fermentation or curing of the raw beans that produces flavor precursors like amino acids, reducing sugars, and other flavanoids (1). The second is the roasting of the fermented cocoa beans to generate aroma compounds via the thermal reaction. The roasted cocoa beans are pressed and ground to produce chocolate liquor, which is further processed to produce cocoa butter, cocoa powder, or confectionery chocolate (2).

With the interest of producing inexpensive replacements for expensive natural flavors, scientists have extensively studied flavors, flavor precursors, and chemical processes. Many model reaction systems that produce chocolate flavors have been reported. Hodge (3) and Herz and Shallenberger (4) produced chocolate aromas in the reaction of valine and leucine with sugars. Pinto and Chichester (5) studied cocoa beans and found that leucine and valine were the predominant free amino acids in fermented cocoa beans. Recently, a few patents have described processes yielding chocolate flavor. One of them was registered by Watterson (6), which was directed toward a means of enhancing the cocoa flavor by roasting phenylalanine, valine, leucine, and other amino acids with reducing sugars in a fat matrix. These research studies have led to the conclusion that chocolate aromas are results of complex chemical reactions of amino acids, sugars, and other flavor precursors during the thermal process.

## Thermal Reactions and Reaction Products

The Maillard reaction, that results from the interaction of amino acids and sugars, is the most important process in the development of cocoa flavor. The initial Maillard reaction to chocolate can be understood by comparing the changes of free amino acids during the roasting process (7). Reineccius (8) indicated that two-thirds of the initial amount of free amino acid are degraded in the thermal process. Lysine, arginine, glutamine, and asparagine degraded significantly, but they are more associated to the browning reaction or color formation.

Strecker aldehydes are those derived from the degradation of amino acids, by a reaction called Strecker degradation. 3-Methylbutanal, 2-methylbutanal, 2-methylpropanal, and phenylacetaldehyde are generated by the Strecker degradation of leucine, isoleucine, valine, and phenylalanine respectively. The main route in the formation of Strecker aldehydes during the roasting process is the condensation of amino acids with carbonyls to form a Schiff's base. This base later enolizes and decarboxylates. The new Schiff's base, with one carbon atom less, is hydrolyzed to an amine and an aldehyde containing one carbon atom less than the initial amino acid. Most of the degradation products of amino acids are not found in cocoa products; they may, in fact, further degrade or enter browning reactions. Arnoldi, et. al. (9) investigated Strecker degradation of leucine and valine in a lipid model system at different temperature conditions. Their results demonstrated that cocoa fat significantly enhanced the formation of 2-methylpropanal and 3-methylbutanal.

Pyrazines are another important class of compounds that are present in great numbers and in high concentrations in cocoa flavor. They impart a desirable aroma to chocolate flavor. More than eighty alkylpyrazines and their derivatives have been found in cocoa products so far. The chemistry of the formation of pyrazines has received considerable attention and their mechanism of formation via the condensation of amino acids and reducing sugars has been investigated quite extensively.

Aliphatic aldehydes, ketones, and aliphatic acids are derived from both enzymatic and nonenzymatic lipid oxidation during the fermentation and roasting processes of cocoa beans due to the fact that cocoa beans are comprised of about 50% fat. The basic mechanism of lipid autooxidation is considered to occur at an elevated temperature as a free radical chain leading to the formation of hydroperoxide intermediates. The intermediates, in turn, break down to form volatile products. A great number of lipid oxidation products have been identified in cocoa flavor at high concentrations.

Phenolic compounds contribute very important odor and taste to chocolate flavor. Surprisingly, only a few phenolic compounds have been identified in cocoa flavor even though cocoa beans contain about 15% non-volatile phenolic compounds such as tannins and purines (10).

Pyrroles, oxazoles, and thiazoles are also present in cocoa flavors. These compounds possess strong and interesting sensory characteristics even though their concentrations are quite low. The chemistry of these heterocyclic compounds has been

extensively investigated and it has been found that most of these classes of compounds are associated with Strecker degradation, deamidation, desulfurization, and the Maillard reaction.

## Control of the Maillard Reaction

The Maillard reaction involves condensation of the carbonyl of a reducing sugar with a free amino group of a peptide or amino acid. This rearranges to form an Amadori product. The Amadori product dehydrates to form furfurals, reductones, and fission products. These reactive species include: aldehydes generated by Strecker degradation, ammonia formed via deamidation, and hydrogen sulfide generated by desulfurization. These species will undergo a series of complex reactions to develop volatile aromatic compounds or non-volatile, brown, nitrogenous polymers known as melanoidins.

In order to produce high quality products, the chemistry of the Maillard reaction and factors that affect the reaction must be understood. The most important determinations of reaction conditions are: composition of the system, temperature, time, pH, and water activity.

Composition of the system is a primary factor to be considered based upon the flavor being developed. Cysteine and pentoses are essential flavor precursors in the development of meaty flavors; valine and leucine greatly contribute to chocolate flavors; proline and hydroxyproline result in bread and cracker aromas; methionine is a key amino acid in the production of a French fry flavor; and lysine results in the darkest color of the Maillard reaction. Certain proteins and protein hydrolysates have been, in recent years, successfully used to develop specific food flavors due to the fact that they contain the correct proportions of amino acids.

Reaction temperature and time are critical control points in the Maillard reaction. An increase in temperature increases the rate of Maillard reaction, while an increase in temperature and/or time of heating results in both aroma compound production and color development. Above a certain temperature, the color development is much faster than the formation of aroma compounds in the Maillard reaction. Finer control of reaction temperature makes it possible to balance flavor profile and control color development in this type of reaction.

Water activity is another factor that influences the flavor profile and color development in the Maillard reaction. A lower water activity is more beneficial to the formation of heterocyclic compounds since the Maillard reaction occurs in the absence of water; however, these compounds could possibly contribute to over-roasted or burnt notes in the final products. Color development may cause a serious problem if the reaction occurs in water solution at an elevated temperature.

Low pH values result in the formation of furans and furan derivatives, while higher pH values result in reductones or fission products. Although there is no clear explanation, the most desirable meaty flavor/aroma is obtained at pH 4-5, while a chocolate flavor/aroma can be developed at a pH above 6.

## Experimental

A cocoa extract was purchased from Sanofi Bio Ingredients while an N&A chocolate flavor and a reaction chocolate flavor were prepared by Universal Flavors. Fifty grams of each of these three flavors were weighed out separately into three 1000 mL flasks that contained 500 grams of water each. The mixtures were then simultaneously steam-distilled and extracted at atmospheric pressure into methylene chloride using a Likens-Nickerson apparatus. The distillates were dried over anhydrous sodium sulfate and concentrated under a stream of nitrogen to a final volume of 0.1 mL.

A Hewlett Packard 6890 gas chromatograph coupled to a Hewlett Packard 5973 mass spectrometer was used to analyze the volatile compounds that were isolated from samples in a quantitative and qualitative manner. GC conditions were as follows: DB-1 column (30 m x 0.25 mm i.d., 1  $\mu$ m, J&W); 250°C injector temperature; 1 mL/min. helium flow rate; split ratio of 100:1; and temperature program 70°C hold for 2 min., then 4°C/min. to 150°C and hold for 3 min., then 8°C to 250°C and hold for 20 min.

## Results and Discussion

Compounds identified from Cocoa Extract, N&A Chocolate Flavor and Reaction Chocolate Flavor are compiled in Tables I, II and III.

Cocoa bean are composed of about 50% fat, 1.5% free amino acids, 10% protein, and 15% carbohydrates (10), all of which are very important flavor precursors. These flavor precursors will undergo Strecker degradation, sugar degradation, lipid oxidation, and Maillard reaction processes during the roasting process. Three major groups of volatile compounds are Strecker aldehydes and their derivatives, lipid oxidation products and their derivatives, and Maillard reaction products. Isovaleraldehyde, valeraldehyde, phenylacetaldehyde are typical Strecker aldehydes. They have very penetrating, chocolate-like, sweet, creamy notes and are found in many processed foods. Lipid oxidation products and their derivatives impart fatty, fruity, warm notes to the extract, while pyrazines contribute nutty, roasted, grassy notes to the flavors. Indole possesses powerful floral notes at a low concentration (<0.1%). Even though 15% tannin was present, no phenolic compounds were identified, probably due to the volatile nature of these particular compounds.

Cocoa extract possesses sweet, brown, roasted, chocolate-like, phenolic notes. It, however, is not commonly used in the food industry as a flavor. In the confectionery industry, sugar, milk, and flavor are added into chocolate liquor in order to produce high quality chocolate flavors.

N&A Chocolate flavor was prepared using cocoa extract as a base, and then compounded with other chemicals to enhance the top notes. Furfural and furfuryl alcohol impart sweet, burnt, cinnamon-almond-like caramellic notes, and vanillin contributes sweet, balsamic, hay-like notes. 5-Methyl-2-phenyl-2-hexenal is an important compound that has sweet, floral, woody, caramellic-fruity, and chocolate-

like notes. Methyl amyl ketone and methyl nonyl ketone tend to enhance fresh, fruity, and creamy notes in the flavor.

**Table I. Volatile Compounds Identified from Cocoa Extract**

<i>Compound Identified</i>	<i>GC Peak %</i>	<i>Compound Identified</i>	<i>GC Peak %</i>
Isovaleraldehyde	0.764	2,5-dimethyl-3-(3-methylbutyl)pyrazine	0.135
Valeraldehyde	0.159	Phenylacetaldehyde PG acetal	0.115
Isobutyric acid	0.281	Decanoic acid	0.240
Isovaleric acid	2.004	5-Hydroxy-2-decenoic acid delta-lactone	1.425
Valeric acid	0.900	Lauric acid	0.105
2,5-Dimethylpyrazine	0.312	Myristic acid	0.718
Isovaleraldehyde PG acetal	0.205	Methyl palmitate	10.982
Trimethylpyrazine	0.462	Palmitic acid	4.526
Phenylacetaldehyde	1.205	Ethyl palmitate	0.442
2-Ethyl-3,(5 or 6)dimethyl- pyrazine	0.068	Ethyl linoleate	5.021
Tetramethylpyrazine	0.529	Ethyl oleate	10.673
Benzeneacetic acid, methyl ester	0.653	Methyl stearate	2.750
Indole	0.128	Ethyl linoleate	0.501

**Table II. Volatile Compounds Identified from N&A Chocolate Flavor**

<i>Compound Identified</i>	<i>GC Peak %</i>	<i>Compound Identified</i>	<i>GC Peak %</i>
Isovaleraldehyde	0.210	Phenylacetaldehyde	2.290
2,3-Pentanedione	0.044	Limonene	0.165
Propyl acetate	0.180	Acetophenone	0.070
Hexanal	0.080	2-Nonenal	0.260
Furfural	0.326	Octanoic acid	17.730
Furfuryl alcohol	0.165	Methyl nonyl ketone	1.870
Methyl amyl ketone	0.460	Decanoic acid	14.210
2,5-Dimethylpyrazine	0.470	Vanillin	47.300
Benzaldehyde	0.990	5-Methyl-2-phenyl-2-hexenal	0.125
Hexanoic acid	1.400	Lauric acid	9.560
Trimethylpyrazine	1.340		

**Table III. Volatile Compounds Identified from Reaction Chocolate Flavor**

<i>Compound Identified</i>	<i>GC Peak %</i>	<i>Compound Identified</i>	<i>GC Peak %</i>
Isovaleraldehyde	1.696	2,3-Dimethylpyrazine	0.124
Valeraldehyde	0.028	Benzaldehyde	0.025
2,3-Pentanedione	0.075	2-Ethyl-5-methylpyrazine	0.502
Pyrazine	0.267	Trimethylpyrazine	0.940
Dihydro-2-methyl- 3(2H)furanone	0.157	2-Ethyl-3-methylpyrazine	0.271
2-Methylpyrazine	1.333	Phenylacetaldehyde	20.019
Furfural	0.602	2-Phenylfuran	1.857
Furfuryl alcohol	0.265	4-Methyl-2-phenyl-2-pentenal	1.025
Acetylfuran	0.181	Vanillin	2.579
2,5-Dimethylpyrazine	2.996	5-Methyl-2-phenyl-2-hexenal	1.849
2-Ethylpyrazine	0.315		

Reaction chocolate flavor has sweet, roasted, caramellic, phenolic, and chocolate-like odors. It can be used as a dark chocolate flavor or be used as a base to develop various natural or N&A chocolate flavors. Since the reaction chocolate flavor is produced directly by the Maillard reaction process, unlike the chocolate liquor or cocoa extract production processes, it has stronger flavor and a lower production cost. The second advantage of reaction chocolate flavor is that the flavor precursors and conditions can be altered in order to produce a variety of desirable flavors based upon their application.

Two important compounds, 4-methyl-2-phenyl-2-pentenal and 5-methyl-2-phenyl-2-hexenal were identified in the reaction flavor. The two compounds impart sweet, floral, woody, caramellic-fruity, chocolate-like, and penetrating top notes to the chocolate flavor. 4-Methyl-2-phenyl-2-pentenal is derived from the interaction of two Strecker aldehydes: isobutanal and phenylacetaldehyde, followed by a loss of a molecule of water. 5-Methyl-2-phenyl-2-hexenal is derived from the aldol-condensation of isovaleraldehyde and phenylacetaldehyde, two Strecker aldehydes from leucine and phenylalanine.

## Conclusion

The aroma of processed food is derived from the degradation and interactions of amino acids, lipids, sugars, and other flavor precursors during processing. Therefore, it is possible to simulate these processes in order to develop flavors with higher qualities, stronger aromas, and at lower costs.

5-Methyl-2-phenyl-2-hexenal and 4-methyl-2-phenyl-2-pentenal, both of which are found in the reaction product, make a significant contribution to the chocolate flavor.

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## Chapter 30

# Differentiating the Flavor Potential of Cocoa Beans by Geographic Origin

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Unique flavors are derived from cocoa beans that originate from different geographic regions. Continually monitoring these flavor differences using a trained sensory panel can be effective; however, this process is time consuming, costly, and subjective in nature. To simplify the screening process and, more objectively, gauge the flavor potential of cocoa beans, this study sought to develop a multivariate statistical model to enable predictions of cocoa bean flavor from analytical measurements of the volatiles emitted during nib roasting. Twelve cocoa bean samples were examined using traditional bean quality tests to assess fermentation, headspace/gas chromatography (GC) to provide analytical characterization, and subsequently, made into chocolate and evaluated by a trained sensory panel. Both the analytical and sensory data matrices allowed the cocoa beans and their corresponding chocolates to be distinguished and similarly grouped using principal component analysis (PCA). When the data matrices were subjected to partial least squares regression analysis (PLS) to test for correlation, a limited predictive model was generated.

Despite the global appeal of chocolate and its long history of consumption, scientists have not fully explained the mechanism by which chocolate flavor arises. Many of the known reactions, which contribute to the chemistry of chocolate flavor, have been described in review articles (1-3). Hoskin and Dimick (3) described the formation of chocolate flavor as dependent upon genotype, geographic origin, proper fermentation, drying, roasting, and processing of the cocoa beans.

Chocolate flavor consists of both volatile and non-volatile chemical compounds that contribute to its complexity. Much of what scientists know about chocolate flavor

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is the result of research on the volatile aroma fraction. Maarse and Visscher (4) reported that as many as 503 volatile compounds occur in cocoa. Flament (5) provided identification for 462 volatile compounds present during chocolate production and new compounds are continually being discovered and identified (6). Differences between the volatiles from one sample to another have been successfully used to identify cocoa beans by origin, understand flavor development, and interpret process changes (7-10). Among the hundreds of volatile compounds present in foods, often a small number, in specific ratios, determine the characteristic odor. In a complex flavor like chocolate, finding this cause-and-effect relationship has been elusive. Even today, the chemist is unable to accurately duplicate this desirable flavor.

With numerous countries supplying cocoa beans, combined with the variation that can occur during fermentation, the task of consistently formulating chocolate according to flavor specifications can be difficult. Besides compositional and physical differences, cocoa beans originating from different supply countries possess their own unique flavor. Traditionally, chocolate manufacturers have considered West African cocoa beans to be the industry flavor standard. However, other supply countries have undergone considerable efforts to improve the flavor quality of their cocoa beans. Today, a common practice among chocolate manufacturers is to select cocoa beans from several countries and blend them.

Economic considerations and changing supply patterns have complicated the selection of cocoa beans and reinforced the importance of performing quality tests on incoming raw materials. Many of the quality tests for cocoa beans assess the degree of fermentation and by themselves provide limited insight into the flavor potential of the beans. Researchers can increase the value of these tests by correlating their results with sensory scores. Sensory tests, for raw materials and finished products, are necessary and effective for assessing their flavor quality. However, cocoa beans are difficult to assess for flavor until they have been roasted or semi-processed making them impractical, costly, and time consuming to evaluate. Because the flavor potential of cocoa beans is difficult to assess and often subjective in nature, the present study served to expand on existing bean quality tests and identify meaningful statistical relationships between analytical and sensory data that could be used for predictive modeling.

## Materials and Methods

### Cocoa Beans

A total of 12 cocoa bean samples were obtained from a commercial supplier. The samples and their *descriptive names*, when available, were from Brazil (*Bahia*), Ecuador (*Arriba*), Ghana, Ivory Coast, Indonesia (*Sulawesi*), Malaysia, and two from Dominican Republic (*Sanchez* and *Hispaniola*). Three lots from Ivory Coast (890P, 864, 889) and Indonesia (891P, 892, 909) were carried through the study as separate samples to assess the variation between cocoa beans of the same origin. All of the samples were, to some extent, fermented and dried (cured) except the Dominican Republic (*Sanchez*) sample, which was unfermented.

## Cocoa Bean Quality Tests

### *Cut-test Score*

The cut-test (11) is a visual assessment of 100 halved cocoa beans. Results are qualitative and based on criteria such as cotyledon color and defects. A modified version of the cut-test (12) was used to generate more quantitative results in the form of cut-test scores. One hundred halved cocoa beans were assessed as either slaty (unfermented), fully purple, three-quarter purple, half brown, three-quarter brown, or fully brown (fermented) and scored 1 through 6, respectively.

### *Fermentation Index*

Fermentation indices (FI) were determined from extracts prepared from ground (40 mesh) raw cocoa. Extracts were prepared with 0.5g cocoa in 50 mL of methanol and hydrochloric acid (97:3). Extracts were cooled to 8°C and held for 16-18 hrs. before the cocoa solids were removed by vacuum filtration. Absorbance values were determined for the extracts from 200 nm to 900 nm using a scanning spectrophotometer. The fermentation index was obtained by calculating the ratio of absorbance at 460 nm to 530 nm (13). Triplicate determinations were conducted and reported as mean  $\pm$  standard deviation. In addition to FI determination, complete spectrophotometric scans were included to provide a more revealing look at the differences between samples.

### *pH and Titratable Acidity*

A 10 g sample of raw cocoa (40 mesh) was added to 90 mL of boiling water. The mixture was stirred vigorously and cooled to 20-25°C in an ice bath. The pH was determined using a pH meter standardized with pH 4.0 and pH 7.0 buffers. Following pH measurement, each sample was titrated with 0.1 N sodium hydroxide to an endpoint of pH 8.1. Triplicate determinations were performed for both pH and titratable acidity and reported as mean  $\pm$  standard deviation.

## Sensory Analysis

Dark chocolates were prepared from the 12 cocoa bean samples using minimal processing and a simplified recipe to allow the cocoa derived flavors to dominate (14). Finished chocolates were 57% cocoa mass, 40% 10X confectionery sugar, and 3% deodorized cocoa butter. A trained sensory panel evaluated the chocolates for 8 attributes. The attributes evaluated were sour, bitter, chocolate, fruity, nutty, smoky, burnt, and astringent mouthfeel. Computer generated 15 point intensity scales (Compusense, Guelph, Ontario), anchored with reference standards, were used to collect the data (14). The 15 point scale went from 0=none to 15=very strong.

## Analytical Characterization

Gas chromatograms of the volatiles emitted during nib roasting were used for analytical characterization. Nibs were roasted in sealed vials under static conditions and the headspace was auto-sampled and injected into a GC with a flame ionization detector. The capillary column used was a 30 M X 0.25 mm id Supelcowax™10 with a film thickness of 0.25  $\mu\text{m}$  (Supelco, Inc., Bellefonte, PA). Column temperature was held at 50°C for 5 min, then increased at 5°C/min to 220°C and held isothermally for 10 min. Chromatograms were integrated and divided into 8 retention time segments (A-H) that were used to quantify the volatiles and differentiate the samples (14). The time segments used to divide the chromatograms were chosen based on inspection. Retention time segments were selected at points where peaks could be clearly grouped and where sufficient peaks fell within each segment. The same 8 retention time segments were maintained throughout all of the samples.

## Statistical Analyses

Sensory data were analyzed by a two-way ANOVA with interaction, for each of the 8 defined flavor attributes. Cocoa bean origin, panelist and their interaction were the factors used in the general linear model (GLM) procedure within the SAS statistical program (SAS Institute, Inc. Cary, NC). Analytical data were analyzed by a one-way ANOVA for each of the 8 GC retention time segments. Cocoa bean origin was the factor used in the GLM procedure. Means for both sensory and analytical data were separated using Duncan's Multiple Range procedure at  $\alpha=0.05$ .

## Multivariate Statistics

Multivariate statistics examine many variables simultaneously, while reducing them to a few factors capable of providing the most information. Multivariate statistical techniques used for this study were Principal Component Analysis (PCA) and Partial Least Squares regression (PLS) (15-16). PCA was the initial step used to plot or map the cocoa bean samples by origin. Plots were generated for both the sensory and analytical data to determine whether the two maps would group the cocoa beans similarly. Secondly, PLS regression was used to identify correlation between the sensory and analytical data that could be used for predictive modeling. Other researchers (17-18) have successfully used PCA and PLS to develop robust statistical models capable of predicting sensory scores from analytical data. However, Chien and Peppard (19) emphasized that seemingly reliable correlations between sensory attributes and analytical data do not necessarily indicate a cause-and-effect relationship. Researchers can only validate these cause-and-effect relationships through further experiments specifically designed to test them.

## Results and Discussion

### Cocoa Bean Quality Tests

Results from the cocoa bean quality tests were used to assess the degree of fermentation and acidity level within the cocoa bean samples. Individually, each of the cocoa bean quality tests provides directional information, but together they provide a more complete picture. The samples have been listed in Table I by cut-test score. Although previous research (12) showed a strong correlation between the cut-test score and fermentation index, the results from this study did not correlate well. In agreement with earlier work (20-21), linear relationships were observed between pH and titratable acidity with a correlation coefficient of  $-0.93$ . Aside from being correlated to one another, pH and titratable acidity were highly correlated with FI. The correlation coefficients which related FI to pH and titratable acidity were  $-0.89$  and  $0.95$ , respectively. In addition to the FI values derived from absorbance at 460 and 530 nm, complete spectrophotometric scans were included to more visually reveal differences between the cocoa bean extracts (Figures 1 and 2). The Ecuador (Arriba), Indonesian, and D.R. (Sanchez) samples, with their higher absorbance values at 530 nm, were determined to be less fermented. Greater variation in fermentation was observed within the 3 lots from Indonesia as compared to the more consistent fermentation of the 3 lots from Ivory Coast (Figure 1).

**Table I. Summarized Results from the Cocoa Bean Quality Tests**

<i>Sample</i>	<i>Cut-test Score</i>	<i>Fermentation Index</i>	<i>pH</i>	<i>Titratable Acidity</i>
I.C. 889	594	$1.1281 \pm 0.0109$	$5.78 \pm 0.08$	$0.141 \pm 0.004$
Braz. Bahia	593	$1.4597 \pm 0.0010$	$5.31 \pm 0.04$	$0.193 \pm 0.008$
I.C. 809P	589	$1.1595 \pm 0.0161$	$5.76 \pm 0.02$	$0.126 \pm 0.004$
Ecu. Arriba	578	$0.7647 \pm 0.0194$	$6.37 \pm 0.11$	$0.101 \pm 0.004$
Ghana	566	$1.2530 \pm 0.0250$	$5.39 \pm 0.05$	$0.149 \pm 0.005$
Malaysia	483	$1.9080 \pm 0.0127$	$5.21 \pm 0.05$	$0.200 \pm 0.009$
I.C. 864	441	$1.0522 \pm 0.0030$	$5.78 \pm 0.05$	$0.129 \pm 0.006$
Ind. 909	438	$0.7827 \pm 0.0239$	$6.10 \pm 0.05$	$0.103 \pm 0.004$
Ind. 892	429	$0.6077 \pm 0.0052$	$6.09 \pm 0.03$	$0.097 \pm 0.004$
D.R. Hisp.	352	$1.4870 \pm 0.0056$	$5.24 \pm 0.01$	$0.176 \pm 0.002$
Ind. 891P	337	$0.7424 \pm 0.0087$	$6.09 \pm 0.05$	$0.086 \pm 0.001$
D.R. San.	231	$0.5929 \pm 0.0280$	$5.91 \pm 0.03$	$0.110 \pm 0.001$

NOTE: Fermentation Index equals the ratio of absorbance at 460 nm to 530 nm. Titratable Acidity expressed as meq NaOH/g sample.

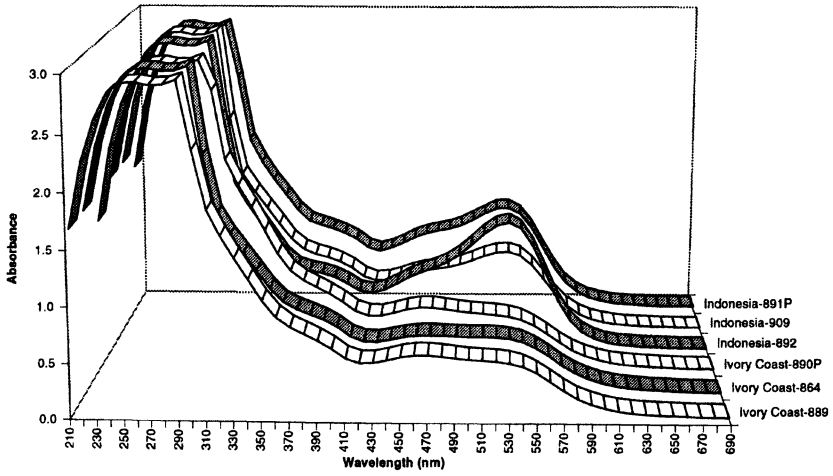


Figure 1. Spectrophotometric scans of FI extracts from Indonesia and Ivory Coast.

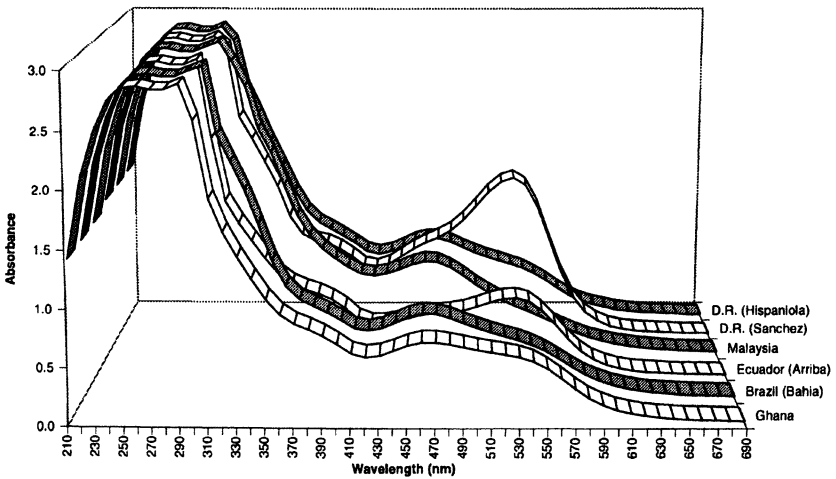


Figure 2. Spectrophotometric scans of FI extracts from Malaysia, Dominican Republic (Hispaniola), Dominican Republic (Sanchez), Ghana, Ecuador (Arriba), and Brazil (Bahia).

Overall, the results from the cocoa bean quality tests served as excellent indicators of cocoa bean fermentation. The well fermented samples included the 3 lots from Ivory Coast along with samples from Brazil (*Bahia*) and Ghana. The moderately fermented samples were from Malaysia and Dominican Republic (*Hispaniola*). The poorly fermented or unfermented samples originated from Ecuador (*Arriba*), Indonesia (*Sulawesi*), and Dominican Republic (*Sanchez*). Although still underfermented, the Indonesia 909 sample was better fermented than the other Indonesian samples.

## Sensory and Analytical Results

Sensory results have been tabulated in Table II. Significant differences were observed at  $P < 0.05$  for 7 of the 8 sensory attributes tested. Although the Ecuador (*Arriba*) sample scored highest for fruity, significant differences were not observed between the chocolates which led to the omission of fruity from Table II. The sensory results from all 8 attributes were considered simultaneously using a PCA plot to spatially arrange or map the samples (Figure 3). Samples in close proximity to one another, within Figure 3, produced chocolates that were more similar in their total sensory profile. Samples from Ghana, Ivory Coast, and Ecuador all clustered within the lower left quadrant meaning their total flavor profile was more similar. The less fermented Indonesian samples were less tightly grouped within the lower right quadrant due most likely to their higher variation with regard to fermentation. Interestingly, the better fermented Indonesia 909 sample was closer in flavor to the West African samples and this was reflected directionally within the PCA plot.

Analytical results were evaluated similarly. Individual retention time segments from the GC profiles were used to differentiate the samples. When differences between the 8 designated retention time segments were considered simultaneously, a PCA plot was generated based on the analytical data (Figure 4). Samples located in close proximity to one another, within Figure 4, were more similar with regard to the volatiles they emitted during nib roasting. In Figure 4, the Ghana and Ivory Coast samples grouped towards the center of the plot while the Indonesian samples clustered towards the upper left quadrant. Once again, the better fermented Indonesian 909 sample tended towards the West African samples.

Similarities were observed between the PCA plots for both the sensory and analytical data. The loading values for the sensory PCA plot, Figure 3, were included as Figure 5 to provide an understanding of how individual sensory attributes influenced this plot. Along principal component 1, samples that were more bitter and astringent tended towards the positive direction while samples that exhibited stronger chocolate, sour, and fruit flavor tended toward the negative direction. Along principal component 2, samples that were more sour, smoky, and burnt were plotted in the positive direction while the more fruity and nutty samples tended towards the negative direction. Loading values for the analytical data were not included because the analytical data were used predominantly as predictor values and their corresponding loading values provided little useful information on their own.

Table II. Mean Sensory Scores for Dark Chocolates

Chocolate		More ↑ Less	Bitter			
Sample	Mean Score		Sample	Mean Score		
I.C. 809P	7.7750 A	More ↑ Less	Ind. 892	4.8937 A		
Ghana	7.6063 A		Ind. 891P	4.7937 A		
I.C. 889	7.2687 AB		D.R. San.	4.1500 AB		
I.C. 864	7.2125 AB		Ind. 909	3.4812 BC		
Braz. Bahia	6.7187 BC		Malaysia	3.3750 C		
Ind. 909	6.6187 BC		I.C. 864	3.1625 C		
D.R. San.	6.5250 BCD		Ghana	3.1437 C		
D.R. Hisp.	6.3875 CDE		I.C. 889	2.7750 CD		
Ind. 891P	6.1625 CDE		D.R. Hisp.	2.7375 CD		
Malaysia	5.9312 CDE		Braz. Bahia	2.7312 CD		
Ecu. Arriba	5.7687 DE		I.C. 809P	2.7250 CD		
Ind. 892	5.5875 E		Ecu. Arriba	2.2062 D		
Sour			Astringent (mouthfeel)		Smoky	
Sample	Mean Score		Sample	Mean Score	Sample	Mean Score
Braz. Bahia	4.2500 A	Ind. 892	5.5375 A	Malaysia	4.7812 A	
Malaysia	3.9562 AB	Ind. 891P	5.2437 A	D.R. San.	1.6562 B	
D.R. Hisp.	3.4312 B	D.R. San.	4.3000 B	Braz. Bahia	1.6562 B	
Ind. 891P	2.4187 C	Ind. 909	3.1437 C	I.C. 864	1.3250 B	
I.C. 889	2.3937 C	Malaysia	3.0750 C	D.R. Hisp.	1.2250 B	
Ghana	2.3875 C	Ghana	2.9562 C	Ind. 891P	0.9688 B	
I.C. 809P	2.2562 C	I.C. 889	2.7437 C	I.C. 890P	0.8938 B	
I.C. 864	2.1937 C	I.C. 864	2.7000 C	Ghana	0.8813 B	
D.R. San.	2.0312 C	Ecu. Arriba	2.6437 C	I.C. 889	0.8250 B	
Ecu. Arriba	2.0062 C	I.C. 809P	2.5062 C	Ecu. Arriba	0.8125 B	
Ind. 892	1.9937 C	D.R. Hisp.	2.3500 C	Ind. 909	0.7875 B	
Ind. 909	1.8250 C	Braz. Bahia	2.2812 C	Ind. 892	0.7438 B	
Nutty		More ↑ Less	Burnt			
Sample	Mean Score		Sample	Mean Score		
Ind. 909	1.8812 A		D.R. San.	4.1250 A		
Ind. 892	1.8125 A		Malaysia	4.0937 AB		
D.R. San.	1.6812 A		D.R. Hisp.	3.9062 ABC		
I.C. 809P	1.6500 A		I.C. 864	3.4625 ABCD		
Ecu. Arriba	1.5875 AB		Ind. 892	2.9625 ABCDE		
Ind. 891P	1.4062 ABC		I.C. 889	2.9312 ABCDE		
Ghana	1.3750 ABC		Ind. 909	2.9000 BCDE		
D.R. Hisp.	1.2875 ABC		Ind. 891P	2.7750 CDE		
I.C. 864	1.2687 ABC		Braz. Bahia	2.4187 DE		
I.C. 889	1.2062 ABC		Ghana	2.3562 DE		
Malaysia	0.9250 BC		Ecu. Arriba	2.3250 DE		
Braz. Bahia	0.8750 C		I.C. 809P	2.1812 E		

NOTE: Means followed by the same letter [A,B,C,D,E] are not significantly different ( $P < 0.05$ ).

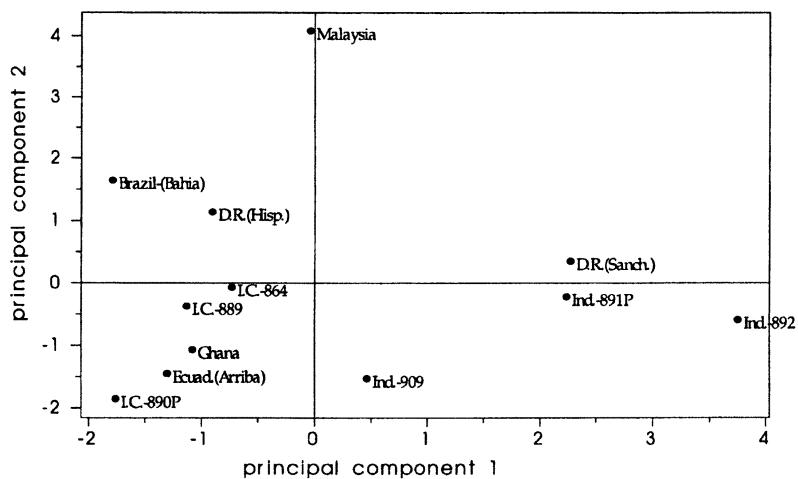


Figure 3. *Principal Component Analysis of the Sensory Data.*

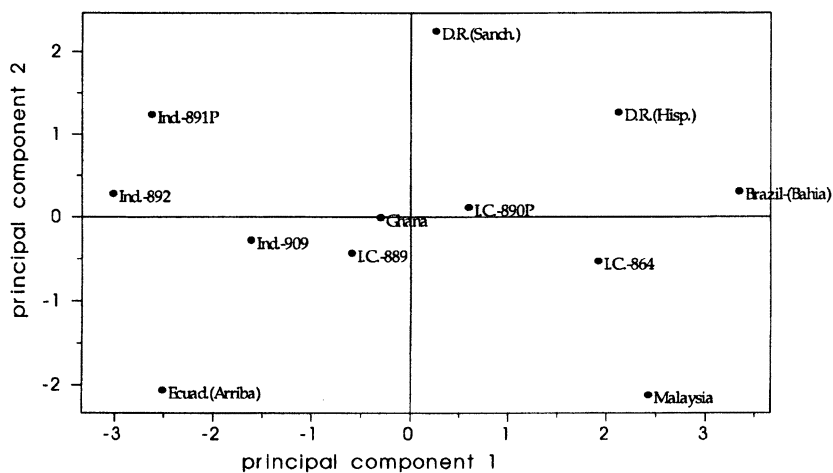


Figure 4. *Principal Component Analysis of the Analytical Data.*



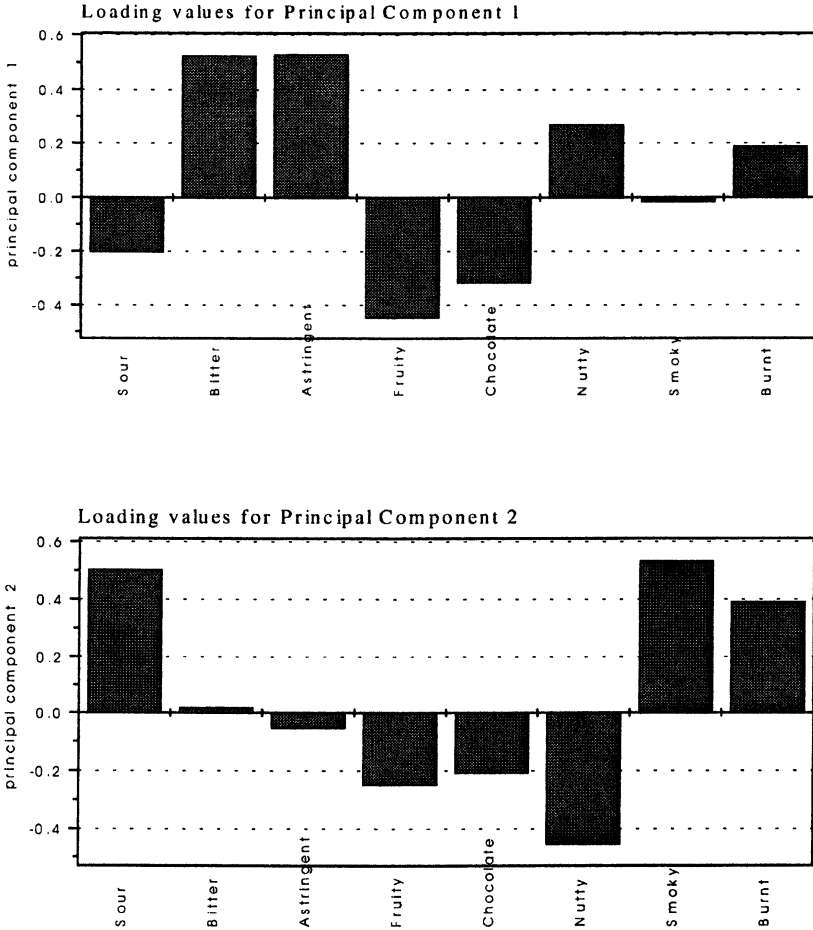


Figure 5. Attribute Loadings for the Sensory PCA plot (Figure 3).

**Correlation between Sensory and Analytical Data**

The sensory and analytical data were subjected to PLS regression to identify relationships between the two data matrices that could be used for predictive modeling. Unfortunately, this analysis revealed a co-linear relationship within the analytical variables limiting the usability of the predictive model. When higher

concentrations of volatiles were quantified within one GC retention time segment, then the other GC retention time segments, for the same sample, were also higher. Due to this co-linearity within the analytical variables only some general conclusions could be made. Cocoa bean samples that emitted higher concentrations of volatiles during nib roasting, across their entire GC profile, correlated with chocolates that scored higher for sour and smoky. Inversely, cocoa bean samples that emitted fewer volatiles during nib roasting, across their GC profile, correlated with chocolates that scored higher for nutty, bitter, and astringency.

## Conclusions

The cocoa bean quality tests resulted in a thorough fermentation assessment of the 12 cocoa bean samples in which relationships between the quality tests were observed. Linear relationships were noticed between pH, titratable acidity and fermentation index. Additionally, all three of these tests correlated with sour flavor in the chocolates. These results were in agreement with Holm *et al.* (22) who concluded that higher concentrations of lactic and acetic acid in the cocoa beans would result in higher sour scores for corresponding chocolates. The cocoa bean samples and their corresponding chocolates were differentiated by sensory and analytical methods that resulted in similar PCA plots. The PLS analysis revealed co-linearity within the analytical variables which resulted in a limited predictive model and some general conclusions. Cocoa bean samples that emitted higher concentrations of volatiles during nib roasting, across their entire GC profile, correlated with chocolates that scored higher for sour and smoky and lower for nutty, bitter, and astringency.

Although the cocoa bean samples examined in this study were believed to be representative of their corresponding origin, the results and conclusions drawn from this study pertain specifically to the 12 cocoa bean samples tested. Additional samples would need to be evaluated to determine whether the samples from this study were accurate representations of the total sample population for a given origin.

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## Chapter 31

# The Chemistry of Guaraná: Guaraná, Brazil's Super-Fruit for the Caffeinated Beverages Industry

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A naturally occurring source of caffeine that has received limited attention is the seed of guaraná fruit. Guaraná, a low growing bush-type plant, is cultivated primarily in the Amazon rain-forest area of Brazil and several other Latin American countries. Guaraná seed contains 3.5-7% caffeine on a dry weight basis and is likely the richest known vegetable source of caffeine. Features of the guaraná plant are presented with an emphasis on uses for the caffeinated beverages industry. Specifics include horticulture of guaraná, treatment of unprocessed product, commercial processing and chemical changes during processing.

\* Approved for publication by the Director for the Louisiana Agricultural Experiment Station. Trade names are used solely to provide specific information. Mention of a trade name does not constitute a warranty by the Louisiana Agricultural Experiment Station of the LSU Agricultural Center of the product nor an endorsement to the exclusion of other products not mentioned.

## Historical

In the year of 1669, a missionary priest named Betendorf reported his findings of the consumption of beverages made from the seeds of a fruit common among tribal members of the Andirás Indians of the Amazon River Basin in Brazil (1). The explorers, Humboldt and Bonpland, collected the plant on an expedition through the upper Orinoco basin in Venezuela in 1810 (2,3). The plant was later classified in 1821 as *Paullinia cupana* (2). The genus gets its name from C. F. Paullini, a German medical botanist who died 1712 (2).

In the 1920's the first chemical analyses of *P. cupana* were conducted by Varg in Cassicourt, England and Theodore von Martius. Martius isolated a chemical component that he called 'guaranine' (4). Of the 180 species of *Paullinia* found strictly in the neotropics (with the exception of *P. pinnata*), nearly 40 have been used for centuries by the indigenous people primarily as medicines and stimulating beverages (5).

Traditional cultivation and processing of guaraná (*P. cupana*) is still practiced by the Saterê-Maué Indians of the Central Amazon basin. The processing steps include picking the ripened, eye-shaped seeds as the fruit shells begin to open, removing the white aril and roasting the seeds to facilitate removal of the hard seed coats (baumann). The seeds are finely ground and made into a paste with the addition of water. The dough-like paste is then shaped and dried slowly by fire for later use. The dough is converted back to powder using a tongue of the Pirarucu fish or "hioide" by a sanding action (6).

## Horticultural Aspects

The wild-type, caffeine-rich variety first discovered by Humboldt and Bonpland was classified as *P. cupana* Ducke. The cultivated plant, *P. cupana* H.B.K. var. *sorbilis* (Mart.) Ducke, is called guaraná. Guaraná belongs to the plant division Angiosperms, class Dicotyledonea, order Spindales, and family Sapindaceae.

Until 30 years ago, guaraná was primarily cultivated in the central Amazon basin of Brazil located in the state of Amazonas (6). Presently, only 60% (or 1500 tons/year) of guaraná in Brazil is cultivated in the state of Amazonas. The remaining 40% is now cultivated in the adjacent states such as Mato Grosso, Bahia, Acre, Pará and as far south as Mato Grosso do Sul (7). Guaraná are also cultivated to a less extent in Venezuela and Uruguay.

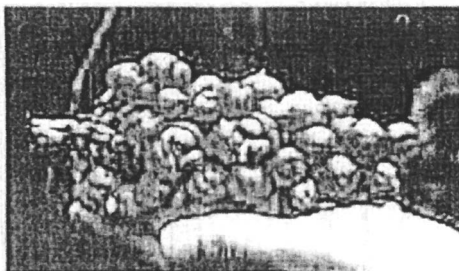
The guaraná plant is low growing, which makes field conditions somewhat challenging. Fruit production occurs after the third year, but is greatest after the sixth year (8). Vegetative or asexual propagation has proven to be the most effective method where advantages include greater productivity after the third year (nearly 1kg dry seeds per plant) and greater disease resistance (9). The guaraná fruit is elongated (2 cm) with a pointed distal extremity. The immature fruit is enclosed in a dark green shell. The mature fruit results in a striking appearance of deep yellow to red-orange pericarp, a white, scentless aril and glossy black seed coat (3).

Plants are typically grown in a nursery for the first nine months in rustic facilities covered with a layer of plastic and palm leaves. The palm leaves are gradually removed after five months to increase sunlight. Cuttings are dipped in indolebutyric acid, set in soil medium and irrigated with approximately 600 mL of water per day. Soils are typically well-drained, heavy clay to sandy clay (6). Fertilizers containing urea, potassium chloride and dipotassium magnesium sulfide are applied every three months during the nursery stage. Approximately 650 plants are required to start one hectare (8).

After the initial nine months in the nursery stage, the healthy plants (typically 60-70%) are adapted to the field where the long growing process is initiated. Methods have been developed to add herbicides periodically (glyphosate or Round Up™ and paraquat) and urea to the soil to eliminate competition (8). Pruning of leaves from the extremities of the terminal branches is recommended after the first harvest of fruit (6). Harvesting of the fruit occur primarily between October and January when nearly 50% of the fruit cluster shows a rupture in the distal extremity (see Figure 1) of the shell. The plant has a peculiar inverted bowl shape making mechanical harvesting of the fruit a challenging task (6). An excellent world-wide-web site for viewing the general cultivation process and ripened fruit is available at the "The Guaraná Homepage" (10). Caffeine content in the leaves average 0.38% compared to 4.4% of that found in the seeds (8).

### Treatment of Unprocessed Product

Treatment of the unprocessed guaraná fruit includes fermentation, pulping and drying. Fermentation accompanied by drying is carried out naturally for a period of three days by spreading the fruit on cloth to a depth of 25 cm, covering for protection



*Figure 1. Guaraná fruit with exposed seeds at harvest.*

and allowing adequate ventilation (6). The shells and pulp are removed either mechanically or manually. The manual pulping process is carried out by traditional means where individuals walk on the fruit with bare feet to separate the fermented fruit from the shells while removing the pulp. The seeds are placed on a screen (5 mm mesh) and washed with water. The seeds are then sun dried for approximately 12 hours before the final drying stage.

Dehydration is carried out by one of three methods: traditional, natural, or industrial. The traditional method used mainly by small farmers include placing the seed on iron plates and roasting the seeds for five hours in wood-fired clay ovens, which are also used in the processing of cassava flour (6). Natural drying occurs in solar driers for up to four days until the seeds obtain a moisture content of 10 to 12% (wet basis). Industrial methods typically use coffee dehydrators that utilize forced heated air for only 15 minutes. Although the forced-convection dehydrators add to the expense, the product is dried more uniformly over short periods. After drying, the seed shells are removed and the seed pounded into a fine powder. The powder is either sold at this point, converted to a syrup, concentrated for the beverage industry, or made into a dried "stick" by traditional methods that require addition of water for conversion to a formed dough that is subsequently dried.

### Commercial Processing Procedures

Commercial processing of guaraná converts the dried seed material to powder, syrups and concentrates for a variety of uses. The syrups must be prepared with a minimum of 1% dried seed. Nearly 80% of guaraná is converted to concentrate for beverages. Guaraná holds about 14% of the total market for soft drinks, which places it behind "cola" beverages (6). The concentrate is prepared with a slow ethanol-water (60:40, v/v) extraction procedure. Concentration ranges for soft drinks must not exceed 0.2% extract with the minimum concentration of 0.02% (6).

Because caffeine is of primary interest in guaraná beverages, decaffeination procedures and caffeine purification are of interest to industry. Several methods exist for caffeine extraction including liquid/liquid extraction, water decaffeination, and supercritical carbon dioxide extraction. Other solvents include ethyl acetate and natural oils. The seeds are first ground (1 mm), steamed and brought to a moisture content of 40% (wb). Further processing involves refining the caffeine to remove waxes, oils, and water-soluble pigments (11). Residual solvents are removed by steam distillation. Activated carbon is added and later filtered to remove pigments. Caffeine crystals are recovered with acetic acid (later removed by steam distillation) followed by centrifugation. A final vacuum-drying procedure produces caffeine at about 80 to 90% purity (11). Figure 2 shows the relative extraction times for methylene chloride (6,12), ethyl acetate, ethanol-water (60:40 v/v) and supercritical carbon dioxide (13) to remove caffeine from ground guaraná seed.

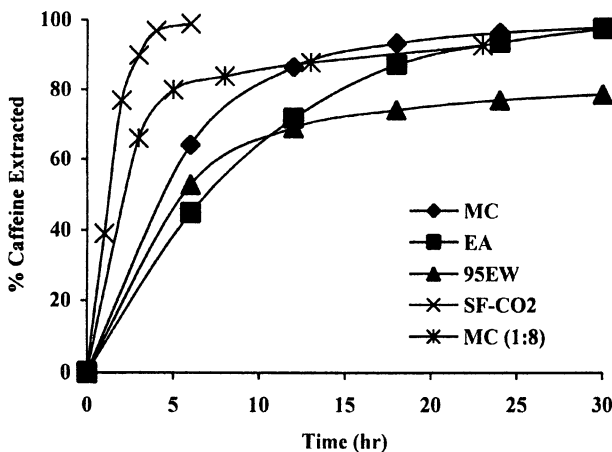


Figure 2. Caffeine extraction from guaraná using various solvents for comparison. MC: methylene chloride; EA: ethyl acetate; 95EW: ethanol: water (95:5 v/v); SF-CO<sub>2</sub>: supercritical CO<sub>2</sub> (55°C, 200 atm); MC(1:8): methylene chloride (1:8 seed:solvent ratio). Note: all solvents other than CO<sub>2</sub> were at room temperature.

## Major Chemical Changes during Processing

The chemical changes of guaraná during processing are not well documented. A proximate analysis was conducted on guaraná seeds processed by solar drying and traditional drying or roasting for 5 hours (6). Table 1 shows the results of this analysis. Data is also shown (drying process not known) (13,14). Differences were seen within all constituents at the 0.05 level for the two types of drying procedures (6).

Table 2 shows the minerals identified for the solar and traditionally-dried seeds by atomic absorption-emission spectrometry (Instrumentation Laboratories, Model 551 AA/AE). Greater amounts of iron seen in seed dried by the traditional method may be explained by the contact of the seeds with the iron heating vessels in the clay oven. Other differences could depend on many factors including cultivation conditions such as soil types, fertilizers used, etc. on different plantations.



**Table 1. Proximate analysis of solar and traditionally-dried guaraná seed.**

<i>Constituent</i>	<i>% Composition Solar Drying</i>	<i>% Composition Traditional Drying</i>	<i>% Compositio n (13)</i>	<i>% Composition (14)</i>
Moisture	6.50	8.7	NA	10.47
Protein	14.12	16.1	9.86	13.23
Fat	1.95	2.2	3.00	2.69
Ash	1.61	1.83	1.42	1.44
Fiber	34.30	41.2	NA	10.23
Carbohydrate	48.02	38.7	NA	61.94
Starch	NA <sup>1</sup>	NA	5.0-6.0	NA
Caffeine	4.07	4.40	2.5-7.6	3.28

1 NA: data not available

**Table 2. Mineral Composition of Solar and Traditionally-Dried Guaraná Seed.**

<i>Minerals</i>	<i>Mg% Composition Traditionally-Dried</i>	<i>Mg% Composition Solar-dried</i>	<i>Mg% Composition (11)</i>
Ca	14.0*	11.9*	29
K	555.2*	524.0*	337
Mg	124.3	111.5	83
Na	6.9	5.9	6
Cu	4.0*	2.9*	0.9
Fe	14.6*	6.1*	2.6
PO <sub>4</sub>	17.5	16.9	344
Mn	2.1	1.7	2.8
Zn	2.8	3.1	1.7

\* means within a factor are significantly different at 0.05 level

## Phytochemicals in Guaraná

Some phytochemicals associated with guaraná include adenine, tannic acid, catechutannic-acid, choline, guanine, mucilage, saponin and timbonine (16). The methylxanthines present in guaraná include caffeine (3,7-dihydro-1,3,7-trimethyl-1H-purine-2,6-dione, or more commonly 1,3,7- trimethylxanthine), theobromine (3,7-dimethylxanthine), theophylline (1,3-dimethylxanthine), and the polyphenols including (+)-catechin and (-)-epicatechin (16). Caffeine is also known as guaranine (from guaraná), coffeine (from coffee), theine (from tea extract) and methyltheobromine. Theophylline is known for its stimulating effects similar to caffeine, but to a lesser extent. This compound is a pharmaceutical compound used as a bronchodilator (18).

Theobromine is the primary compound in cocoa that simulates the effects of caffeine, but also to a lesser extent. Theobromine is widely known as the principle compound toxic to dogs (19). Figure 3 shows the chemical structures of the principle alkaloids present in guaraná. The primary difference is the number and location of the methyl groups. Table 3 shows the alkaloid content including the caffeine content of various raw materials and beverage products containing the raw materials. Guaraná is the richest known natural source of caffeine.

The main components of the essential oils of guaraná (greenish fixed oil) were identified as (2) methylbenzenes, (1) cyclic monoterpene and (2) cyclic sesquiterpene hydrocarbons, (2) methoxyphenylpropenes and (2) alkylphenol derivatives (20). Tannins that are common to guaraná and kola nuts are associated as a carcinogen and inhibitor to protein function (21).

**Table 3. Alkaloid content of different products.**

<i>Product</i>	<i>Caffeine (% dry basis)</i>	<i>Beverage Product (per serving)</i>	<i>Caffeine (mg)</i>	<i>Theobromine (mg)</i>	<i>Theophyllin e (mg)</i>
Guaraná	3.5-7.0	Guaraná	30-40	0.3	0.5
Coffee	1.0-2.0	Coffee	90-150	0	NA <sup>1</sup>
Tea	2.5-4.0	Tea	30-70	NA	3-4
Cocoa	0.07-0.39	Cocoa	NA	250	NA
Kola	2.5-3.5	Soft drink	30-55	0	0

<sup>1</sup> NA: data not available

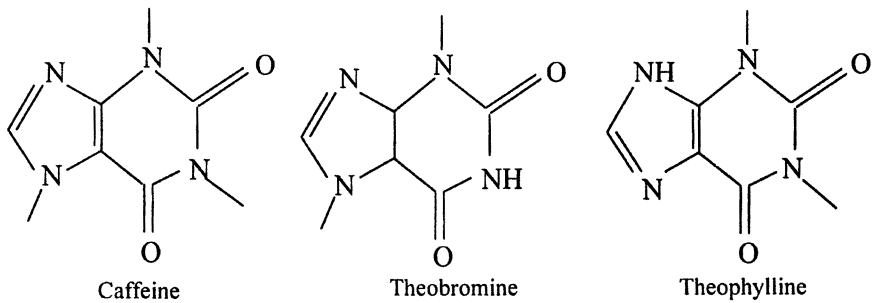


Figure 3. Alkaloids in guaraná.

### Toxicity of Guaraná

The genotoxic and mutagenic effects of guaraná aqueous extracts have been reported (7). Genotoxic effects were found for guaraná at high concentrations by lysogenic induction in *Escherichia coli*. Related to this activity was the formation of a caffeine-flavonoid complex (identified by NMR) in the presence of potassium (7). Mutagenesis was also found in *Salmonella typhimurium* as identified by the Ames test, again at high concentrations (7).

The toxicological effects of guaraná and ginseng extracts were tested in mice with results showing no toxicity as demonstrated by histopathological examination (22). The study did show that antioxidant effects were evident. The results showed inhibition of lipid peroxidation even at concentrations as low as 1.2  $\mu\text{g/mL}$  (22). The ability of guaraná to induce *in vitro* cytotoxic effects were also studied in Chinese hamster ovaries (23) and mice (24). The results suggested that cytotoxic effects may occur at high doses, but no toxicity was evident at low levels (similar to that found in guaraná drinks).

The primary benefit of guaraná as advertised by the commercial beverage industry is the high natural caffeine content. The drinks are promoted to consumers as “high energy” and “natural” beverages and have recently become a commercial success. Guaraná, once considered a drug in the United States, is now classified by the Food and Drug Administration as a diet aid and food additive (17). Mair (25) indicated concern over the most recent introduction of guaraná (and ginseng extract) drinks by Coca Cola Industries in New Zealand, Lift Plus®, which contains nearly three times the caffeine of the average soft drink. The concern was that the diuretic effect of caffeine results in dehydration during sports activities, while the “energy” beverages are perceived to increase performance. The second major use of guaraná is the dried extract in form of capsules for energy boost, diet aids, and headache remedies. The claim by commercial vendors is that the caffeine is absorbed into the body at slower rates causing the effect of less “shock” or stress to metabolic systems

in the body. Presently scientific evidence is generally lacking to validate this claim, but there may be some merit for further investigation.

### Flavor Changes during Processing

As with chemical changes during processing, literature is sparse concerning flavoring volatile changes. Therefore, a qualitative study was conducted to identify the compounds from ground, dried, guaraná seeds (6). The seeds (150 g) were ground with a Viking hammer mill to pass through a 1.5 mm sieve, homogenized for 5 minutes with 700 mL water (HPLC grade) at 60°C. Volatiles were steam distilled and the aqueous residue extracted for two hours with methylene chloride. The extract was concentrated to 1 mL (26). The volatiles were analyzed qualitatively with a Shimadzu Model 9AM GC-MS. The flavor volatiles are shown with matching probabilities in Table 4.

Guaraná seed extract is used in soft drinks at low level amounts varying from 0.02 to 0.2%. Therefore, the flavoring capacity is limited. In other products, however, the volatiles that contain guaraná seed extract in greater amounts such as syrups and concentrates may have a significant flavor contribution.

**Table 4. Flavor Volatiles in Guaraná Extracted with Methylene Chloride and Analyzed by GC-MS.**

<i>Retention Time (minutes)</i>	<i>Volatile Component</i>	<i>Matching Probability (%)</i>
10.1	1-octen-3-ol	52
21.2	2,5, dymethylpyrazine	52
24.2	Carophyllene	45
27.1	Pyrrole	39
30.2	Nerolidol	47
33.1	Allyl benzoate	32
36.1	Nerol	39
38.3	Hexadecanal	58
41.8	Acetophenone	63
42.0	Dimethyl heptane	22
42.7	Isoamylbenzyl ether	22
50.9	Decadienal	78
51.6	Musk xylol	22
59.0	Ethyl caproate	25

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## Chapter 32

# The Chemistry of Tea

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Tea is one of the most popular beverages consumed worldwide. It is the brew prepared from the leaves of the plant *Camellia sinensis*. Freshly harvested tea leaves require processing to convert them into green, oolong and black teas. An estimated 2.5 million metric tons of dried tea are manufactured annually. The distinctive flavor and color of various teas are due to the chemical changes that occur during processing. The two major groups of compounds in tea leaves are catechins and methylxanthins.

Tea refers to the plant *Camellia sinensis*, its leaves, and the extracts and infusions thereof. *Camellia sinensis* was first cultivated in China and then in Japan. With the opening of ocean routes to the East by European traders during the fifteenth to seventeenth centuries, commercial cultivation gradually expanded to Indonesia and then to the Indian subcontinent, including Sri Lanka (*1*). Tea is now second only to water in worldwide consumption. Annual production of about 1.8 million tons of dried leaf provides world per capita consumption of 40 liters of beverages (*2*).

Black and green teas are the two main types, defined by their respective manufacturing techniques. Green tea is consumed mostly in Asian countries such as China and Japan, while black tea is more popular in North America and Europe. Oolong tea is an intermediate variant (partially fermented) between green and black tea. Its production is confined to some regions of China including Taiwan.

## Commercial Tea Processing

Tea leaf, like all other plant leaf matter, contains carbohydrate, protein, lipids, full complement of genetic material, enzymes and secondary metabolites. In addition, tea leaf is distinguished by its high content of methylxanthins and polyphenols, in particular catechins (flavanols). Table 1 shows a representative analysis of fresh tea leaf. 25% of its dry weight are catechins, 3.0% are flavonols and flavonol glycosides, 3.0% are caffeine and 0.2% are theobromine (2).

**Table 1. Composition of Fresh Tea Leaf**

<i>Components</i>	<i>% of dry weight</i>
Flavanols	25.0
Flavonols and flavonol glycosides	3.0
Polyphenolic acids and depsides	5.0
Other polyphenols	3.0
Caffeine	3.0
Theobromine	0.2
Amino acids	4.0
Organic acids	0.5
Monosaccharides	4.0
Polysaccharides	13.0
Cellulose	7.0
Protein	15.0
Lignin	6.0
Lipids	3.0
Chlorophyll and other pigments	0.5
Ash	5.0
Volatiles	0.1

SOURCE: Reproduced from Reference 2. Copyright 1998 CRC Press.

### Green Tea

In the processing of green tea, the tea flesh is first steamed in the case of Japanese green tea "sen-cha" or pan-fired to produce Chinese green tea (3). These heat treatments inactivate enzymes in the tea leaves. The temperature of pan-firing can reach as high as 230°C which is much higher than the steaming temperature of 100°C. Steaming, therefore, results in fewer chemical changes than pan-firing. Following heat treatment, tea leaves are subjected to subsequent rolling and drying processes to achieve a dry product exhibiting the desired twisted leaf appearance.

## Black Tea

More than 75% of the world tea production is black tea. The steps involved in the processing of black tea include withering, leaf disruption, fermentation, drying and grading. All steps are designed to achieve optimal oxidation of tea catechins and produce tea products with good flavor and color.

Withering step is used for the partial removal of moisture in the tea leaves. The moisture is reduced to about 60-70% of the leaf weight. There are many methods used for leaf disruption such as rolling, cutting, crushing and tearing. The basic requirements are size reduction and cell disruption. This initiates the fermentation process.

Fermentation in tea processing simply refers to an enzymatic browning reaction catalyzed by the polyphenol oxidase. After the leaf disruption stage, the leaves are allowed contact with the surrounding air for about 1-3 hours. Fermentation has great impact on the quality of brew. The final step of black tea processing is drying. In this step, tea is dried by exposing the leaves to a stream of hot air. Moisture is reduced to 2-3%.

## Oolong Tea

Oolong tea is prepared by firing the leaves after rolling to terminate the oxidation process. It is only partially oxidized and retains a considerable amount of the original polyphenols (4).

## Major Chemical Changes During Tea Processing

Catechins are the predominate form of flavonoids in fresh tea leaves. Catechins are characterized by di- or trihydroxy group substitution of the B ring and the meta-5,7-dihydroxy substitution of the A ring of the flavonoid structure (Figure 1). The principal catechins presented in fresh tea leaves are (-)-epicatechin (EC), (-)-epigallocatechin (EGC), (-)-epicatechin gallate (ECG) and (-)-epigallocatechin gallate (EGCG) (Figure 2).

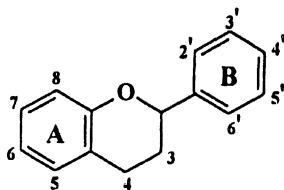
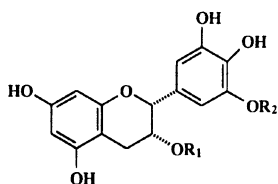


Figure 1. Basic flavonoid structure.





		R <sub>1</sub>	R <sub>2</sub>
(-)-Epicatechin	EC	H	H
(-)-Epicatechin gallate	ECG	Gallate	H
(-)-Epigallocatechin	EGC	H	Gallate
(-)-Epigallocatechin gallate	EGCG	Gallate	Gallate

Figure 2. Major catechins in tea.

The major chemical reaction during tea manufacturing is the oxidative conversion and polymerization of catechins. The oxidative fermentation of catechins in tea results in the development of appropriate flavor and color of oolong and black teas. It will cause a darkening of the leaf and a decrease in astringency. The initial step of fermentation is the oxidation of catechins to reactive quinones catalyzed by polyphenol oxidase. Polyphenol oxidase can use any of the catechins as a substrate to form the complex polyphenolic constituents found in oolong and black teas. The major condensation compounds are theaflavins and thearubigins. Other products, such as theaflavic acids, theaflagallins, theasinensins, oolongtheanin, and theaflavate A have also been found to be present in black and oolong teas. The possible oxidation pathways of tea catechins during fermentation can be divided into (a) pyrogallol-catechol condensation (Figure 3) and (b) pyrogallol-pyrogallol condensation (Figure 4) (5).

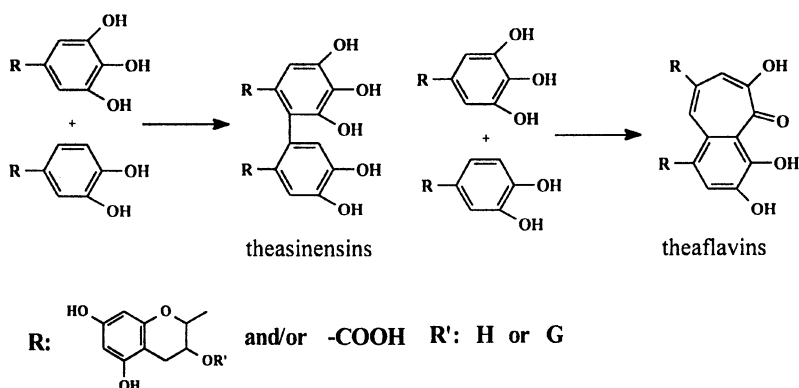


Figure 3. Possible pyrogallol-catechol condensation pathway of tea catechins during fermentation.

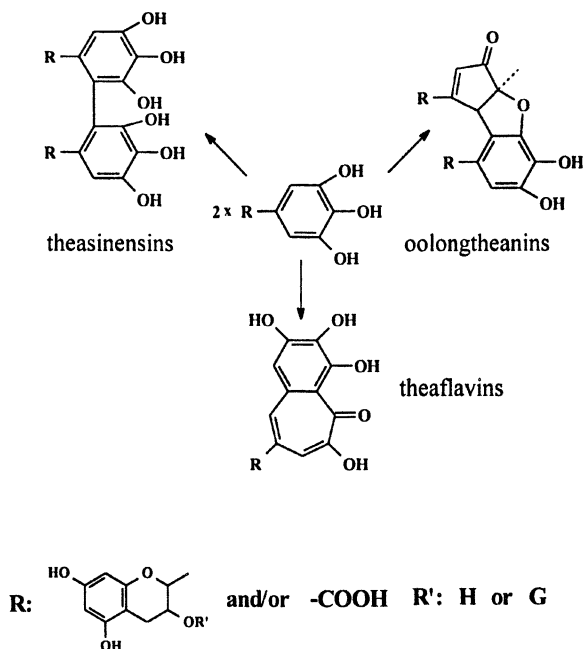
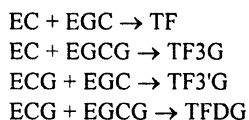


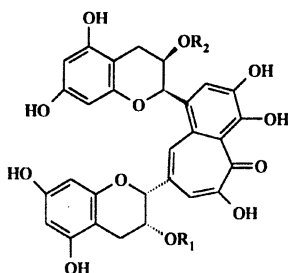
Figure 4. Possible pyrogallol-pyrogallol condensation pathway of tea catechins during fermentation.

### Formation of Theaflavins

Theaflavins, which give the characteristic bright orange-red color of black tea, account for 1-2% dry weight of the water extractable fraction. Theaflavins consist of four major components (Figure 5): theaflavin (TF), theaflavin-3-gallate (TF3G), theaflavin-3'-gallate (TF3'G) and theaflavin-3,3'-digallate (TFDG), which are formed by the pair oxidation of catechins as follows (6):



This series of reactions has been established by the use of model tea fermentation systems (7). Total theaflavin content has been shown to have correlation with the quality of black tea (6).



		R <sub>1</sub>	R <sub>2</sub>
Theaflavin	TF	H	H
Theaflavin 3-gallate	TF3G	Gallate	H
Theaflavin 3'-gallate	TF3'G	H	Gallate
Theaflavin 3,3'-gallate	TFDG	Gallate	Gallate

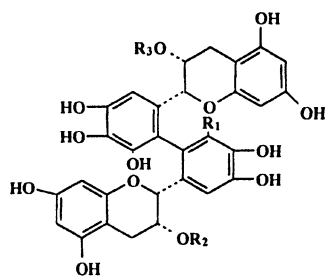
Figure 5. Structures of theaflavins.

### Formation of Thearubigins

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. They are the major oxidation product of catechins during fermentation, however, due to the difficulty encountered in their separation, the thearubigin chemistry is poorly characterized.

### Theasinensins and Oolongtheanin Formation

Theasinensins are compounds formed by the coupling of quinone with catechol or the pyrogallol ring of the flavan-3-ol derivatives. Seven theasinensins (Figure 6) have been isolated and identified from oolong tea (5). They occur only in very small quantities in black tea, presumably because of high reactivity (2). Another condensation product of catechin, with a two fused furan ring structure has been identified in oolong tea and was named oolongtheanin (Figure 7).



	R <sub>1</sub>	R <sub>2</sub>	R <sub>3</sub>
A	OH	G	G
B	OH	G	H
C	OH	H	H
D	OH	G	G
E	OH	H	H
F	OH	G	G
G	H	G	G

Figure 6. Structures of theasinsins A-G identified in Oolong tea.

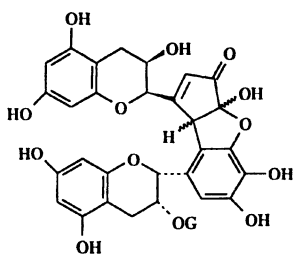


Figure 7. Structure of Oolongtheanin identified in Oolong tea.

## Flavor Changes During Tea Processing

Flavor is the most important factor on determining the quality of tea. Flavor involves both taste and aroma. The balance of astringency, bitterness and brothy taste is important to the characteristic taste of tea (8). The major contributors to astringency and bitterness of tea are catechins and caffeine. Table II lists the threshold levels for the astringency and bitterness of tea polyphenols (9).

**Table II. Threshold Levels for Astringency and Bitterness of Tea Polyphenols**

Phenolic compound	Threshold level (mg/100mL)	
	Astringency	Bitterness
(-)-EC	Not astringent	60
(-)-ECG	50	20
(-)-EGC	Not astringent	35
(-)-EGCG	60	30
Theaflavin	80	75-100
Theaflavin monogallate (natural mixture)	36	30-50
Theaflavin digallate	12.5	Not determined
Gallic acid	Not astringent	Not bitter

SOURCE: Modified from Reference 9.

Table III shows the concentrations of catechins, theaflavins, thearubigins and highly polymerized substances in green, oolong and black teas. These differences contribute significantly to the taste differences among these teas.

**Table III. Concentrations (%) of Major Polyphenols in Different Teas (8)**

Compound	Green tea	Oolong tea	Black tea
(-)-EC	0.74-1.00	0.21-0.33	-
(-)-ECG	1.67-2.47	0.99-1.66	0.29-0.42
(-)-EGC	2.60-3.36	0.92-1.08	-
(-)-EGCG	7.00-7.53	2.93-3.75	0.39-0.60
Theaflavins	-	-	0.98-2.12
Thearubigins	-	-	7.63-8.03
HPS	-	-	7.27-7.66

Note: HPS, Highly polymerized substance. SOURCE: Reproduced from Reference 8. Copyright 1995 Marcel Dekker, Inc.

Pure caffeine is bitter and the detection threshold is about 3 ppm in water (8). The caffeine concentration in tea brew ranges from 2-4% (10). Part of the caffeine

**Table IV. Odorants of Green and Black Teas Identified on the Basis of AEDA (13)**

<i>Compound</i>	<i>Odor quality</i>	<i>FD</i>	
		<i>Green tea</i>	<i>Black tea</i>
( <i>Z</i> )-Octa-1,5-dien-3-one	Geranium-like, metallic	512	256
3-Hydroxy-4,5-dimethyl-2(5H)-furanone	seasoning-like	512	512
3-Methylnonane-2,4-dione	strawy, fruity	512	256
( <i>Z</i> )-Hept-4-enal	biscuit-like	256	128
( <i>Z</i> )-Hex-3-enal	leaf-like	128	32
( <i>E,Z</i> )-Nona-2,6-dienal	cucumber-like	128	128
Oct-1-en-3-one	mushroom-like	128	128
( <i>E,E</i> )-Deca-2,4-dienal	deep-fried	128	64
( <i>E</i> )- $\beta$ -Damascenone	boiled, apple-like	128	512
4-Hydroxy-2,5-dimethyl-3(2H)-furanone	caramel-like	64	512
2-/3-Methylbutanoic acid	sweaty	64	128
trans-4,5-Epoxydec-2-enal	metallic, green	64	32
( <i>E,E</i> )-Nona-2,4-dienal	deep-fried	64	16
Unknown	coconut-like	64	64
2-Phenylethanol	honey-like	32	64
( <i>E</i> )-Non-2-enal	fatty, green	32	64
2-Methoxyphenol	burnt	16	8
Vanillin	vanilla-like	16	128
$\delta$ -Decalactone	coconut-like	16	16
Linalool	floral	8	512
Butyric acid	buttery, rancid	8	8
Phenylacetaldehyde	honey-like	8	16
Bis(2-methyl-3-furyl)disulfide	boiled, meat-like	<4	256
Phenylacetic acid	sweet	<4	16
Hexanal	green	4	4
3-Methylbutanal	malty	4	4
Butane-2,3-dione	buttery	4	4
Octanal	fatty	<4	4

must complex with the tea polyphenol to reduce its bitterness and play an important role to the tea taste (8).

The brothy taste of tea is mainly due to amino acids. The most important amino acid in tea leaves is theanine, it has sweet umami taste quality.

### Aroma of Teas

More than 600 volatile compounds have been identified in the aroma of various teas. A complete list of compounds reported in tea aromas has been compiled (8).

The aroma of Japanese green tea has been characterized as a refreshing aroma with a green note. (*Z*)-3-Hexen-1-ol, (*Z*)-3-hexenyl acetate and linalool are proposed to contribute to the aroma of green tea (8). Compounds such as linalool, geraniol, nerolidol, indole and  $\beta$ -esquiphellandrene identified in oolong tea are thought to be responsible for its floral characteristics (11).

The difference in volatile compositions between semi-fermented tea and black tea has been studied (12). In general, semi-fermented tea contains higher amounts of *cis*-jasmine,  $\beta$ -ionone, nerolidol, jasmine lactone, methyl jasmonate and indole, while the black tea contains higher amount of *trans*-2-hexenal, *cis*-3-hexenol, *trans*-2-hexenyl formate, monoterpene alcohols and methyl salicylate

### Determination of Potent Odorants in Green and Black Teas

Aroma extract dilution analysis (AEDA), an important gas chromatography-olfactometry (GC-O) method for the determination of potent odorants in foods has been applied to green and black teas (13). According to the results listed in Table IV the same odorants were detectable in the two tea extracts. A significant difference was found for (*Z*)-hex-3-enal and (*E,E*)-nona-2,4-dienal, both of which predominated on the basis of the high FD-factor, in the extract obtained from green tea. On the other hand, the higher FD-factors of linalool, 4-hydroxy-2,5-dimethyl-3(2H)-furanone, (*E*)- $\beta$ -damascenone, phenylacetic acid and bis(2-methyl-3-furyl)-disulfide in the black tea showed that their levels were higher in black tea (13). In the same report, the most potent odorants occurring in the air above the tea powders were also evaluated. It indicated that the difference in the odors of green and black tea was mainly due to a higher concentration of (*Z*)-hex-3-enal, (*Z*)-octa-1,5-dien-3-one and butane-2,3-dione and the much lower concentration of linalool in the air above the former (13).

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## Chapter 33

# $\beta$ -Primeverosidase Relationship with Floral Tea Aroma Formation during Processing of Oolong Tea and Black Tea

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Flavor is one the most important factors to determine the quality of beverages. Tea aroma, especially floral aroma liberated from brewed oolong tea and black tea, is so attractive. This study covers the molecular basis of the floral aroma formation during the tea processing. Previous work demonstrated the tea aroma precursors of geraniol, linalool, *etc.* as  $\beta$ -primeverosides (6-*O*- $\beta$ -D-xylopyranosyl- $\beta$ -D-glucopyranosides) from the tea leaves (*Camellia sinensis* var. *sinensis* cvs. Shuixian and Maoxie) which can be processed to oolong tea. We have also purified  $\beta$ -primeverosidases from fresh leaves of cv. Yabukita for Japanese green tea, cv. Shuixian for oolong tea and a cultivar of *C. s.* var. *assamica* for black tea. The molecular weight of each enzyme was shown to be 60,500, 60,200 and 60,300 by TOFMS, respectively. The enzymatic characteristics (optimum temperature, 45 °C; stable temp., 40-45 °C; optimum pH, 4; pH stability, pH 3-5; specific activity, 0.90-0.99 unit/mg) were very similar to each other. The enzyme was confirmed to effectively hydrolyze the aroma precursors,  $\beta$ -primeverosides as well as 6-*O*- $\beta$ -D-apiofuranosyl- $\beta$ -D-glucopyranoside, into disaccharides and each aglycon without further hydrolysis. Most of the alcoholic tea aroma, which contribute to the floral tea aroma, are primarily stored as disaccharide glycosides ( $\beta$ -primeverosides and 6-*O*- $\beta$ -D-apiofuranosyl- $\beta$ -D-glucopyranosides) and generated by the action of a specific enzyme,  $\beta$ -primeverosidase, during the fermentation process of tea manufacturing.

## Introduction

Any tea leaves can be processed to yield green tea, oolong tea or black tea (Figure 1), although suitable cultivars for each made tea have been developed. Floral

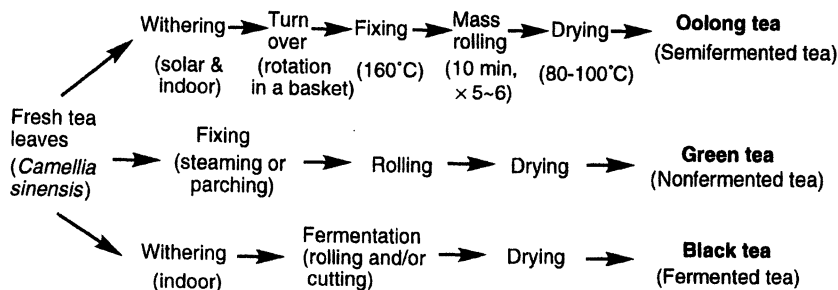


Figure 1. Tea manufacturing process.

tea aroma is known to be generated during the fermentation process in the tea manufacturing (Withering and Turn over processes in oolong tea production and Withering and Fermentation processes in black tea production; Figure 1). The process used is one of the most important factors to determine the quality of each made tea, especially oolong tea and black tea (1).

Takeo *et al.* subjected the fresh leaves of cv. Benihomare to the processing for three types of tea (two types of green tea and oolong tea) and compared the GC analytical results among them (Figure 2) (2). It is evident that larger quantities of alcohols such as geraniol are generated during the processing for oolong tea.

It was a goal to study the molecular basis of the alcoholic aroma formation in oolong tea. Initially we isolated the aroma precursors of the floral alcohols of tea aroma, and found that most of them were  $\beta$ -primeverosides (6-*O*- $\beta$ -D-xylopyranosyl- $\beta$ -D-glucopyranosides (3-7). The aroma precursors of linalool oxides III and IV (*cis*- and *trans*-linalool 3,7-oxides) and (*Z*)-3-hexenol were found to be 6-*O*- $\beta$ -D-apiofuranosyl- $\beta$ -D-glucopyranosides and  $\beta$ -D-glucopyranoside, respectively (6). Quite recently we isolated and identified (*Z*)-3-hexenyl  $\beta$ -primeveroside and 7,8-dihydro- $\beta$ -ionyl 6-*O*- $\beta$ -D-apiofuranosyl- $\beta$ -D-glucopyranoside as aroma precursors from cv. Yabukita (8). We also isolated and identified prunasin [(*R*)-2- $\beta$ -D-glucopyranosyloxy-2-phenylacetonitrile] as an aroma precursor of benzaldehyde from fresh leaves of cv. Yabukita (9).

Kobayashi *et al.* had isolated and identified (*Z*)-3-hexenyl and benzyl  $\beta$ -D-glucopyranosides as aroma precursors from cv. Yabukita (10,11) when we launched

our study. They have also found geranyl  $\beta$ -primeveroside as well as geranyl  $\beta$ -vicianoside (6-*O*- $\alpha$ -L-arabinopyranosyl- $\beta$ -D-glucopyranoside) in cv. Yabukita (12). Figure 3 summarizes all the alcohol aroma precursors isolated from tea leaves by our and Kobayshi's group.

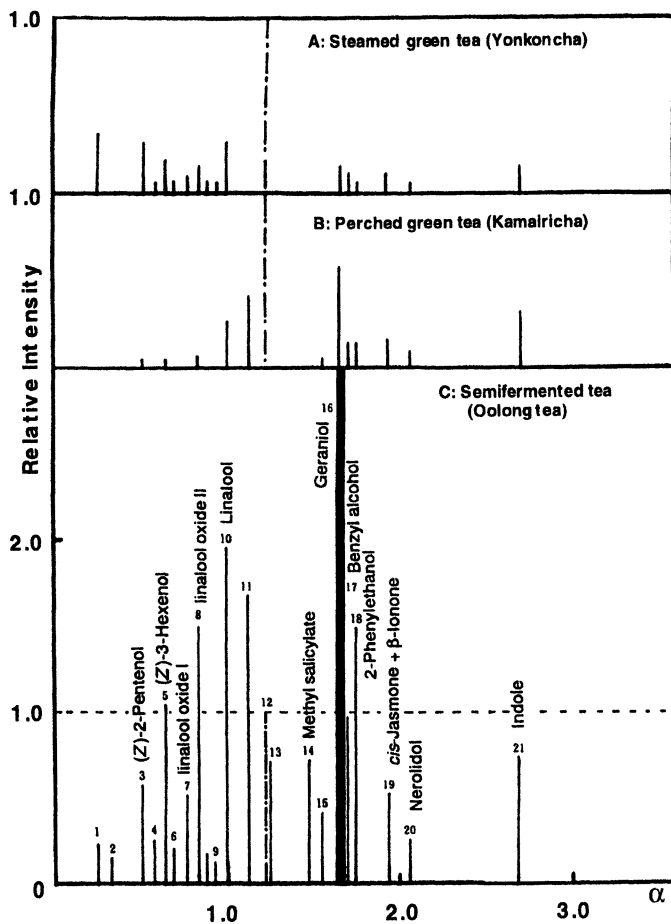


Figure 2. Comparison of volatiles from three types of tea prepared from the same material fresh leaves of cv. Benihomare plucked on the same day (2). (Reproduced with permission from reference 2. Copyright 1985.)

## Preliminary Characterization of the Glycosidase Involved with the Alcohol Compounds in Tea Aroma

Fresh tea shoots (cv. Shuixian) with the 1st to the 3rd or 4th were subjected to the conventional acetone powder preparation followed by ammonium sulfate precipitation (40-80%) as previously reported (13). A crude enzyme sample extracted with 0.1 M citrate buffer (pH 6.0) from the precipitate was fractionated by CM-Toyopearl column chromatography (Figure 4 upper). Each fraction was tested for the activities toward *p*-nitrophenyl (*p*NP)-Glc, *p*NP-Xyl, *p*NP-Gal, and *p*NP-Ara, and then was combined into 8 groups (Frs. A~H) according to these activities (Figure 4). This elution pattern is similar to that in the case of cv. Yabukita (13).

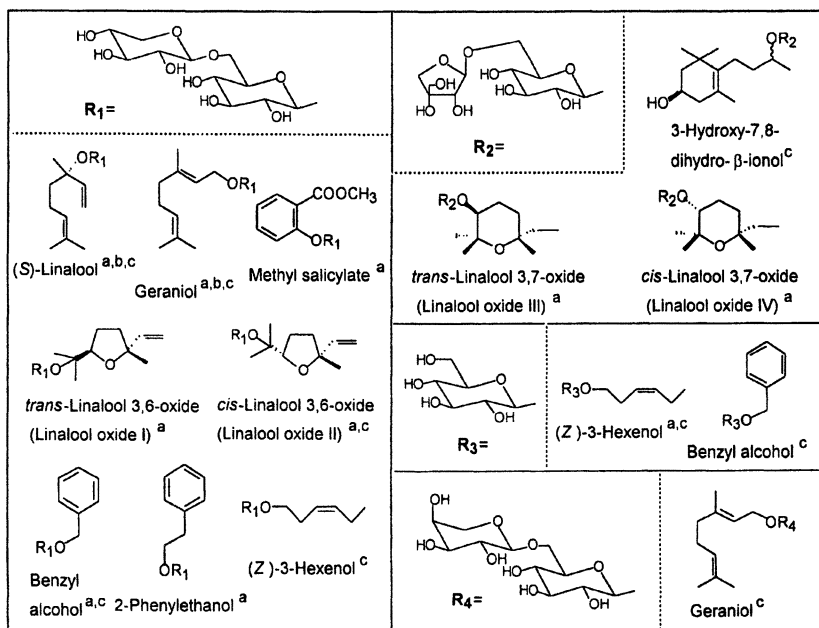


Figure 3. Glycosidic aroma precursors isolated from tea leaves (22).

<sup>a</sup> from oolong tea leaves (cv. Maoxie), <sup>b</sup> from oolong tea leaves (cv. Shuixian), <sup>c</sup> from green tea leaves (cv. Yabukita)  
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Each fraction A to H was treated with a crude aroma precursor solution from tea leaves (cv. Benihomare: a cultivar for black tea) and the generated aroma constituents were analyzed by GC (Figure 4). Geraniol and linalool were generated in Fraction D and E as well as B which apparently contains various glycosidases. Since there was

little glycosidase activities toward monosaccharide glycosides in Fraction D (Figure 4 upper), we expected the presence of primeverosidase in Fractions D and E. Because we had isolated several kinds of  $\beta$ -primeverosides as main aroma precursors (Figure 3).

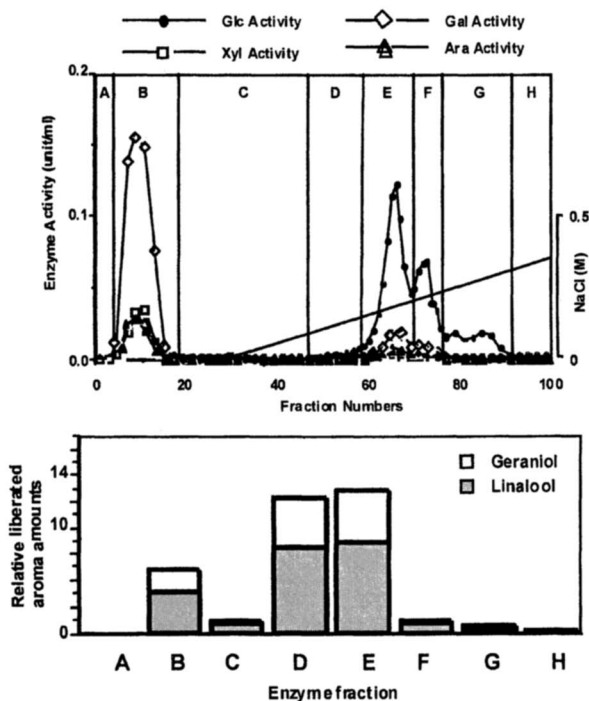


Figure 4. Glycosidic activities of Fractions A-H towards several kinds of *pNP*-glycosides (upper) and their aroma formation ability (lower) from the crude glycosidic fractions of tea leaf extracts (*cv. Benihomare*) (15).

Fractions A-H: from *CM-Toyopearl 650M* column chromatography (20 mM citrate buffer, pH 6.0; NaCl gradient) of the crude enzyme preparations from fresh leaves of *cv. Shuixian*

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### Demonstration of a $\beta$ -Primeverosidase as the Main Glycosidase Responsible for the Alcohol Compounds of Tea Aroma

At this stage we obtained *pNP*  $\beta$ -primeveroside by enzymatic transglycosylation from commercially available xylobiose to *pNP*  $\beta$ -D-glucopyranoside (Figure 5) (23). Glycosidase activities of each fraction from the *CM-Toyopearl* column was measured

with *p*NP  $\beta$ -D-glucopyranoside and *p*NP  $\beta$ -primeveroside (Figure 6).  $\beta$ -Primeverosidase was eluted just before the main glucosidase, suggesting that the primeverosidase from cv. Shuixian was eluted mainly in Fraction E and partly in Fraction D (Figure 4). This explains the reason why a lot of tea aroma were liberated when the enzyme sample of Fraction D was reacted with a crude tea aroma precursor solution, although Fraction D did not show any glycosidase activities toward any *p*NP monosaccharide glycosides (Figure 4).

The elution profiles of  $\beta$ -primeverosidase and glucosidases of each tea leaf sample (cvs. Yabukita (13) and Shuixian (Figure 4) and a cultivar of var. *assamica* (14) from a CM-Toyopearl column were quite similar to each other.

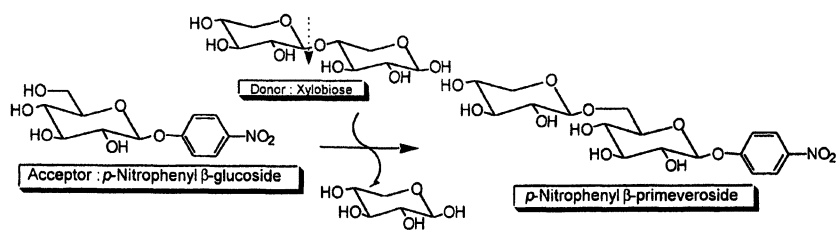


Figure 5. Enzymatic synthesis of  $\beta$ -primeveroside.

## Purification of the $\beta$ -Primeverosidase

The  $\beta$ -primeverosidase fractions almost devoid of other glycosidic enzymes (Fractions 62-64 in Figure 6) were finally purified by FPLC with a column of Mono S-HR (20 mM citrate buffer, pH 6.0; 0-0.2 M NaCl gradient). Primeverosidase activity with a very low level of the apparent  $\beta$ -glucosidase activity overlapped with the absorption at 280 nm. The purified enzyme was found to be 61 kDa by the SDS-PAGE analysis and identical to the enzyme of cv. Yabukita (13). Its molecular weight was confirmed to be 60,300 by TOFMS analysis.

$\beta$ -Primeverosidase from *C. s.* var. *assamica* that is mainly used for black tea manufacturing was also purified (14) in the same manner as previously reported (15). Enzymatic properties of the  $\beta$ -primeverosidase from fresh leaves of each tea plant are summarized in Table I; the results suggest that these enzymes are identical or quite similar to each other.

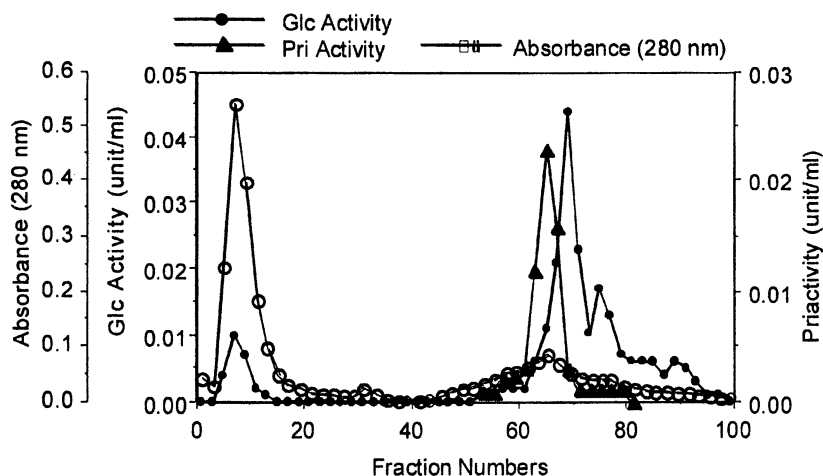


Figure 6. CM-Toyopearl 650M column chromatography of crude enzymes from tea leaves (cv. Shuixian). The elution conditions were the same as in Figure 1. Glucosidase (Glc) and primeverosidase (Pri) activities were measured with pNP  $\beta$ -D-glucopyranoside and  $\beta$ -primeveroside, respectively.

**Table I. Enzymatic Characteristics of the  $\beta$ -primeverosidases from Fresh Tea Leaves**

<i><math>\beta</math>-Primeverosidase</i>			
	<i>From black tea<sup>a</sup></i>	<i>oolong tea<sup>b</sup></i>	<i>green tea<sup>c</sup></i>
M.W. (TOFMS)	60,300	60,200	60,500
PI	9.5	9.5	9.4
Optimum temp. ( $^{\circ}$ C)	45	45	45
Satable temp. ( $^{\circ}$ C)	40	40	45
Optimum pH	4	4	4
pH Stability	4 – 5	3 – 5	4 – 5
Specific activity (unit/mg)	0.99	0.98	0.90

<sup>a</sup>*Camellia sinensis* var. *assamica*; <sup>b</sup>*C. s. var. sinensis* cv. Shuixian; <sup>c</sup>*C. s. var. sinensis* cv. Yabukita

## Substrate Specificity of the $\beta$ -Primeverosidase from Tea Plants

$\beta$ -Primeverosidases from fresh leaves of the three types of teas were shown to be enzymatically identical (Table I). A  $\beta$ -Primeverosidase fraction (from cv. Yabukita) almost devoid of  $\beta$ -glucosidase activity (cf. Fr. 62 and 63 in Figure 4) was reacted with several disaccharide glycosides which have been isolated as aroma precursors [(*S*)-linalyl and benzyl  $\beta$ -primeverosides from *C. sinensis* var. *sinensis* cv. Yabukita (16), (*R*)-linalyl  $\beta$ -vicianoside (6-*O*- $\alpha$ -L-arabinopyranosyl- $\beta$ -D-glucopyranoside from *Gardenia jasminoides* (17), geranyl 6-*O*- $\alpha$ -L-arabinofuranosyl- $\beta$ -D-glucopyranoside from *Rosa damascena* var. *bulgaria* (18) and benzyl 6-*O*- $\beta$ -D-apiofuranosyl- $\beta$ -D-glucopyranoside from *Epimedium grandiflorum* MORR. var. *thunbergianum* (19)]. Liberated aroma was analyzed by GC in the same way as previously reported (15). The primeverosidase enzyme showed rather high substrate specificity toward linalyl and benzyl  $\beta$ -primeverosides as well as to benzyl 6-*O*- $\beta$ -D-apiofuranosyl- $\beta$ -D-glucopyranoside. (*R*)-Linalyl  $\beta$ -vicianoside and geranyl 6-*O*- $\alpha$ -L-arabinofuranosyl- $\beta$ -D-glucopyranoside were hydrolyzed much less effectively (14).

The remaining saccharide constituents in the aqueous layer after the enzymatic hydrolysis were analyzed by HPLC (Figure 7). The  $\beta$ -primeverosidase was clearly shown to recognize disaccharide units of benzyl  $\beta$ -primeveroside and benzyl 6-*O*- $\beta$ -glucosides into each aglycon and disaccharide. Neither disaccharide was further hydrolyzed into a monosaccharide by the  $\beta$ -primeverosidase.

As shown above,  $\beta$ -primeverosidase was shown to be the most important glycosidase in the floral aroma formation during tea processing.

Quite recently we have succeeded in gene cloning the  $\beta$ -primeverosidase from cv. Yabukita and also causing its overexpression in *E. coli*, although the detected primeverosidase activity was lower than that from tea leaves. The amino acid sequence shows fairly high similarity with amygdalin hydrolase from mature black cherry (*Prunus serotina*) (20) (58 %) and myrosinase from white mustard seed (*Sinapis alba*) (21) (45%). Further biochemical and molecular biological studies on the  $\beta$ -primeverosidase is now in progress.

### Acknowledgement

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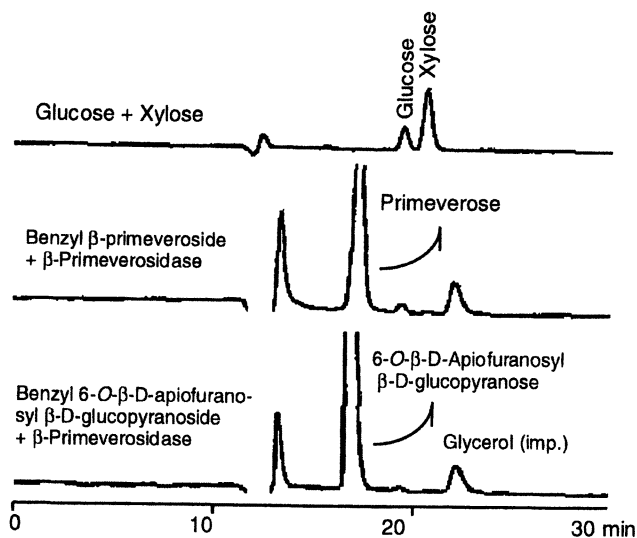


Figure 7. HPLC sugar analysis of the enzymatic hydrolysates of each disaccharide glycoside with  $\beta$ -primeverosidase from cv. Yabukita.

Analyzed by HPLC equipped with a Shodex SUGAR KS-801 ( $\Phi 8 \times 300$  mm) column ( $H_2O$  at flow rate of 0.5 mL/min; 70 °C). Detection of sugars was performed at 415 nm by the post labeling method using 0.1 M  $H_3BO_3$ , 50 mM guanidine hydrochloride, 0.5 mM  $NaIO_4$  and 20% MeCN (pH 12) at flow rate 0.5 mL/min at 170 °C.

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Submitted for publication.

## Chapter 34

# The Change in the Flavor of Green and Black Tea Drinks by the Retorting Process

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The retorting process is responsible for the off-flavor of canned drinks. The quantitative change in the volatile components of the green and black tea canned drinks by the retorting process is not sufficient for explaining the results of sensory evaluation. In this study, the potent components responsible for the off-flavor were found using an aroma extract dilution analysis (AEDA). Many off-flavor components were proposed to be generated from the nonvolatile precursors by a nonenzymic reaction.

Drinks of green, black, and oolong teas together with coffee beverages are very popular in Japan. In general, the manufacturing process of a canned tea drink is as follows: extraction with hot water, filtration, cooling, addition of ascorbic acid, pH adjustment, filling, seaming, retorting, and cooling. The off-flavor of the tea drink is mainly generated by the retorting process (1-3). The amounts of the off-flavor components in a tea drink have been analyzed by GC and GC-MS. However, the quantity of the off-flavor components alone is not a satisfactory explanation to support the difference in the sensory evaluation by the retorting process. Recently, aroma extract dilution analysis (AEDA) has been used to study the characteristic odors of green and/or black teas (4,5). This current study focuses on the flavor change in green and black tea canned drinks during the retorting process using AEDA.

## Experimental

Leaves of the green (sencha, 200 g) and black teas (darjeeling tea, 200 g) were collected in 1998. Extraction was carried out with hot water (4 L, 70°C) for 5 min, followed by filtration. The extract (about 3 L) was immediately cooled to 20 and filled into cans (190 mL/can). The pH's of green and black tea extract were 5.7 and 5.4, respectively. The cans were retort-sterilized at 121 °C for 10 min, followed by cooling to room temperature. The pH's of green and black tea were 5.6 and 5.1, respectively.

The contents were distilled under reduced pressure (40 °C/20 mm Hg). The distillates (about 600-700 mL) were concentrated by the adsorptive column method (column: Porapak Q, 10 g, eluate: dichloromethane). The internal standard solution (100 µL) prepared from methyl undecanoate (5.15 mg) in dichloromethane (10 mL) was added to the concentrate. The column concentrate was evaporated (less than 40 °C/460 mm Hg) and concentrated in a stream of nitrogen.

A GC-MS analysis was performed using a Hewlett-Packard 5890 Series II gas chromatograph connected to a HP-5972 mass spectrometer. A DB-WAX fused-silica capillary column (60 m x 0.25 mm i.d., film thickness: 0.25 µm) was employed. The operating conditions were as follows: initial oven temperature, 80°C or 40°C then to 210°C at 3°C/min; injection temperature, 250°C; carrier gas, 1 mL/min He; split ratio, 1/50 or split-less.

An AEDA was performed using a Hewlett-Packard 5890 (GC) fitted with a glass sniffing apparatus and TCD. A DB-WAX fused-silica capillary column (30 m x 0.53 mm i.d., film thickness: 1 µm) was employed. The operating conditions were as follows: initial oven temperature, 40°C then programmed to 210°C at 5°C/min; injector temperature, 250°C; carrier gas, 4.4 mL/min He; splitless injection. The FD-factors were obtained using AEDA (6). The extract was stepwise diluted with dichloromethane until the odorous compounds were no longer detected by GC sniffing.

## Results and Discussion

Figure 1 shows the sensory descriptive analysis for the green and black tea canned drinks. The retort green tea had more a floral, sweet, clove-like, and heavy odor compared to the nonretort green tea. However, the green odor which is the characteristic odor of fresh green tea, was found to be decreased by the retorting process. On the other hand, in the retort black tea, the sweet, clove-like, heavy, and putrid odor increased compared with the nonretort black tea.

Table I shows the odorous components of the canned green and black tea drinks which were detected by GC sniffing. The black tea had more detectable peaks than the green tea. In addition, the number of components with increased FD-factors in black tea was more than that of the green tea. These results seem to be mainly attributable to the different manufacturing process between the green and black teas. The characteristic manufacturing process of the green tea includes steaming, during which the oxidizing enzyme in the tea leaves is inactivated and the green color of tea leaves is maintained. Therefore, most of the volatile components of the green tea are considered to be originally contained in the fresh leaves (7). On the other hand, black tea is produced by withering and fermentation process during which the enzyme reaction and nonenzymic browning reaction occur (8-10). Therefore, most of the volatile components of the black tea are believed to be formed during the course of manufacturing. Consequently, many more flavor precursors are contained in the green tea than in the black tea.

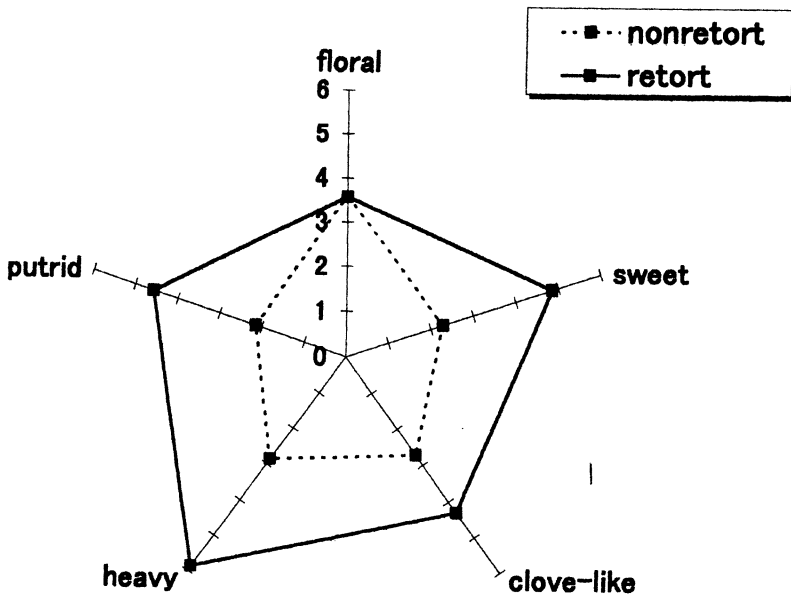
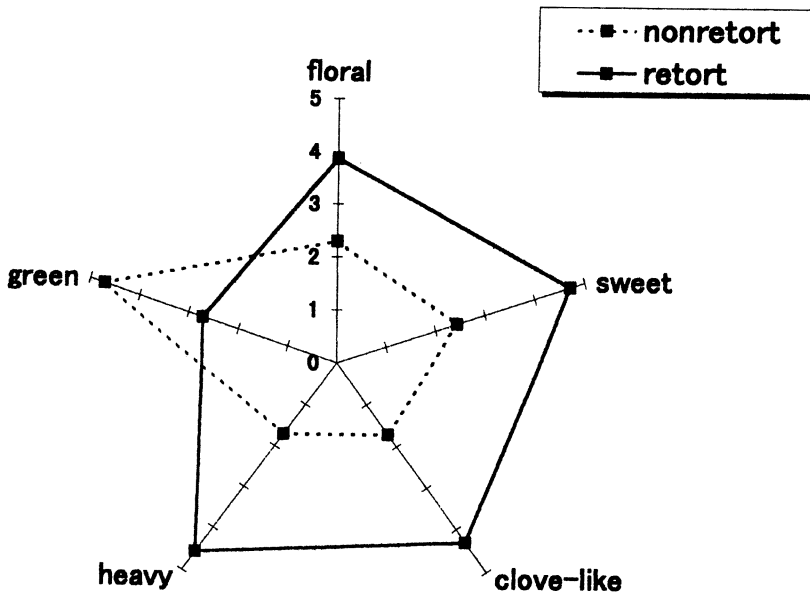


Figure 1. Sensory descriptive analysis of the green (top) and black tea canned drinks (bottom).

Table I. Odorous Components<sup>a</sup> of the Canned Green and Black Drinks

Peak No.	RI <sup>b</sup>	Odor Description	Component	FD-NG <sup>c</sup>	FD-RG <sup>d</sup>	FD-NB <sup>e</sup>	FD-RB <sup>f</sup>
1	927	stimulus	3-methylbutanal	10	100	500	1000
2	972	milk-like	2,3-butanedione	10	100	500	500
3	1013	green	unknown	nd <sup>g</sup>	nd <sup>g</sup>	100	100
4	1050	milk-like	2,3-pentanedione	10	10	10	10
5	1082	green	hexanal	nd <sup>g</sup>	nd <sup>g</sup>	500	500
6	1112	green	unknown	nd <sup>g</sup>	100	10	100
7	1133	green	unknown	nd <sup>g</sup>	nd <sup>g</sup>	10	10
8	1216	meaty	4-methoxy-2-methyl-2-butanethiol <sup>h</sup>	10	10	nd <sup>g</sup>	nd <sup>g</sup>
9	1218	green	unknown	nd <sup>g</sup>	nd <sup>g</sup>	10	100
10	1247	withered grass-like	(Z)-4-heptenal	10	10	500	500
11	1300	orange-like	octanal	10	10	nd <sup>g</sup>	nd <sup>g</sup>
12	1309	mushroom-like	1-octen-3-one	100	100	10	10
13	1349	popcorn-like	unknown	100	1000	100	100
14	1379	metallic	(Z)-1,5-octadien-3-one	500	500	500	500
15	1389	meaty	4-mercapto-4-methyl-2-pentanone <sup>h</sup>	1000	1000	10	10
16	1391	putrid	dimethyl trisulfide	nd <sup>g</sup>	nd <sup>g</sup>	nd <sup>g</sup>	5000
17	1395	green	(Z)-3-hexenol	nd <sup>g</sup>	10	500	500
18	1411	nutty	unknown	nd <sup>g</sup>	nd <sup>g</sup>	10	10
19	1436	nutty	unknown	10	10	10	10
20	1456	raw potato-like, nuty	methional, 2-ethyl-3,6-dimethylpyrazine	500	5000	500	5000
21	1476	nutty	2-ethyl-3,5-dimethylpyrazine <sup>i</sup>	10	10	500	500
22	1505	fatty, green	(E,E)-2,4-heptadienal	10	10	100	100
23	1520	fruity	benzaldehyde	nd <sup>g</sup>	nd <sup>g</sup>	10	10
24	1535	burdock-like	unknown	10	10	10	10
25	1550	green, floral	(Z)-4-decenal <sup>h</sup> , linalool	10	1000	10000	10000
26	1570	roasty	tetrahydrothiophen-3-one	nd <sup>g</sup>	100	10	500
27	1598	green	(E,Z)-2,6-nonadienal	100	100	1000	1000

Table I. Continued

Peak No.	R <sub>f</sub> <sup>b</sup>	Odor Description	Component	FD-NG <sup>c</sup>	FD-RG <sup>d</sup>	FD-NB <sup>e</sup>	FD-RB <sup>f</sup>
28	1608	camphora-ceous	unknown	nd <sup>g</sup>	nd <sup>g</sup>	100	100
29	1626	roasty	2-acetylpyrazine, 2-acetyl-3-methylpyrazine	nd <sup>g</sup>	10	100	100
30	1648	honey-like	phenylacetaldehyde	100	500	1000	1000
31	1675	Sour	isovaleric acid	nd <sup>g</sup>	nd <sup>g</sup>	500	500
32	1699	Fatty, sweet	unknown	nd <sup>g</sup>	nd <sup>g</sup>	500	500
33	1715	Green	(Z)-3-hexenyl-(Z)-3-hexenoate	500	500	100	100
34	1743	sour	valeric acid	nd <sup>g</sup>	nd <sup>g</sup>	10	10
35	1762	powdery	Unknown	10	10	nd <sup>g</sup>	10
36	1776	green	Unknown	nd <sup>g</sup>	nd <sup>g</sup>	100	10
37	1786	minty	methyl salicylate	nd <sup>g</sup>	nd <sup>g</sup>	1000	1000
38	1814	fatty	(E,E)-2,4-decadienal	nd <sup>g</sup>	nd <sup>g</sup>	100	100
39	1824	honey-like	β-damascone <sup>h</sup> , β-damascenone	100	1000	1000	10000
40	1849	green, burnt	hexenoic acid	100	100	1000	1000
41	1858	rosy	Geraniol	100	1000	5000	5000
42	1868	floral, burnt	geranyl acetone, guaiacol, α-ionone	10	10	500	500
43	1878	floral, sweet	Unknown	nd <sup>g</sup>	nd <sup>g</sup>	10	10
44	1908	sweet	Unknown	nd <sup>g</sup>	10	10	10
45	1959	woody, green	β-ionone, (Z)-jasmone	nd <sup>g</sup>	nd <sup>g</sup>	10	10
46	1963	green	heptanoic acid, (Z)-3-hexenoic acid	nd <sup>g</sup>	nd <sup>g</sup>	100	100
47	1980	sweet, lactonic	maltol, 1,5-octanolide	10	10	10	10
48	2010	sweet	Unknown	nd <sup>g</sup>	nd <sup>g</sup>	10	10
49	2047	lactonic	1,4-nonanolide	100	100	500	500
50	2074	sour	octanoic acid	nd <sup>g</sup>	nd <sup>g</sup>	10	10
51	2090	spicy	Unknown	nd <sup>g</sup>	nd <sup>g</sup>	10	10
52	2167	clove-like	Eugenol	100	100	100	100
53	2195	clove-like	2-methoxy-4-vinylphenol <sup>h,j</sup>	100	1000	100	1000

Continued on next page.

Table I. Continued

Peak No.	R <sub>i</sub> <sup>b</sup>	Odor Descriptio n	Component	FD-NG <sup>c</sup>	FD-RG <sup>d</sup>	FD-NB <sup>e</sup>	FD-RB <sup>f</sup>
54	2223	grasy	2-aminoaceto-phenone <sup>h</sup>	10	100	10	10
55	2274	sweet	jasmin lactone	nd <sup>g</sup>	10	10	10
56	2351	floral	(E)-methyl jasmonate	10	10	10	10
57	2400	floral	(Z)-methyl jasmonate	100	100	100	100
58	2444	animalic	Indole	1000	1000	100	500
59	2467	camphora- ceous, sweet	Coumarin	10	10	10	10
60	2502	animalic	Skatole	nd <sup>g</sup>	nd <sup>g</sup>	10	10
61	2588	vanilla- like	Vanillin	100	100	500	500

<sup>a</sup> FD factors of 10 or above were measured. <sup>b</sup>Calculated Kovat's retentions on DB-WAX. <sup>c</sup>FD factors of nonretorted green tea canned drink. <sup>d</sup>FD factors of retorted green tea canned drink. <sup>e</sup>FD factors of nonretorted black tea canned drink. <sup>f</sup>FD factors of retorted black tea canned drink. <sup>g</sup>Not detectable. <sup>h</sup>Newly identified components in green tea. <sup>i</sup>Newly identified components in black tea.

Linalool (no. 25) and geraniol (no. 41) which increased during the retorting process seemed to be mainly responsible for the floral odor of the retort green tea. These potent components are reported to be formed from the corresponding precursors by nonenzymic hydrolysis during the retort processing (11-14).

On the other hand, the retort black tea had a characteristic putrid odor. Dimethyl trisulfide (no. 16) contributes significantly to the off-flavor of the black tea because of the extremely low threshold value: 0.005-0.01 ppb (15). Dialkyl trisulfide was reported to be derived from the corresponding dialkyl disulfide that was formed from the S-alkylcysteine sulfoxide by disproportionation in *allium* plants (16). It has been further reported that in cruciferous vegetables, dimethyl trisulfide was formed from methyl methanethiosulfinate that was derived from S-methylcysteine-L-sulfoxide, and hydrogen sulfide via a nonenzymic reaction (17).

Interestingly, we found 2-methoxy-4-vinylphenol (no. 53), which is a frequent component responsible for off-flavors to be very important because of its clove-like odor. 4-Vinylphenol has been previously reported to be one of the significant components responsible for the off-flavor in the retort green tea (18). However, in our study, 2-methoxy-4-vinylphenol was found to be the potent off-flavor component instead of 4-vinylphenol. The lower threshold value of 2-methoxy-4-vinylphenol (3 ppb) compared with that of 4-vinylphenol (10 ppb) is considered to be the major reason (15). 2-Methoxy-4-vinylphenol is believed to be formed from ferulic acid in turn generated from its glycoside by decarboxylation during heating (19,20).



Furthermore, other common components for the off-flavor,  $\beta$ -damascone (no. 39),  $\beta$ -damascenone (no. 39) and methional (no. 20) were assumed to be responsible for the sweet and heavy odors, respectively.

Figure 2 shows the quantitative changes in the off-flavor components which showed increased FD-factors by the retorting process in the canned green and black tea drinks. The other off-flavor components were not detectable because of their lower concentrations. Except for 2-ethyl-3,6-dimethylpyrazine which showed no change in the retorting process, other off-flavor components showed similar increases in the amount. This agreed with the result of AEDA study.

## Conclusions

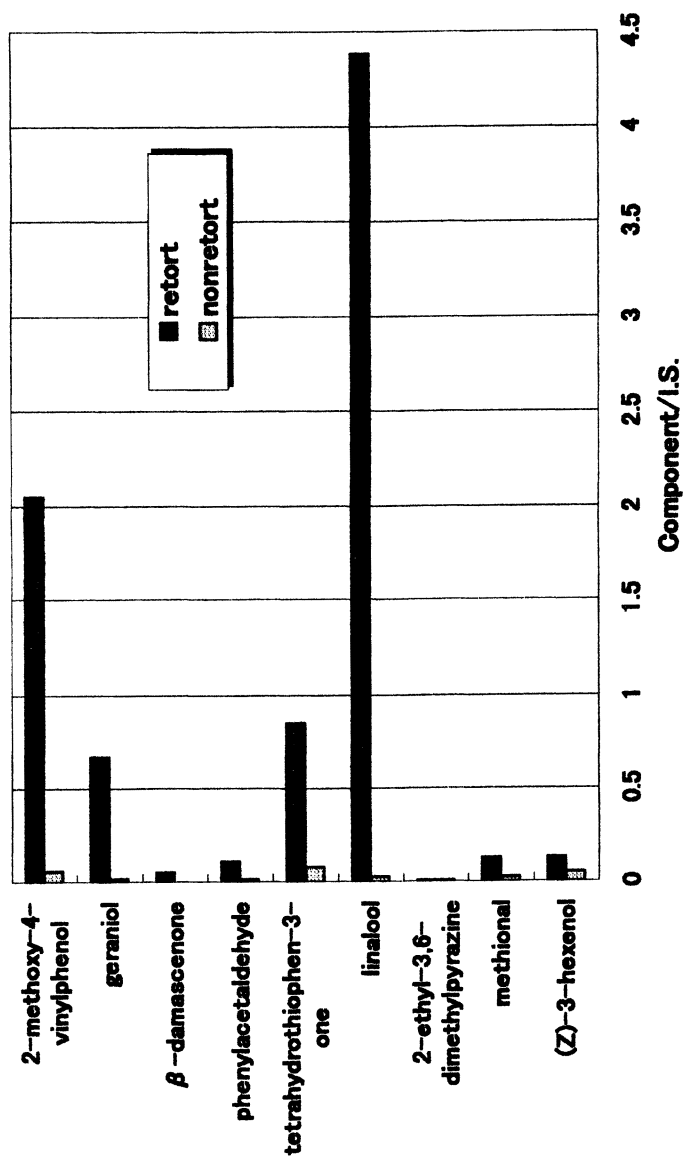
The off-flavor of the green and black tea canned drinks was mainly generated by the retorting process. The characteristic off-flavors of the retort green and black teas were floral and putrid, respectively. The potent off-flavor components were found using AEDA. Linalool and geraniol seemed to be mainly responsible for the off-flavor of the retort green tea. Dimethyl trisulfide was considered to significantly contribute to the off-flavor of the retort black tea. Furthermore, 2-methoxy-4-vinylphenol, one of the common off-flavor components, was important for the clove-like odor.

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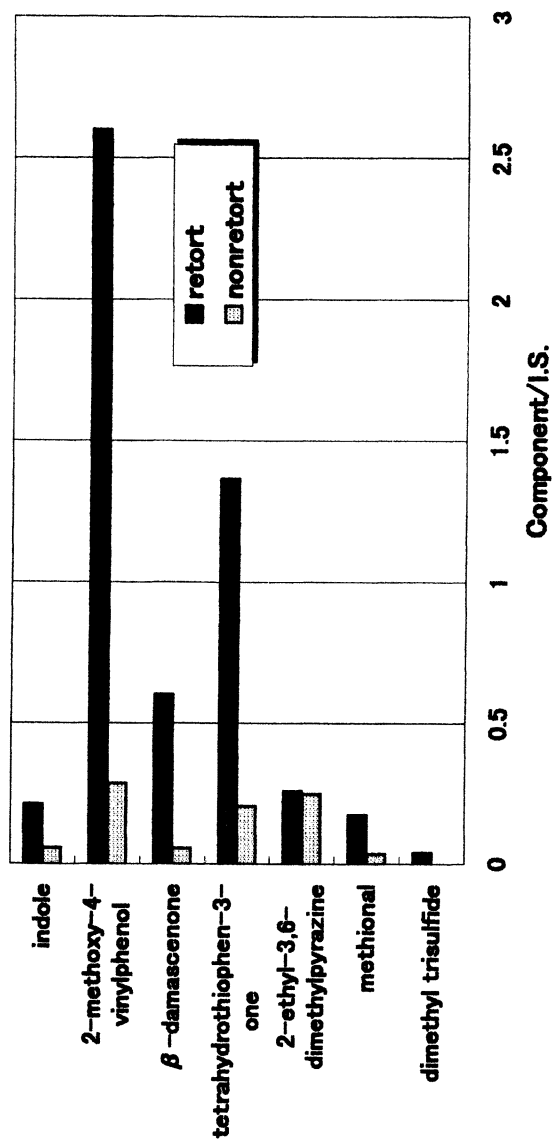


Figure 2. The quantitative ratios of the off-flavor components to the internal standard in the green (top) and black tea canned drinks (bottom).

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## Chapter 35

# Effect of pH and Tea Concentration on Extraction of Catechins from Japanese Green Tea

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Factors that affect the extraction of green tea catechins were studied. Extraction efficiencies of major catechins, EC, EGC, ECg and EGCg, were decreased with use of a higher pH extractant because of epimerization of these catechins to the minor catechins, C, GC, Cg and GCg. Extraction efficiencies of gallo catechins, EGC and EGCg, were more affected by tea concentration than those of non-gallo catechins, EC and ECg. In water extracts of green tea, the pH decreased with increasing tea concentrations. Extraction efficiencies of non ester catechins, EC and EGC, increased with an increase in tea concentration, but for gallate catechins, ECg and EGCg, the efficiencies dropped at higher tea concentrations.

Teas are produced mainly in Asian and some African countries and usually classified into three groups. The first is unfermented teas, the second is fermented teas, and the third is late fermented teas. Usual black teas and semi-fermented Chinese teas belong to the second group and no microorganisms are involved in their fermentation. Late fermented teas are produced using different microorganisms depending on teas. Unfermented teas, usually called green tea, are produced in China, Japan and some Asian countries. In Japan, green teas are one of the most popular beverages for both family and public use (1). Green teas are now receiving considerable attention because of specific health claims related to the presence of certain constituents, such as catechins, caffeine and ascorbate (2-4). Among these constituents, catechins are present at 8 to 15% of dried leaf weight of Japanese green tea (5).

The green tea catechins are composed of a family of four major catechins: epicatechin (EC), epigallocatechin (EGC), epicatechin gallate (ECg) and epigallocatechin gallate (EGCg); and four minor catechins: catechin (C), gallo catechin (GC), catechin gallate (Cg) and gallo catechin gallate (GCg), which are epimers of their respective major catechins. Catechins, along with some amino acids such as theanine,

glutamic acid and arginine are important components in the taste associated with green tea (6).

Studies of the precise composition of green tea catechins and methods for their extraction are needed in order to use catechins efficiently. For quantitative extraction of catechins from green tea, organic solvents such as methanol and acetonitrile have been used. Also, Suematsu *et al* (7) suggested acetonitrile-water (1:1, v/v) as an efficient extraction solvent to analyze catechins in tea. This method was useful for measuring catechin content of leaves without epimerization of catechins during extraction. However, for studying human consumption of tea, extraction using organic solvents may not reflect actual levels of catechins in the tea beverages. There have been some reports on the extraction conditions of green tea using hot water. However, the purpose of these studies was to find the best condition for sensory evaluation of tea quality (8) or to produce the most tasty tea (9). In the present study we demonstrate the effects of extractant pH and tea concentration on the efficiency of the extraction of catechins from green tea as well as effect of filter materials for quantitative analysis of catechins.

## Materials and Methods

### Samples and Chemicals

Eight catechin standards and Polyphenon 60, a partially purified green tea extract, used in this study were products of Mitsui Norin Co. Ltd. (Tokyo, Japan). All other chemicals, including caffeine, were purchased from chemical suppliers. Green tea was prepared at Tokyo Metropolitan Agricultural Experiment Station in May 1997 using first crop of 'Yabukita' leaf of the year. The catechins and caffeine contents of this tea were measured by the method of Goto *et al.* (10). The levels of these constituents in this tea were: EC, 9.90 mg/g; EGC, 40.58 mg/g; ECg, 12.42 mg/g; EGCg, 71.66 mg/g; C, 1.17 mg/g; GC, 2.81 mg/g; Cg, 0.87 mg/g; GCg, 1.72 mg/g; and caffeine, 25.51 mg/g dry weight. In a portion of this study, ten commercially available green teas were also used. These teas consisted of five high grade teas, which contained more than 6% of total nitrogen, and five low grade teas, which contained less than 4% of total nitrogen by dry weight. Bower & Bates buffer (11) was prepared using 0.2 M sodium hydroxide and 0.2 M potassium dihydrogen phosphate.

### Extraction and Analysis

Green tea samples were milled with a small batch grinder and passed through a 1 mm mesh screen. The milled samples were stored at  $-30^{\circ}\text{C}$  until used. Powdered tea samples were extracted with 100 mL of water or buffer at  $80^{\circ}\text{C}$  with constant gentle shaking for 20 min. The extracts were filtered through a filter cartridge with a hydrophilic PTFE membrane (0.45  $\mu\text{m}$ , 13 mm) prior to HPLC analysis.

The amounts of catechins and caffeine in the extract were measured

simultaneously by the method previously developed (10). Briefly, extracts were separated on a high quality reversed phase (ODS) column using water-acetonitrile-phosphoric acid mixture as mobile phase with a gradient for acetonitrile concentration.

## Results

### Absorption of some catechins by filter membranes

Solutions of partially purified green tea extracts were passed through several types of disposable cartridges containing different filtration membranes, and recovery of each chemical was determined. Recovery of non-ester type catechins, EC and EGC, and caffeine were quantitative under all conditions. However the recovery for gallate catechins, ECg and EGCg, was different depending on the condition (Table 1). From these results, we decided to use hydrophilic PTFE filters for sample preparation.

**Table 1. Recovery of Chemicals from Filter Cartridges [%]**

<i>Membrane</i>	<i>Solvent</i>	<i>EC</i>	<i>EGC</i>	<i>ECg</i>	<i>EGCg</i>	<i>Caffeine</i>
PTFE	water	100.3	99.8	97.8	99.2	99.7
RC	water	96.3	94.1	87.1	86.5	98.9
PVDF	water	97.3	96.5	8.6	30.2	99.2
CA	water	100.0	99.2	92.6	96.6	99.7
PTFE	5% CH <sub>3</sub> CN	100.5	101.0	100.5	100.0	101.0
RC	5% CH <sub>3</sub> CN	95.5	93.5	93.0	91.5	100.0
PVDF	5% CH <sub>3</sub> CN	98.0	97.5	21.0	43.5	99.5

2 mL of Polyphenon 60 solution (15 mg/100 mL) was filtered through each cartridge and recovery of chemicals was measured by HPLC (10).

PTFE: Polytetrafluoroethylene with hydrophilic coating, RC: Regenerated cellulose, PVDF: Polyvinylidene fluoride, CA: cellulose acetate

### Epimerization of catechins during extraction

A typical chromatogram of green tea catechins and caffeine is shown in Figure 1. One per cent green tea extracts (1 g of tea extracted by 100 mL of extractant) were prepared under three different pH conditions, pH 6.0, 7.0 and 8.0, and the level of extracted catechins and caffeine were compared to that extracted using a 50% acetonitrile solution (7). The level of caffeine was practically the same in all these extracts (Figure 1), but in buffered solutions, the amounts of the various catechins were different from the levels detected in the 50% acetonitrile extract.

The levels of individual catechins in each buffer were different by extractant. When the pH of the extractant was increased, the levels of the major catechins

decreased. In contrast, the levels of the minor catechins dramatically increased with an increase in pH. However, the total amounts of all eight catechins in the extracts were stable at about 70% of the amount extracted with 50% acetonitrile. Small differences were observed in the total amounts of each paired epimer, EC + C, EGC + GC, ECg + Cg and EGCg + GCg, among these three buffer extracts.

Similar changes between epimers were observed after 20 min of incubation of catechin standards in the buffer at 80°C. These results suggest that the major catechins epimerize to the minor catechins during extraction in high pH buffers.

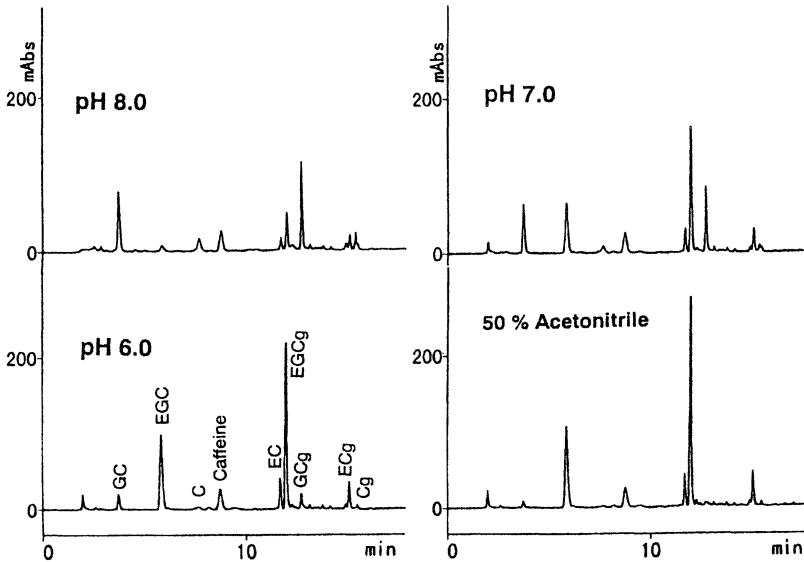


Figure 1. Chromatogram of tea extracts. One gram of tea was extracted by 100 mL of buffer solutions or 50% acetonitrile solution.

### Effect of tea concentration on extraction of major catechins.

Green tea catechins were extracted with distilled water at five different tea concentrations: 0.25, 0.5, 1.0, 2.0 and 3.0%. Before extraction, the pH of the water was approximately neutral (pH 7.2), and after extraction the pH decreased to 6.7 at 0.25%, 6.4 at 0.5%, and 6.3 at 3%. The extraction efficiencies of the four major catechins at different tea concentrations were determined. Varying the tea concentration did not affect the high extraction efficiency of non-ester type catechins, EC and EGC. For both catechins, the extraction efficiency was consistently 85 to 90% of that of the 50% acetonitrile extracts regardless of tea concentration. However, the extraction efficiencies for gallate ester catechins, ECg and EGCg, decreased with



increasing tea concentration, going from 80-85% at 0.25% tea concentration to 65-70% at 2% tea concentration (Table 2).

**Table 2. Effect of Tea Concentration for the Extraction Efficiency. [%]**

Concentration [%]	EC	EGC	ECg	EGCg
0.25	87.5	89.2	85.9	80.9
0.50	88.6	90.8	83.0	79.2
1.00	91.3	90.9	78.0	75.1
2.00	88.2	86.7	66.7	62.9
3.00	89.8	87.3	67.2	63.5

Extraction efficiencies of each major catechins were determined by the amounts of those extracted in a 50% acetonitrile solutions.

### Effects of extractant pH and tea concentration on extraction of major catechins.

To demonstrate the effects of both extractant pH and tea concentration on extraction efficiency, different concentrations of tea were extracted with 14 different pH buffers, including pH 6.0 and pH 6.4-7.6 at intervals of 0.1. As pH increased, the extraction efficiencies of most major catechins decreased. At the same pH, more differences were observed at lower tea concentrations (Figure 2). At the same

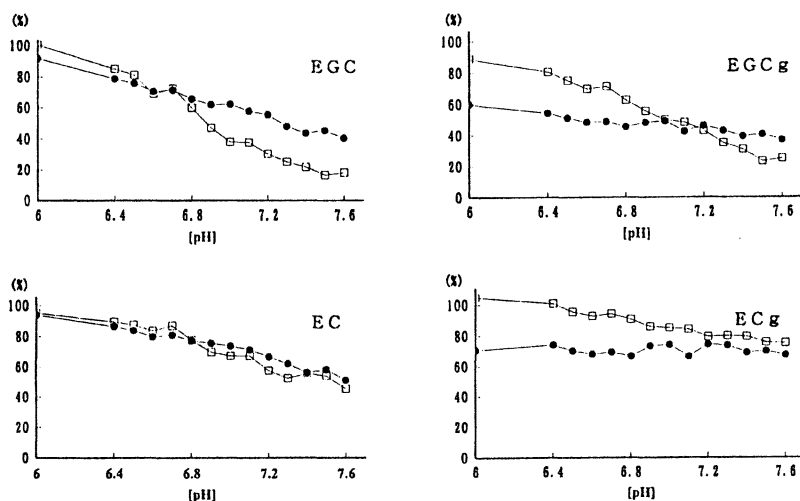


Figure 2. Effects of extractant pH and tea concentration. --□-- :0.25 %, --●-- :3.0 %

tea concentrations, the changes in extraction efficiencies of non-ester type catechins (EGC and EC) were greater than those of ester type catechins (EGCg and ECg) with the increase of extractant pH.

In comparing extraction efficiencies between non gallo catechins (EC and ECg) and gallo catechins (EGC and EGCg), larger changes associated with extractant pH and tea concentration were observed with gallo catechins (Figure 2). For example, the extraction efficiency of EC at pH 6.0 was about 95% and decreased to 55 to 60% at pH 7.6, and the extraction efficiency of EGC at pH 6.0 was 95-100% and decreased to 20-40% depending on tea concentration. Similar relations also were observed between ECg and EGCg.

### Extraction of catechins by tap water

The pH of extracts were measured using five different tap waters at six different tea concentrations (Table 3). The pH of tap waters were 7.15-8.54 before heating and increased to 8.71-9.03 after heating and before addition of tea. Once extraction started, the pH of waters decreased with larger decreases corresponding to increasing tea concentration. At 0.75% concentration, the pH of the all five extracts became less than 7.0, and at 3% concentration they reached 6.35-6.52.

Using two different grades of tea with five teas in each grade, the pH of tap water extracts was measured at tea concentrations ranging from 0.1-3%. There were no differences in average pH between these two grades when tea concentrations ranged between 0.1 and 0.5%. For example, the average pH of tea extracts at 0.25% concentration for both grades was 7.1. When concentrations were 0.75 and 3%, the average pH of low grade tea extracts (6.8 and 6.3, respectively) became lower than that of high grade teas (6.9 and 6.5, respectively).

**Table 3. pH of tea extract by the difference of water and tea concentration**

Water	Hardness of water	Before heating	After heating	Tea concentration (% w/v)					
				0.1	0.25	0.5	0.75	2.0	3.0
A	160	8.54	9.03	7.41	7.17	6.99	6.87	6.59	6.47
B	70	8.19	9.04	7.59	7.28	7.29	6.93	6.65	6.52
C	40	7.57	8.71	7.15	6.95	6.82	6.66	6.41	6.35
D	120	7.20	8.93	7.20	6.99	7.09	6.78	6.51	6.41
E	50	7.15	8.76	7.30	7.03	6.91	6.74	6.52	6.41

Water hardness was measured by PONAL KIT-WH (Wako Pure Chem., Osaka, Japan)

Amounts of catechins in tap water extracts are shown in Figure 3. The amounts of minor catechins in low tea concentrations were always larger than those of high tea

concentrations. However, in the case of major catechins, there were differences between gallate ester type catechins and non-ester type catechins. For non-ester catechins, the amounts increased with an increase in tea concentration with the exception of a slight decrease at 3% tea concentration in some water. In contrast, for gallate catechins at low tea concentration, 0.1 to 0.5%, the highest extraction efficiency was shown and decreased gradually with an increase in tea concentration. The total amounts of epimers of non-ester type catechins, EGC + GC or EC + C, were stable throughout these tea concentrations. In contrast the total amounts of epimers of gallate catechins, EGCg + GCg or ECg + Cg decreased depending on the increase in tea concentration except for total gallo catechins (EGC + GC, EGCg + GCg) at very low (0.1%) tea concentration

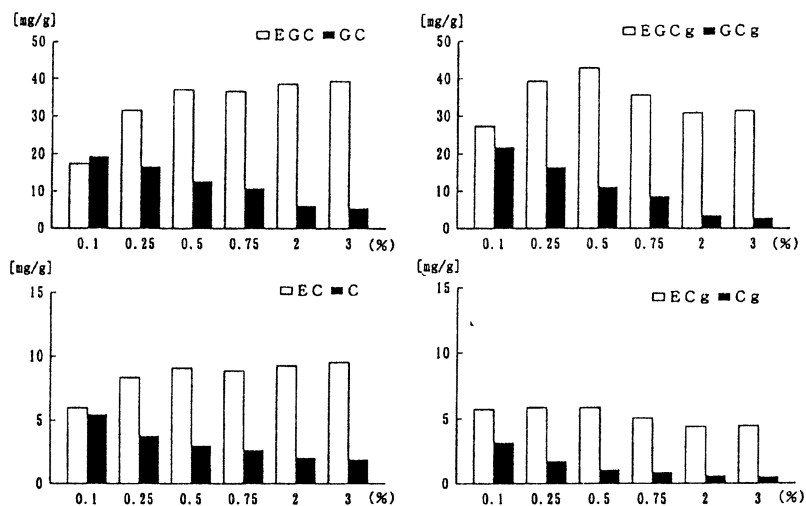


Figure 3. Catechin composition of a tap water extract. Tea was extracted by tap water D in table 3.

## Conclusion

In general, extraction efficiencies of major catechins in green tea were higher with lower pH and the effect of extractant pH on extraction efficiency was smaller with higher tea concentrations. The reason for the lower extraction efficiency of major catechins at high pH is epimerization of these catechins to minor catechins. However, the total amount of two epimers, for example EGC + GC, did not change appreciably depending on extractant pH.

In extracting green tea catechins with water, tea concentration is the most important factor in extraction efficiency because the pH of extracts is dependent on

tea concentration. For all extraction conditions in this study, the extraction efficiency of caffeine was always high and stable.

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## Chapter 36

# The Analysis of Coffee Phenols and Phenolic Acids

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A comparison of high performance liquid chromatography systems one with a photodiode array detector (HPLC/UV PDA) and the other with dual detection, a single wavelength ultraviolet and DC voltametric detectors (HPLC/UV/DCV) yielded mixed performance results. Ultraviolet detection, whether a PDA or single wavelength UV, was able to analyze a series of phenolic compounds with more accuracy and sensitivity than DCV. DCV suffered from electrode passivation. However, DCV was more selective, detecting only phenols making it ideal for identification.

The major phenolic compounds in coffee are the chlorogenic acids. They consist of 3-caffeoylquinic acid (3-CQA), 4-caffeoylquinic acid (4-CQA), 5-caffeoylquinic acid (5-CQA), 3-feruloylquinic acid (3-FQA), 4-feruloylquinic acid (4-FQA), 5-feruloylquinic acid (5-FQA), 3,4-dicaffeoylquinic acid, 4,5-dicaffeoylquinic acid and 3,5-dicaffeoylquinic acid. The total concentration in green coffee beans is between 6 and 9 percent, depending upon variety and type. The chlorogenic acids in foods decompose from physical, chemical and biological abuse. Hydrolysis is the first stage of its decomposition. The chlorogenic acids form caffeic, ferulic and quinic acids. Fiddler *et al* (1) uses a ferulic acid model to measure thermal degradation. Secondary pathways include bacterial(2), fungal(3), yeast(4), and enzymatic reactions. Demethylation of ferulic acid can produce additional caffeic acid. Further hydrolysis, demethylation, oxidation, reduction, and decarboxylation can produce many different phenolic compounds. Some of these compounds found in coffee include 4-vinylguaiacol, 4-methylguaiacol, guaiacol, 4-methylcatechol, catechol, isoeugenol, vanillin, and vanillic acid. Each compound impacts the flavor of coffee producing flavors ranging from musty, metallic or medicinal taste to floral or fragrant. Thus, phenol and phenolic acid assays of coffee are important factors as a measure of its quality.

The most common method for analyzing the phenolic acids and phenols in coffee is high performance liquid chromatography with UV detection (HPLC/UV). Prior to HPLC methodology the measuring of phenols in coffee was usually reported as total

chlorogenic acid. It required separation of the phenol from the matrix, and analysis by UV spectrometry. Gas chromatography(5) employing trimethylsilation was first reported in 1958. HPLC analysis of phenolic acids in coffee was described in 1980(6). More recently, HPLC electrochemical detection has been used to analyze phenols in wine(7-9). In the present work the HPLC UV/PDA and UV/DCV methods were used to determine the major chlorogenic acids, phenolic acids and phenols in green coffee extracts.

## Experimental Section

### Reagents.

Standard phenol compounds were purchased from Aldrich-Sigma Chemical Company with the exception of 4-ethylguaiaicol purchased from CA Aromatics Company and 5-feruloylquinic acid was synthesized. These were used without further purification. Primary standard solutions were prepared to 1000 mg/L in water. This was followed up with a further dilution of a mixed standard at 25 and 50 mg/L in water. All of the water used was HPLC grade. Maxwell House Research provided the robusta and Brazil arabica green coffee samples.

### Sample Preparation.

Fifteen grams of green coffee and 150 grams of water were weighed in to a 500 mL Erlenmeyer flask along with boiling stones to prevent bumping. The samples were boiled on a hot plate for 30 minutes with a condenser, then cooled. They were then reweighed, and any weight loss was corrected by the addition of water. The samples were further diluted by a factor of five before analysis.

### Instrumental Procedure.

Samples were analyzed on a Water Alliance HPLC 2690 System with a 996 PDA and Millenium32 data system and controller, and a Dionex 500 HPLC consisting of a GP-40 gradient pump, an ED-40 electrochemical detector, AD-20 absorbance detector and a AS-3500 autosampler. The ED-40 employed a glassy carbon working electrode and a Ag/AgCl reference electrode. The voltage was set to 1.12 volts. This was obtained empirically. Both systems used the Megachem Intersil ODS-3, 5  $\mu$ , 150 x 2.1 mm, P/N 0396-150X021, column for the separation. The analysis was performed on a 10  $\mu$ L sample. A solvent gradient was used to perform the separation. Mobile phase A is 1% acetic acid in water and mobile phase B is 1% acetic acid in acetonitrile. The following gradient profile was used for all analyses.

Table I. Gradient and Flow Parameters

<i>Time, min</i>	<i>Flow, mL/min</i>	<i>%A</i>	<i>%B</i>	<i>Curve</i>
0	0.2	0	100	
10	0.2	6	94	linear
90	0.2	54	46	linear
95	0.2	70	30	linear
100	0.2	0	100	linear

## Results and Discussion

### Characterization

Identification of the phenolic acids was determined by three criteria. The primary assignment was made using the retention times of the standards. The two detection method, UV PDA and DCV, improve the selectivity of the detectors by selecting wavelengths sensitive to specific types of compounds or adjusting the oxidation potential for phenols. Finally, isomers of the chlorogenic acids were identified by their identical UV spectra and literature reference spectra.

Selection of the best wavelengths was determined by reviewing the spectra obtained on the HPLC/UV PDA. Two monitoring wavelengths were required. Figure 1 is a composite of all the standard spectra. The chlorogenic acids, ferulic acid and caffeic acid absorb at 325 nm. All of the phenols absorb at 280 nm, as do caffeine and theobromine. Caffeine has strong absorption at 280 nm and a peak maximum at 272 nm. Since the concentration of caffeine in coffee is high, it has the potential to

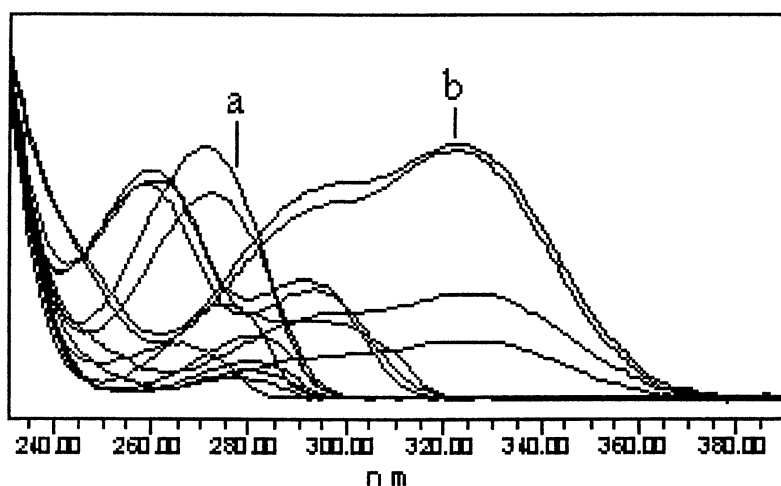


Figure 1. Individual UV spectra of standard mixture. All phenols absorb at 280 nm, a. Chlorogenic, ferulic and caffeic acids have absorbance maxima at 325 nm, b.

interfere with the analysis of phenols, caffeic and vanillic acids, with similar retention times. However, caffeine does not absorb at 325 nm, thus the phenolic acids can be measured without interference at that wavelength.

Figure 2. is a chromatogram of a standard mixture derived from the PDA at 280 nm. Two alkaloids, caffeine and theobromine, were added to the standard mixture because they absorb at 280 nm as do the phenols and phenolic acids. In this chromatogram adequate resolution was obtained for vanillic acid, caffeic acid, caffeine and 5-CQA. However, when a sample is analyzed, the caffeine and 5-CQA are much larger than the other two phenolic acids. To illustrate this point, Figure 3. is

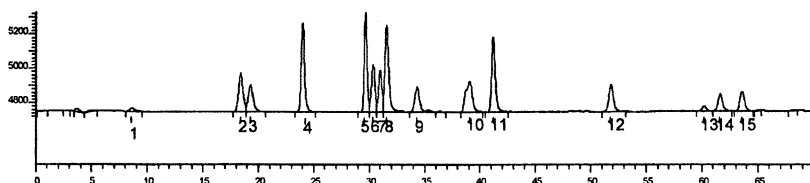


Figure 2. Chromatogram of standard mixture, 25 mg/L, derived at 280 nm 1) pyrogallol, 2) protocatechuic acid, 3) catechol, 4) theobromine, 5) vanillic acid, 6) caffeic acid, 7) caffeine, 8) 5-CQA, 9) guaiacol, 10) 5-FQA, 11) ferulic acid, 12) 4-methylguaiacol, 13) 4-vinylguaiacol, 14) 4-ethylphenol, and 15) 4-ethylguaiacol.

a comparison of two derived chromatograms one at 280 nm and the other at 325 nm. The chromatograms are expanded 25X to reveal some of the minor components in the

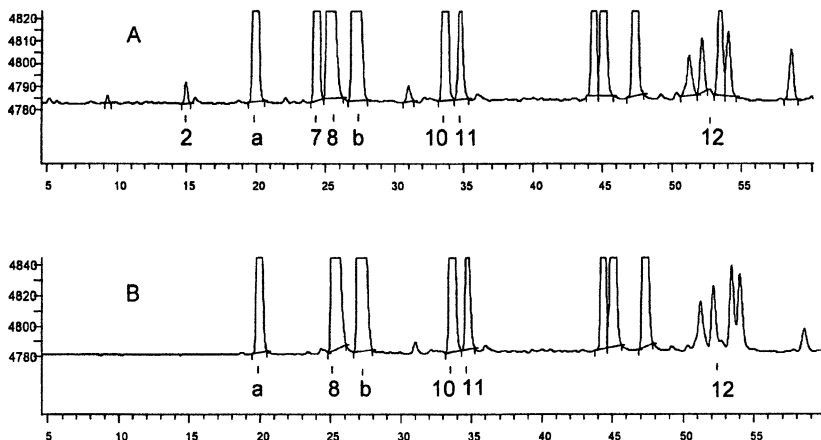


Figure 3. Comparison of green robusta derived chromatograms at 280 nm, A, and 320 nm, B. (a) 3-CQA, (b) 4-CQA based on spectra and literature retention times. Component numbers 2 through 12 are identified in Figure 2.



extract. The striking difference between the two chromatograms is the disappearance of the caffeine (7) and protocatechuic acid (2) peaks at 325 nm.

In contrast to the chromatograms obtained by UV, the same standard mixture was analyzed using DCV detection, as shown in Figure 4. The response to pyrogallol is much more intense. This compound does not absorb at 280 nm. The literature reports oxidation potential for phenol at approximately 0.7 volts (10). Woodring *et al* (9) found 1 volt produces the maximum signal response. After measuring the signal response of 5-CQA at 0.7, 1.0 and 1.12 volts, the maximum relative response was found at 1.12 volts.

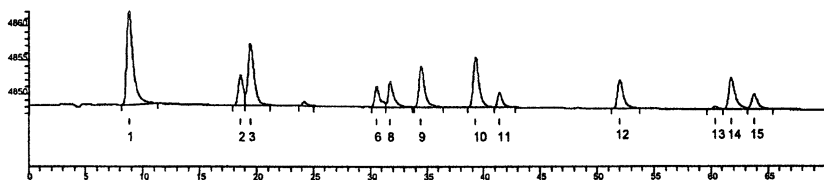


Figure 4. Chromatogram of phenol standard using DCV detection at 1.12 volts.

## Quantitation

After peak identification was made, the data were quantified to determine the concentration of the phenols in a green Brazil and green robusta coffee. The response factors (RF) for each of the standard phenols was calculated using the ratio of its concentration in mg/L to area response, Table II. With the exception for pyrogallol, the RF values for UV detection at 280 nm were 10 to 100 times more sensitive than the DCV. The UV RF values were calculated from an average of 9 injections. The RF values for the standard mixture analyzed by the DCV detector were calculated from the first injection. The average values were not calculated because the response of the electrode decreased by a factor of 2 as compared to the last standard injection. Figure 5 plots the change in RF for the DCV detector over the duration of a 3 day run. Passivation of the glassy carbon electrode produced the loss of sensitivity. The process of passivation will be investigated in a future study. This effect does not seem to be observed in the analysis of wines or beer (7-9).

A robusta and Brazil arabica green coffee were analyzed to test the performance of the method. Quantitative data were obtained using the average RF values obtained with the UV detector. Standard bracketing was used to calculate the concentration of phenols using DCV detection. A mixed standard was injected every fifth vial. Between each standard four replicate sample injections were made. A total of 32 sample and 9 mixed standard injections were made over a 78 hour continuous run. This approach minimized the error due to the rapid change of the electrode

Table II. Response Factors for UV and DCV

	Pyrogallol	Protocatechuic acid	Catechol	Vanillic acid
RF UV 280	5.47E-05	4.15E-06	5.25E-06	2.21E-06
RF DCV	4.55E-05	1.76E-04	7.16E-05	ND
	Caffeic Acid	5-CQA	Guaiacol	5-FQA
RF UV 280	3.91E-06	2.13E-06	6.33E-06	3.80E-06
RF DCV	2.56E-04	2.00E-04	1.19E-04	9.10E-05
	Ferulic Acid	4-Methylguaiacol	4-Vinylguaiacol	4-Ethylphenol
RF UV 280	2.50E-06	5.32E-06	2.81E-05	8.74E-06
RF DCV	3.73E-04	1.59E-04	1.87E-03	1.31E-04
	4-Ethylguaiacol			
RF UV 280	7.74E-06			
RF DCV	2.96E-04			

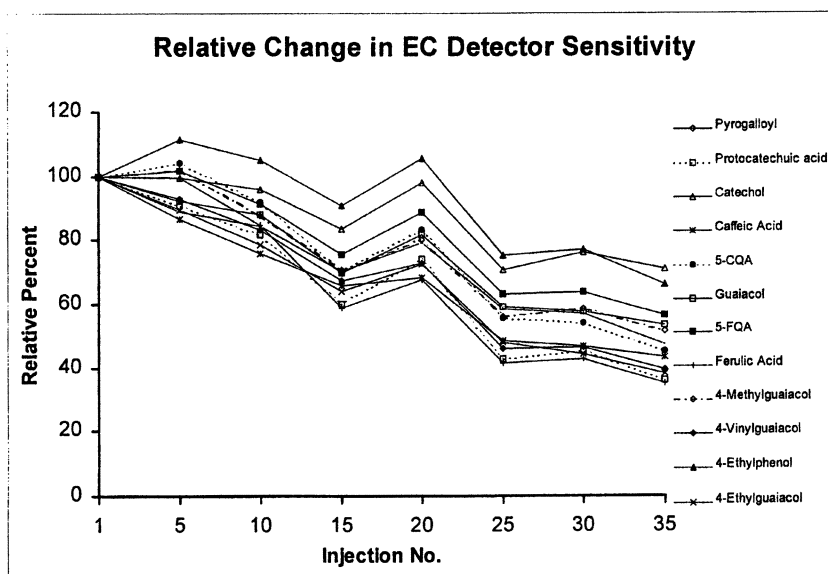


Figure 5. Loss of sensitivity of standard phenols over an extended time, 78 hours. The standard compounds 1 through 15 are described in Figure 2.

**Table III. Analysis of Green Coffee - UV vs. DCV Detection**

% Component	<i>UV Detector</i>			
	<i>Robusta</i>	<i>SD</i>	<i>Brazil</i>	<i>SD</i>
% Catechol	0.08	0.01	-	-
% 5-CQA	5.50	0.17	5.68	0.21
% 5-FQA	0.50	0.01	0.25	0.01
% Ferulic Acid	-	-	0.11	0.01
% Component	<i>DCV Detector</i>			
	<i>Robusta</i>	<i>SD</i>	<i>Brazil</i>	<i>SD</i>
% Catechol	0.19	0.04	0.06	0.01
% 5-CQA	4.46	0.42	5.18	1.1
% 5-FQA	0.44	0.04	0.22	0.05
% Ferulic Acid	-	-	0.12	0.01

sensitivity. The performance of the DCV detector was significantly worse than the UV detector. The results of this analysis are in Table III. The relative standard deviation for the UV detector was 3 to 9% while between 10 and 23% for the DCV detector.

### Conclusion

Both UV photodiode array and DC voltametry detection accomplished the identification of phenolic acids and phenols in green coffee. Each detection system has its advantages and disadvantages. Spectral selectivity of chlorogenic, caffeic and ferulic acids at 325 nm eliminates most interference from other phenols, alkaloids, aliphatic acids and carbohydrates additionally extracted from the matrix. Optimizing the oxidation potential of the DCV detector for phenol oxidation eliminates the interference of other compounds in the coffee extract.

Performance behavior of the UV detector is both substantially more sensitive and stable than the DCV detection. However, the selectivity of the DCV detector beckons further investigation to slow or eliminate the passivation of the glassy carbon electrode.

### Acknowledgement

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electrochemical detector. Finally, the author would like to thank S. McGarty, Technical Information, for scouring the literature for reference materials.

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## Chapter 37

### Capillary Electrophoresis of Roasted Coffees

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Arabica coffee beans were roasted either at 240-290°C for 3 min or for 2-5 min at 265°C. Aqueous extracts prepared from them, and from unroasted beans, were separated into high molecular weight (HMW) and low molecular weight (LMW) fractions by ultrafiltration, using a membrane with a nominal molecular weight cut-off of 5 000 daltons. Absorption at 460 nm for the aqueous extracts increased with degree of roast. Extracts and fractions were analyzed by capillary zone electrophoresis (CZE) using different buffers. Beans roasted to similar colors gave very similar CZE data, regardless of roasting conditions, the electrophoretograms (e-grams) being characteristic of the degree of processing. A broad peak was obtained for the HMW fraction which increased with degree of roast and which was indicative of a complex mixture of compounds. It contained the majority of the colored material. The data suggest that the aqueous extracts of the roasted coffees are qualitatively similar, regardless of the roasting regime.

Capillary electrophoresis (CE) is a family of powerful separation techniques which has several advantages over alternative better established methods. These advantages include a very high separation efficiency, typically 4 000 000 theoretical plates per meter, compared to around 100 000 plates per meter for an HPLC column (*1*), injection of nanolitres of sample, separation in aqueous buffer and short run times. The absence of organic solvent means that detection at 200 nm is possible with no loss of sensitivity, permitting the detection of analytes with weak chromophores. The most frequently used mode of CE is capillary zone electrophoresis (CZE), in which positively and negatively charged analytes are separated in buffer according to their charge to mass ratios.

The application of CE to the analysis of food components has been reviewed (2-4). The determination of caffeine (5) and phenolics in coffee (6) has been reported.

Roasting is an essential processing step for the conversion of green coffee beans to a desirable beverage. The purpose of roasting is to develop the characteristic flavor and color of roast coffee. Additional consequences of roasting include loss of dry matter (typically 5-8%) (7), evolution of carbon dioxide, loss of water, reduction in levels or loss of components (including sugars, amino acids, chlorogenic acids and trigonelline), and formation of melanoidins (which give roast coffee its characteristic color) (8).

All melanoidins are notoriously difficult to characterize (9-10). Their structures are unknown, but recent work by Hofmann (11) suggests that small colored molecules may play a crucial role in their formation. Matrix assisted laser desorption mass spectrometry (MALDI-TOF-MS) has been successfully used to elucidate the structures of polymers prepared from pyrrole monomers (12), while solid state nuclear magnetic resonance spectroscopy (NMR) has been used to characterize some functional groups of melanoidins (13).

Although melanoidins constitute at least 20% of the solids of a roast coffee brew, relatively little is known about their structure and properties. In a series of detailed studies, Maier (14) used resin and gel permeation chromatography to separate coffee melanoidins into fractions and a wide range of functional groups was identified. Amino acids and monosaccharides were released from the melanoidins on acid hydrolysis. Steinhart and Packert (15) have used gel permeation chromatography and thin layer chromatography to separate coffee melanoidins into distinct fractions.

CZE has the potential to separate coffee melanoidins according to their charge:mass ratios and to monitor changes in charge:mass ratio with degree of roasting. CZE with pH 9.3 borate buffer has already been used to profile total Maillard reaction products from model systems. Components of lower molecular weight migrated as sharp peaks while the higher molecular weight material migrated as a single broad band (16).

The aim of this study was to apply CE to the analysis of two series of coffees to ascertain if the technique could distinguish any differences that might exist between fractions from beans subjected to different regimes but to the same color. The first series was roasted for different times at the same temperature, and the second was roasted for the same time at different temperatures.

## Experimental

### Sample preparation

Colombian Arabica coffee beans were roasted using the conditions given in Table I. The colors of the beans were measured using a Color-Tester LK100 unit (Dr Bruno Lange GmbH, Berlin) and are shown in Table I.

**Table I. Roasting regimes and Lange units for the coffee samples**

<i>Sample Code</i>	<i>Temperature (°C)</i>	<i>Time (min)</i>	<i>Lange Units</i>
C0/T0	-	-	43
C2	240	3	24.5
C3	260	3	13.3
C4	280	3	7.1
C5	290	3	4.5
T2	265	2	25.8
T3	265	3	11.8
T4	265	4	6.3
T5	265	5	3.9

Beans (10 g) were ground in a coffee grinder for 1 min. Aqueous extracts were prepared by adding boiling water (50 mL) to grounds (2.5 g), stirring for 10 s and standing for 10 min. Extracts were filtered through paper and then a 0.2 µm PVDA filter. Aqueous extracts (200 µL) were separated into high molecular weight (HMW) and low molecular weight (LMW) fractions using Microcon ultrafiltration units with low binding regenerated cellulose membranes (nominal molecular weight cut-off of 5 000 daltons) (Millipore, Watford, UK) at 5 000g for 50 min. The retained sample (<20µL) was washed with water (100 µL) and centrifuged. Washing was repeated three times and the resulting retained material was made up to a total of 50 µL with water to give the high molecular weight (HMW) fraction. HMW fractions were four times more concentrated than the total samples. Absorbance measurements at 460 nm were taken of 1:10 dilutions of the aqueous extracts in water. Reverse osmosis water was used throughout.

### CZE

Separations were carried out using a Hewlett Packard (Bracknell, UK) <sup>3D</sup>Capillary Electrophoresis instrument equipped with Chemstation software. The capillary was 48.5 cm long (40 cm to the detector), 50 µm i.d. and with a x3 bubble cell. The temperature was 25°C and injection was at 50 mbar for 5s. Separations were monitored at 200 and 460 nm and spectra were collected from 190-600 nm. When pH 2.5, 30mM phosphate buffer was used, the voltage was 30 kV and preconditioning involved a 3 min flush with 20mM phosphoric acid followed by 3 min with buffer. When pH 9.5, 50 mM borate buffer was used, the voltage was 20 kV and conditioning involved a 3 min flush with 0.1M NaOH followed by 3 min with buffer. Benzyl alcohol was used as a neutral electroosmotic flow (EOF) marker.

## Results and Discussion

The unroasted coffee beans were green-brown while the roasted beans were all brown, the intensity of color increasing with degree of roasting, as indicated by the decreasing color unit values. Samples C3 and T3 represent normal roast coffees, while samples C4 and T4 have colors typical of dark roast coffees. The organic roast losses of the samples are shown in Table II. Values increased with degree of roasting and similar values were obtained for samples of similar color unit values (Table I). Absorbance values for the aqueous extracts increased with the degree of roasting and were also very similar for samples roasted to about the same color value (Table II).

**Table II. Organic roast losses for roasted coffee beans and absorbance values at 460 nm for the aqueous extracts**

<i>Sample Code</i>	<i>Organic roast loss (%)</i>	<i>Absorbance</i>
C0/T0	-	0.034
C2	3.54	0.244
C3	5.96	0.376
C4	10.19	0.520
C5	14.92	0.598
T2	3.39	0.258
T3	6.58	0.404
T4	11.29	0.529
T5	16.85	0.525

### CZE

The EOF marker migrated at 15.7-16.9 and 3.6 min, respectively, in the phosphate and borate buffers. Positively charged and negatively charged components migrated, respectively, with shorter and longer times than the EOF marker, while uncharged compounds all migrated with the EOF. Positively charged, neutral and negatively charged components were separated using phosphate buffer, while the vast majority of components were negatively charged in borate. A wavelength of 200 nm was routinely used to monitor separations since this gave most information. All the components, including the colored ones, absorbed at this wavelength. An outstanding feature of the electrophoretograms (e-grams) of the aqueous extracts and of the LMW and HMW fractions was the similarity between traces prepared from beans with approximately the same color unit values. Thus, the e-grams for both the temperature series and the time series of samples were virtually identical and only e-grams for the time series are presented here.



## Aqueous extracts and LMW fractions

E-grams obtained for the total sample and LMW fraction of the same coffee run in pH 2.5 phosphate buffer were often very similar to each other and contained three main peaks. A positively charged peak migrating at 7.8 min decreased from T2 to T5 (with degree of roasting, Figure 1a). Its migration time matched that of standard trigonelline. The remaining two peaks migrated close to the EOF. The second of these, migrating at 20.3 min., increased in size with degree of roasting. Standard caffeine migrated with the EOF.

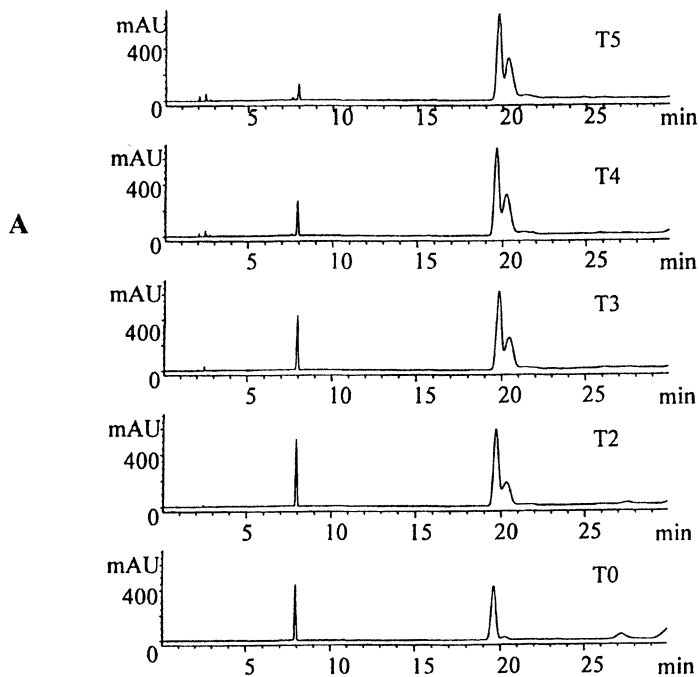
In pH 9.5 borate buffer, around ten peaks migrated within 15 minutes for both the total samples and LMW fractions. The profile varied and the overall intensity of the e-grams decreased with degree of roasting, although some peaks increased in size with heating (Figure 1b). The LMW fraction of T0 contained seven peaks, with spectra almost identical to that of 5-chlorogenic acid, that migrated between 5 and 12 minutes, in line with them being negatively charged. They all decreased in size with progressive heat treatment and only the largest could be detected in T5. (At low pH these peaks were not observed since, being negatively charged, they migrated against the direction of the EOF and their mobility was greater than the EOF. Thus, they never reached the detector.) The identities of the peaks that increased in size with degree of roasting are unknown. In pH 9.5 buffer, standard caffeine and trigonelline both migrated with the EOF.

## HMW fractions

The spectral data showed that most of the color of the total samples resided in the HMW fractions. In pH 2.5 phosphate buffer, T0 gave several very sharp peaks, migrating between 3 and 5 minutes (Figure 2a). Being positively charged and having nominal molecular masses of greater than 5000 daltons, they could be due to peptides and proteins. They were not observed in fractions from the roasted coffees, even the one subjected to the mildest treatment (T2). In the roasted samples run at low pH, two broad peaks were observed with migration time ranges of 5-18 minutes (positively charged) and 18-30 minutes (neutral or possibly slightly negatively charged), respectively. The first peak gave no absorbance at 460 nm and its size, relative to that of the second peak, decreased with roasting (although it was not present in T0). The second peak absorbed at 460 nm as well as at 200 nm and thus contained colored material. It appeared to be comprised of two poorly resolved components, the mean migration time of which increased with roasting, implying an increase in overall negative charge/mass ratio.

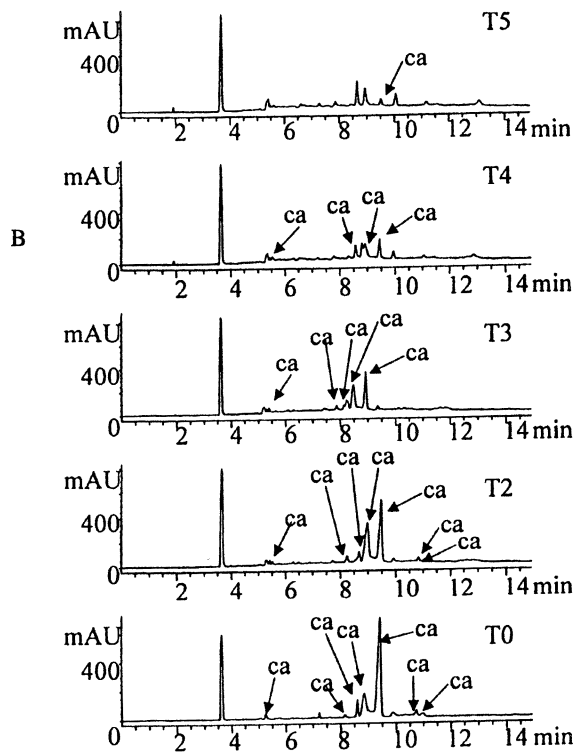
Sharp peaks in the HMW fraction of T0 migrated between 3.8 and 6 minutes in pH 9.5 borate buffer and thus were negatively charged. At high pH, the HMW fraction of T2-T5 showed a broad peak with a shoulder which increased in size up to T4 (Figure 2b) and which gave featureless absorption (typical of brown colored compounds) between 200 and 600nm.

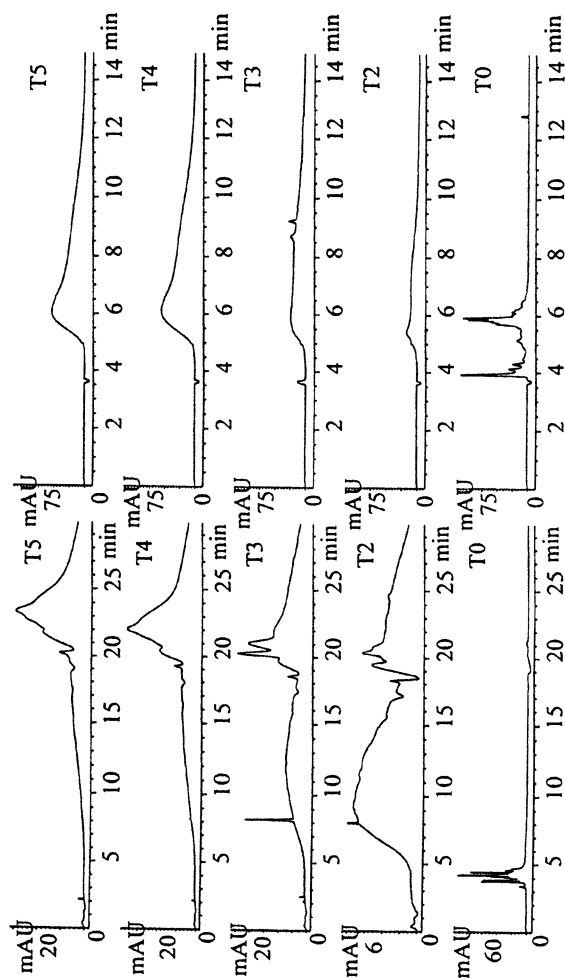
The absence of peaks, which are likely to be due to proteins and peptides, together with the development of broad bands (one of which contains



*Figure 1. E-grams of LMW fractions of samples T0-T5 run in (a) pH 2.5 phosphate buffer and (b) pH 9.5 borate buffer, with detection at 200 nm. 'ca' indicates that the peak had a spectrum identical or very similar to that of 5-chlorogenic acid.*

*Continued on next page.*

Figure 1. *Continued.*



*Figure 2. E-grams of HMW fractions of samples T0-T5 run in (a) pH 2.5 phosphate buffer and (b) pH 9.5 borate buffer, with detection at 200 nm.*

colored material), in the roasted coffees supports the proposal by Hofmann (11) that melanoidins may form from proteins which have become cross-linked by small colored molecules. The broadness of the bands on the e-grams may be attributed to several components that possess very similar charge:mass ratios, due to cross-links involving different side-chains on amino acid residues.

## Conclusion

Samples from the unroasted beans give e-grams that are different from those from the roasted beans. Among the roasted coffees, samples from beans roasted to the same color unit values give very similar e-grams, regardless of the roasting conditions. CE is a useful means of rapidly profiling roast coffees since the samples give e-grams that are characteristic of the degree of roasting. CE shows potential for the determination of low molecular mass coffee components, such as trigonelline, chlorogenic acids and caffeine, and well as for monitoring the development of colored compounds.

## Acknowledgments

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## Chapter 38

# HPLC–MS Analysis of Flavonoids in Foods and Beverages

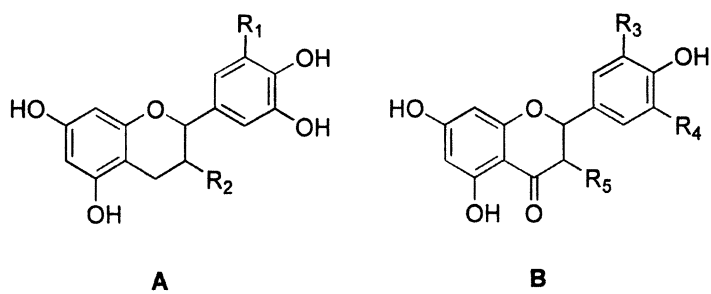
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In the current study, we investigated the usefulness of reversed-phase and normal-phase chromatography for comparing the separation of low molecular weight flavonoids in green tea versus the oligomeric procyanidins in cocoa. The results of this study demonstrated that the reversed-phase technique was better suited for the separation of the flavan-3-ols and flavonols in green tea while the normal-phase method was superior for separation of flavan-3-ol oligomers in cocoa. Therefore, it was concluded that both techniques are required for a comprehensive survey of the flavonoid classes that are ubiquitous in nature.

Since the presence of (-)-epicatechin was first described in tea (1), researchers have isolated and identified structurally similar components which led to the elucidation of the other flavan-3-ol monomers (2). Eventually the growth of this field resulted in the discovery of similar catechins in other plant materials such as cocoa (3,4). To date, detailed studies into the polyphenolic composition of plant materials have successfully elucidated more than 4000 structurally different compounds found ubiquitously in nature (5). As a result, it is not surprising that the types and quantities of polyphenols found in different plant-derived foods vary considerably. For example, in addition to the monomeric catechins (Figure 1A), other low molecular weight flavonols (Figure 1B) and phenolic acids have been identified in green tea whereas a series of complex oligomers have been found in cocoa (6,7). Consequently, the need to develop separation techniques to improve upon the tedious methods historically employed to generate more efficient and effective isolation techniques such as high performance liquid chromatography (HPLC) became evident.

Traditionally, reversed-phase HPLC methods have been employed for the analysis of various flavonoid classes in foods. For example, Salagoity-Auguste and Bertrand used a C<sub>18</sub> hydrocarbon polymer column for the separation of phenolic acids in wine (8). Hertog *et al.* also used a similar method for the separation and quantification of low molecular weight flavonols in fruits and vegetables (9). More recently, Bronner and Beecher used a C<sub>18</sub> bonded silica column for the separation of



**Figure 1.** Backbone structures for a flavan-3-ol (A) and a flavonol (B).



monomeric catechins in tea infusions (10). From the outset, it was shown that reversed-phase (RP) methods had limited application to flavonoids since RP methods are ineffective in separating oligomeric proanthocyanidins as demonstrated by Wilson who showed that oligomers could only be partially resolved (11). Furthermore, he also demonstrated the difficulty in determining degree of polymerization using RP methods since the oligomers elute in non-sequential order.

With this limitation identified, normal-phase methodologies have been explored for the separation of discrete oligomeric classes in foods. Early efforts to separate oligomeric proanthocyanidins by Wilson in apple juice were effective only through the hexamers (11). Although showing better resolution, Rigaud *et al.* was only successful in separating through the pentamers in cocoa and tetramers in grape seeds (12). Recently, an improved normal-phase method has been reported by Hammerstone *et al.* in which a silica column was used to separate oligomers as discrete groupings through the decamers in cocoa and chocolate (13).

In the current study, we investigated the usefulness of reversed-phase and normal-phase chromatography for comparing the separation of low molecular weight flavonoids in green tea versus the oligomeric procyanidins in cocoa. Spectral data from a diode array detector and mass spectrometer were collected to aid in assessing the effectiveness of these two separation techniques.

## Materials and Methods

### Reference Compounds

(+)-Catechin, (-)-epicatechin, (-)-epigallocatechin, (-)-epicatechin gallate, (-)-epigallocatechin gallate, caffeine and theobromine were purchased from Sigma Chemical (St. Louis, MO).

### Samples

Green tea (*Camellia sinensis*) was purchased commercially at a local grocery store. Cocoa (*Theobroma cacao*) mix for milk containing Cocoapro™ was provided by Master Foods Interamerica (Mars, Inc., Puerto Rico).

### Sample Preparation and Polyphenol Extraction

The cocoa drink mix powder (10 g) was extracted twice with 45 mL of hexane to remove lipids. The resulting defatted material (~9 g) was extracted with 40 mL of acetone:water:acetic acid in a ratio by volume of 70:29.5:0.5, respectively. The solids were pelletized by centrifuging for 5 mins at 1500 x g then the supernatant decanted and the organic solvent removed by rotary evaporation under partial vacuum at 40°C. As previously reported (14), the resulting aqueous solution was loaded onto a Supelcosil Envi-18 20 mL Solid Phase Extraction (SPE) Column (Supelco, Inc., Bellefonte, PA) previously conditioned with 3x20 mL of methanol followed by 5x20

mL of deionized water. After sample loading, the SPE column was rinsed with 3x20 mL of deionized water before eluting the polyphenols with 7 mL of acetone:water:acetic acid (70:29.5:0.5 v/v).

Green tea beverage was prepared by boiling 1 tea bag in 250 mL of deionized water for 2 minutes. The beverage was cooled prior to loading onto a SPE column conditioned with methanol and water as described above. After sample loading, the SPE column was rinsed with 3x20 mL of deionized water before eluting the polyphenols with 5 mL of acetone:water:acetic acid (70:29.5:0.5 v/v).

### Reversed-phase HPLC/MS Analysis of Proanthocyanidins

Chromatographic analyses were performed using a HP 1100 Series HPLC (Hewlett Packard, Palo Alto, CA) equipped with an auto-injector, quaternary HPLC pump, column heater, fluorescence detector and HP ChemStation for data collection and manipulation. Reversed-phase separations were performed using a HP (Palo Alto, CA) 5  $\mu$ m Hypersil ODS column (200 x 2.1 mm) at 26°C. The binary mobile phase consisted of A) 0.5% acetic acid in water and B) 0.5% acetic acid in acetonitrile. Separations were affected by a series of linear gradients of B into A at a flow rate of 0.4 mL/min as follows: elution starting with 8% B in A, 0-5 mins isocratic; 8-40% B in A, 5-60 mins; 40-100% B in A, 60-65 mins; 100% B, 65-70 mins isocratic. Data were collected using the UV detector at 280 nm for flavan-3-ols, 320 nm and 360 nm for flavonols. Fluorescence detection (FLD) at  $\lambda_{ex}$  = 276 nm and  $\lambda_{em}$  = 316 nm was also used for non-gallated flavan-3-ols. Other FLD conditions included a photomultiplier tube gain of 12, frequency of 110 Hz and response time of 2 seconds.

HPLC/MS analyses of polyphenol extracts were performed using a HP 1100 Series HPLC as described above and interfaced to a HP Series 1100 mass selective detector (model G1946A) equipped with an API-ES ionization chamber. Methanol was delivered with a HP 1100 series HPLC pump (0.4 mL/min) via a tee into the eluant stream of the HPLC just prior to the mass spectrometer in order to aid ionization. Conditions for ionization in the negative mode include a capillary voltage of 3.5 kV and a fragmentation voltage of 100 V. Spectra were scanned over a mass range of  $m/z$  200-2000 at 2.12 s per cycle.

### Normal-phase HPLC/MS Analysis of Proanthocyanidins

Chromatographic analyses were performed using a HP 1100 Series HPLC (Hewlett Packard, Palo Alto, CA) equipped with an auto-injector, quaternary HPLC pump, column heater, fluorescence detector and HP ChemStation for data collection and manipulation. Normal-phase separations of the proanthocyanidin oligomers were performed using a Phenomenex (Torrance, CA) 5 $\mu$ m Lichrosphere silica column (250 x 4.6 mm) at 37°C. The ternary mobile phase consisted of A) dichloromethane, B) methanol and C) acetic acid and water (1:1 v/v). Separations were achieved by a series of linear gradients of B into A with a constant 4% C at a flow rate of 1 mL/min as previously described by Hammerstone et al. (13). In brief, elution started with 14% B in A then increased to 28.4% B over 30 mins; to 50% B over 30-60 min; to 86% B

over 60-65 min; and finally isocratic to 70 min. Data were collected using the UV detector at 280 nm for flavan-3-ols, 320 nm and 360 nm for flavonols. Fluorescence detection (FLD) at  $\lambda_{\text{ex}} = 276$  nm and  $\lambda_{\text{em}} = 316$  nm was also used for non-gallated flavan-3-ols. Other FLD conditions included a photomultiplier tube gain of 12, frequency of 110 Hz and response time of 2 seconds.

HPLC/MS analyses of polyphenol extracts were performed using a HP 1100 Series HPLC as described above and interfaced to a HP Series 1100 mass selective detector (model G1946A) equipped with an API-ES ionization chamber. Ammonium acetate (10 mM) was delivered with a HP 1100 series HPLC pump (0.1 mL/min) via a tee into the eluant stream of the HPLC just prior to the mass spectrometer in order to aid ionization. Conditions for ionization in the negative mode include a capillary voltage of 3.5 kV and a fragmentation voltage of 85 V. Spectra were scanned over a mass range of  $m/z$  220-2020 at 2.12 s per cycle.

## Results and Discussion

Reversed-phase HPLC techniques are most commonly employed for the separation of low molecular weight flavonoids such as the flavan-3-ol monomers. In order to develop an effective reversed-phase method, the five most predominant monomers and the two abundant xanthine alkaloids, caffeine and theobromine, found in tea and/or cocoa were purchased commercially and used as standards. An HPLC method which was successful in separating the monomers and xanthine alkaloids was achieved using a Hypersil ODS column. Interestingly, it was observed during method development that the xanthine alkaloids, the prodelphinidin monomer, and the gallated catechins gave little or no fluorescence signal in contrast to the two procyanidin catechins. Therefore, all analyses were performed using both the UV and fluorescence (FLD) detectors. Furthermore, the mass spectrometer conditions were optimized in the negative ion mode using the catechin standards in order to aid peak identification. It should be noted that the mass range scanned excludes the xanthine alkaloids since our primary interest is in the flavan-3-ols.

Next, the polyphenol extract of the cocoa drink mix was analyzed using the reversed-phase method with fluorescence detection since cocoa contains predominantly the procyanidin class and the FLD has increased sensitivity over the UV detector at 280 nm (3,4,14). The FLD trace can be seen in Figure 2 and using the extracted ion chromatogram (EIC) at  $m/z$  289, the two monomer peaks can be identified with (+)-catechin eluting significantly earlier than (-)-epicatechin as confirmed using authentic standards. Since it is well known that cocoa contains a complex series of procyanidin oligomers (6), EICs were generated which correspond to the molecular ions for dimers through tetramers and the doubly charged ion for pentamers in order to determine their elution times. As can be seen in Figure 2, the major dimer elutes between the two monomer peaks while other dimer isomers elute after the primary pentamer peak. This non-sequential elution order along with multiple isomers for each oligomeric class makes interpretation difficult when using reversed-phase techniques.

For comparison, the polyphenol extract of the cocoa drink mix was also analyzed using the normal-phase HPLC method previously developed with fluorescence detection and modified mass spectrometer conditions to eliminate the use of a strong base (13). Similar to previous results for cocoa and chocolate, the drink extract yielded a FLD trace in which the procyanidin oligomers elute according to degree of polymerization (Figure 3). Additionally, the multiple isomers for each oligomer class are still evident, but elute in close proximity to each other in contrast to the reversed-phase method. For example, (+)-catechin elutes just prior to the (-)-epicatechin with less than a minute separating the two, whereas on the reversed-phase column, the monomers eluted approximately five minutes apart. Therefore, it can be concluded that for a sample containing complex oligomers, the normal-phase method is superior to the reversed-phase since separation as discrete oligomeric groupings can be achieved in sequential order.

With the normal-phase method preferable for the cocoa polyphenol extract, green tea beverage was analyzed in a similar manner. However, since it is known that tea contains substantial quantities of galled flavan-3-ols (7,15), the UV detector was used exclusively since the FLD is insensitive to this class of monomers. Furthermore, UV data at 320 and 360 nm were collected in addition to the traditional 280 nm in order to selectively detect the flavonols which are also a major flavonoid class found in tea (7,15,16). The UV traces from green tea beverage extract at 280 and 360 nm can be seen in Figure 4. Using a combination of mass spectrometry and authentic standards, it is evident that not only are the monomers incompletely resolved, but also the (-)-epigallocatechin gallate elutes at the same time as the dimers in cocoa. Furthermore, the UV trace at 360 nm indicates that the flavonols elute as a large, mostly unresolved hump late in the separation. Due to these inadequacies, the more traditional technique using the reversed-phase method developed above was employed for comparison.

The UV traces at 280 and 320 nm for the reversed-phase separation of the green tea beverage extract can be seen in Figure 5. Immediately, it is evident that greater resolution is achieved for green tea using the reversed-phase method compared to the normal-phase chromatography. Table 1 lists the spectral data used to tentatively identify the complex mixture of peaks observed in the reversed-phase chromatograms. As can be seen, six of the commonly described flavan-3-ol monomers were identified in the green tea beverage extract in addition to two obscure methylgallate catechins which is consistent with previous reports (7,17). Interestingly, the flavan-3-ol monomers have an unusual ionization pattern in which they tend to form the (2M-H)<sup>-</sup> ion readily. For example, in addition to the molecular ion ( $m/z$  441) for (-)-epicatechin gallate, the (2M-H)<sup>-</sup> ion with  $m/z$  883 was also observed. Furthermore, the spectrum was characterized by the loss of the gallic acid ester resulting in an ion with  $m/z$  289. In addition to the monomers, oligomers through trimers were also observed in the green tea beverage which is consistent with previous results (14).

While the flavan-3-ols account for the major peaks in the UV trace at 280 nm, the substantial number of peaks detected at 320 nm suggests that additional flavonoids are also present in the green tea beverage as expected (7). Traditionally, UV data at 320 nm and 360 nm are used to elucidate other classes of flavonoids such as the phenolic

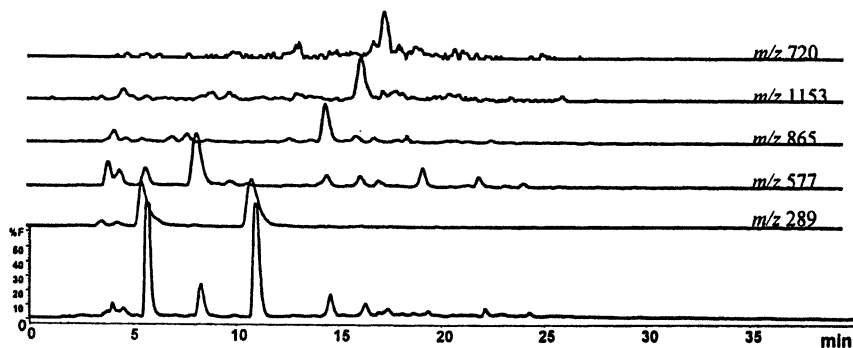


Figure 2. FLD trace (bottom panel) and EIC (top panels) of a reverse-phase separation of cocoa drink extract.  $m/z$  289, 577, 865, 1153 and 720 correspond to monomer through pentamer, respectively.

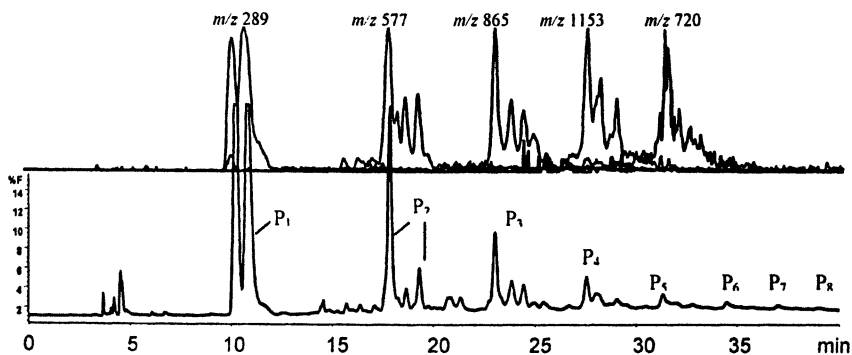


Figure 3. FLD trace (bottom panel) and EIC (top panel) of a normal-phase separation of cocoa drink extract.  $m/z$  289, 577, 865, 1153 and 720 correspond to monomer through pentamer, respectively.

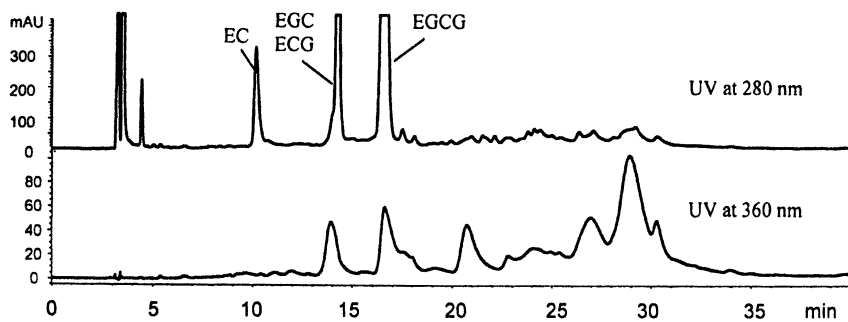


Figure 4. UV traces at 280 and 360 nm for a normal-phase separation of tea extract.

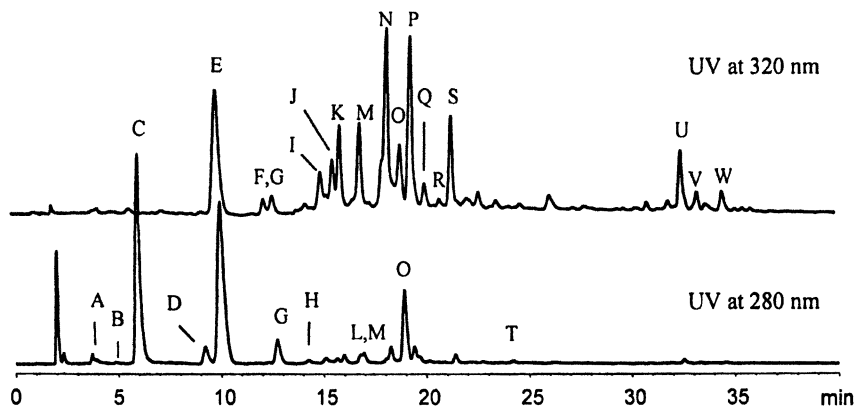


Figure 5. UV trace at 280 and 320 nm for a reversed-phase separation of tea extract. A through W corresponds to peaks identified in Table 1.

Table 1. Flavonoids in Green Tea by Reversed-Phase HPLC/MS.

<i>Retention Time</i>	<i>Peak</i>	<i>Major Ions (m/z)</i>	<i>UV<sub>max</sub> (nm)</i>	<i>Tentative ID</i>
3.70	A	305, 611	270	(-)-Epigallocatechin* (EGC)
4.84	B	289	278	(+)-Catechin*
5.86	C	577	N/A <sup>†</sup>	Caffeine* and Dimer
9.20	D	289	278	(-)-Epicatechin* (EC)
9.89	E	457, 915	274	(-)-Epigallocatechin gallate*
12.28	F	593, 729	272, 335	Dimers
12.71	G	457, 915	274	(+)-Gallocatechin gallate
14.25	H	897, 729, 787	274	Dimers
15.10	I	479, 635, 625	266, 358	Myricetin glycoside + HT
15.64	J	479	262, 356	Myricetin glycoside
15.97	K	563	270, 338	Hydrolyzable Tannin (HT)
16.78	L	943, 471, 287	272	EGC-3-(3- <i>O</i> -methylgallate)
16.93	M	771	258, 356	Quercetin glycoside
18.24	N	593, 771	256, 354	Quercetin glycoside
18.90	O	441, 883, 289	278	(-)-Epicatechin gallate*
19.39	P	609, 755	264, 350	Quercetin glycoside
19.64	Q	577	272, 339	Dimer
20.12	R	463	256, 356	Quercetin glycoside
21.40	S	755, 597	266, 346	Kaempferol glycoside
24.21	T	425, 455	277	EC-3-(3- <i>O</i> -methylgallate)
32.52	U	1049, 524, 917	268, 317	Trimer
33.32	V	1049, 524, 1063, 531	266, 318	Trimers
34.55	W	1033, 516	266, 322	Trimer

\*Confirmed with authentic standard. <sup>†</sup>Not applicable – signal off-scale.

acids and flavonols which have also been reported in tea (7,15,16). However, under the current mass spectral conditions, the phenolic acids are not detected since their molecular weights fall below the mass range scanned. Therefore, flavonol glycosides appear to account for the majority of the components detected at 320 nm as indicated in Table 1. It has been reported that the major flavonols in tea are myricetin, quercetin and kaempferol which is consistent with the UV and mass spectral data used to tentatively identify their glycosides (7,15). However, further confirmation of the aglycone would require collision-induced dissociation experiments in addition to hydrolysis or NMR experiments to elucidate the sugar moiety. Moreover, other minor phenolic constituents were detected in the UV chromatograms and tentatively identified as hydrolyzable tannins (HT) since they are known to occur in tea (7). Finally, it should be noted that the range of phenolic compounds observed in the current study may be limited since the extract was prepared from a tea infusion rather than from direct extraction of the leaves.

## Conclusion

This study sought to compare the effectiveness of reversed-phase and normal-phase chromatography for the separation of flavonoids in tea and cocoa beverages. Since the reversed-phase technique was capable of separating the low molecular weight flavonoids, this method was found to be better suited for the tea beverage extract than the normal-phase technique. In contrast, better resolution for the cocoa extract was achieved using the normal-phase method that was capable of separating the complex series of flavan-3-ol oligomers as discrete groups in increasing order of polymerization. In conclusion, a combination of reversed-phase and normal-phase chromatography along with multiple detection systems (e.g., fluorescence, DAD, MS) are necessary for a comprehensive survey of the various classes of flavonoids in foods and beverages.

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## Chapter 39

# Methyluric Acids: Chemical Markers of Oxidation in Coffee, Tea, and Cocoa

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Recent studies have shown that C-8 hydroxylated methylxanthines, such as 8-oxocaffeine (1,3,7-trimethyluric acid), can reflect free radical mediated reactions in coffee and tea, during processing and in the finished product. In order to further extend the usage of uric acids as markers of oxidation, the C-8 hydroxylated analogue of theobromine, namely 8-oxotheobromine, is determined in unsweetened cocoa powders using LC-ESI/MS/MS. The generation of oxotheobromine is dependent on a number of factors, such as transition metal availability, oxygen, and pH. Such stable and non-volatile chemical markers of oxidation may assist in industrial quality control screening of raw materials and also aid in assessing the shelf life of methylxanthine-rich finished products.

## Introduction

Purine alkaloids are major dietary constituents of a number of beverages such as coffee, tea, cocoa, guarana, and cola-based refreshments, and contribute to the positive organoleptic perception enjoyed globally by these popular drinks. Raw cocoa beans are rich in methylxanthine derivatives, and theobromine - also termed 3,7-dimethylxanthine - is the major alkaloid in *Theobroma cacao*. Its concentration is dependent on the botanical and geographical origin of the beans and ranges from 2.8 - 3.4 % w/w (1). Cocoa beans also contain a plethora of polyphenolic compounds, principally catechins and procyanidin mono-, di-, and trimers, with a (-)-epicatechin concentration of approx. 2% on a w/w basis in the unfermented bean (2,3). The widespread public and scientific interest in health-beneficial dietary constituents in the past years has more and more focused on food commodities that are abundant in

natural polyphenols, such as cocoa and tea. Flavonoid-rich extracts and pure chemical constituents of green and black tea provide antioxidant protection *in vitro* (4,5) and *in vivo* (6-8). In the case of cocoa solids, previous studies have demonstrated that the antioxidant capacity is primarily related to the flavonoid (-)-epicatechin (9). Recently, cocoa liquor constituents, identified as flavans, have been isolated and shown to be potent antioxidants in bulk oil and hydrophilic model systems (10), probably contributing to the chemical stability of chocolate and in general decreasing the need for added preservatives (11). In fact, cocoa extracts rich in polyphenols and procyanidins have been claimed to exhibit antioxidant, antineoplastic and preservative properties (12), and inhibition of LDL oxidation by cocoa powder extracts is comparable to that of pure catechin (11).

The antioxidant activity described for many foods and beverages is not only restricted to the action of polyphenolics. Model studies have demonstrated that caffeine is an efficient scavenger of hydroxyl (13) and superoxide (14) radicals. Furthermore, *in situ* oxidation of caffeine in soluble coffee solution procures 1,3,7-trimethyluric acid, also designated 8-oxocaffeine, as the major reaction product (15), whose formation involves a C8-OH radical intermediate as recently shown by EPR spectroscopic studies (16). This chemical marker reflects the "oxidative status" of the brew and its formation is dependent on a number of physico-chemical parameters such as oxygen tension, pH, and transition metal availability (15). Analogously, theobromine subject to Fenton reagents in model systems furnishes the 8-hydroxy analogue, 3,7-dimethyluric acid, as a major reaction product (17).

This study now addresses the free radical scavenging activity of inherent theobromine in cocoa powders, and the detection/quantification of the major reaction product, 8-oxotheobromine, by LC-ESI/MS/MS techniques. The impact of food additives and preservatives as well as the major physico-chemical properties that influence the *in situ* formation of the oxidized analogue are also discussed.

## Experimental Procedures

### Chemicals

All reagents were prepared fresh before use. L(+)-ascorbate was purchased from Merck (Darmstadt, Germany). The chemicals 3,7-dimethyluric acid, theobromine, and EDTA were from Sigma (Buchs, Switzerland). Chromabond (500 mg) C18ec cartridges were from Macherey & Nagel.  $MnCl_2$ ,  $FeCl_3 \cdot 6H_2O$ , and  $CuSO_4$  were from Aldrich (Buchs, Switzerland). PRS solid phase extraction columns (1g) were from IST. The internal standard 7-methyl-3-(trideuteromethyl)uric acid was custom synthesized by Toronto Research Chemicals Inc, Canada, isotopic purity > 98%.

## Cocoa Powders

Alkalised unsweetened cocoa powders with 12% or 20% fat were employed in this study. Theobromine contents of the powders was determined by HPLC with UV detection, showing 2.23 and 1.82 % for defatted and 20% fat powders, respectively. Iron and copper contents of the defatted powders was determined as 15.7 ppm and 5.4 ppm, respectively.

## Cocoa Powder Preparation

Cocoa powder solution was prepared by dissolving 1.68 g cocoa powder in 10 mL boiling distilled water. After 10 minutes under slow stirring (magnetic) at rt, aliquots were removed and incubated as described. For  $t_0$  samples, aliquots (1 mL) were removed and immediately acidified with 1M HCl (50  $\mu$ L) in Eppendorf tubes. Deuterated 3,7-dimethyluric acid was added as an internal standard (final concentration 2ng/ $\mu$ L). The tubes were then centrifuged (14 000 rpm, Eppendorf systems), and the clear supernatant applied to preconditioned (each 2 bed-volumes of methanol, water, and 10 mM HCl) Isolute PRS and Chromabond C18ec columns coupled in series. After penetration of the samples under slight suction (Vasiprep, Supelco), the columns were washed with 2 bed volumes 10 mM HCl. The PRS columns were then removed and discarded, and 8-oxotheobromine eluted from the C18ec columns with 4 mL of a solvent comprised of 30% methanol acidified with acetic acid (final conc. 2 mM). The clear effluent was lyophilised and taken up in 100  $\mu$ L of the same elution solvent and directly analysed by LC-MS/MS.

## Oxidation Experiments with Cocoa Powder

Cocoa powders were prepared as described above and an aliquot incubated in a potassium phosphate buffer (final buffer concentration 75 mM), pH 6.8 (unless otherwise stated) in a total assay volume of 1 mL (Eppendorf tubes). A typical reaction mix comprised of FeCl<sub>3</sub>·6H<sub>2</sub>O (100  $\mu$ M), EDTA (500  $\mu$ M) and 16.8 mg cocoa powder. The reactions were terminated after 1h at 37°C by addition of 1 M HCl, centrifugation at 14000 rpm, 2 min. and immediate extraction over solid phase columns as described above.

## Spectroscopic analyses

HPLC-MS analyses were done with a Finnigan TSQ 7000 mass spectrometer using a Vydac C-8 reverse phase column (5  $\mu$ m, 1 X 100 mm) coupled to a Hewlett-Packard 1100 pump. The flow rate was 0.50 mL/min and was split 1:10 with a LC Packings Acurate microflow processor prior to the injector port so that the effective flow rate through the column was 0.05 ml/min. The solvent conditions were 0.1%

CH<sub>3</sub>COOH and 5% CH<sub>3</sub>CN at T<sub>0</sub>, which increased with a linear gradient to 25% acetonitrile at 15 min, and then to 100% CH<sub>3</sub>CN at 17 min, and held for 3 min prior to column re-equilibration. The electrospray interface was operated with a high voltage of 3.5 kV and a capillary temperature of 250 °C. Nitrogen was used as the sheath gas at a pressure of 80 psi. MS/MS analyses were done by collision-induced dissociation (CID) of the protonated molecular ions at a collision energy of 40 eV. Argon was used as the collision gas at a pressure of 3 mTorr. Quantitation was done by tandem MS/MS using multiple reaction monitoring (MRM) mode measuring two transitions of the protonated oxotheobromine *m/z* 197 (M+H)<sup>+</sup> at *m/z* 126 and 182 and the two transitions of the protonated *d*<sub>3</sub>-oxotheobromine *m/z* 200 (M+H)<sup>+</sup> at *m/z* 129 and 185. A calibration curve was constructed at 10 concentration levels of oxotheobromine ranging from 0.05 to 37.5 ng injected on column, with the internal standard *d*<sub>3</sub>-oxotheobromine set at 10 ng (*r*<sup>2</sup>=0.9995).

## Results

### LC-MS/MS Analysis of 8-Oxothobromine in Cocoa Powders.

The LC-ESI-MS/MS daughter ion spectrum of protonated oxotheobromine *m/z* 197 (M+H)<sup>+</sup> is shown in Figure 1, with multiple fragments which include: *m/z* 182 (197-CH<sub>3</sub>), 179 (197-H<sub>2</sub>O), 169 (197-CO), 151 (197- H<sub>2</sub>O, -CO), 139 (197-CH<sub>3</sub>, -HNCO), 126 (197 - HNCO, -CO), 111 (197 - HNCO, -CO, -CH<sub>3</sub>). The daughter ions of oxotheobromine at *m/z* 182 and 126, and 185 and 129 for the trideuterated analogue were chosen for MRM analyses because of their abundance and specificity.

### Relative Abundance

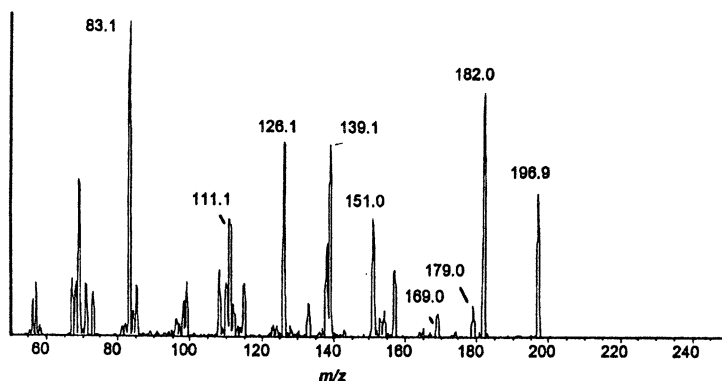


Figure 1. LC-ESIMS/MS daughter ion spectrum of 8-oxotheobromine [M+H]<sup>+</sup>.

## Influence of EDTA and Transition Metals on 8-Oxotheobromine Formation

Supplementation of cocoa powder with Fe<sup>3+</sup>-EDTA chelate augments the level of 8-oxotheobromine nearly 10-fold vs that of the control, whereas iron or EDTA alone had only a minor effect (Table 1). An important property of many dietary phenolics when exposed to transition metals is the reduction of oxygen to generate superoxide radicals and hydrogen peroxide (18,19), the latter compound measured in our laboratory in the same cocoa powder and reaching up to 120 μM after 1h. Reductive cleavage of H<sub>2</sub>O<sub>2</sub> by Fe<sup>2+</sup>-EDTA then procures the reactive hydroxyl radical which indiscriminately reacts with cocoa constituents, including theobromine to form principally 8-oxotheobromine. A recent study with caffeine/soluble coffee has shown a similar increase in 8-oxocaffeine levels when fortified with Fe<sup>3+</sup>-EDTA (15). Both observations - in cocoa and coffee - corroborate the ability of iron-EDTA to promote oxidative damage *in situ*, most probably by increasing the solubility of iron and its redox potential (20).

**Table 1. Influence of EDTA and Transition Metals on 8-Oxotheobromine Formation in Cocoa Powder.**

<i>Conditions<sup>a</sup></i>	<i>8-Oxotheobromine<sup>b</sup></i> (ppm)
Control <sup>c</sup>	4.4
EDTA	7.5
Fe <sup>3+</sup> (5.6 μg Fe <sup>3+</sup> /μL)	6.1
Fe <sup>3+</sup> - EDTA (0.56 μg Fe <sup>3+</sup> /mL)	37.1
Fe <sup>3+</sup> - EDTA (2.8 μg Fe <sup>3+</sup> /mL)	52.2
Fe <sup>3+</sup> - EDTA (5.6 μg Fe <sup>3+</sup> /mL)	54.8
Cu <sup>2+</sup> - EDTA (5.6 μg Cu <sup>2+</sup> /mL)	6.0
Mn <sup>2+</sup> - EDTA (5.6 μg Mn <sup>2+</sup> /mL)	6.1

<sup>a</sup> Defatted cocoa powder (16.8 mg) incubated for 1h at 37°C, pH 6.8, as described in "Experimental Procedures". <sup>b</sup> Entries are averages of duplicate determinations and quantified by LC-MS/MS using deuterated 8-oxotheobromine as internal standard. <sup>c</sup> Only cocoa powder.

Fortification of cocoa powders with equivalent amounts of copper(II) or manganese(II) revealed no effect on theobromine oxidation, substantiating the minor role of Cu-EDTA complexes in redox cycling (21), and inability of manganese to participate in Fenton reactions (22).

### Influence of Ascorbate on 8-Oxotheobromine Formation

Numerous cocoa-based products are fortified with the common water soluble antioxidant ascorbate, often for health-beneficial, organoleptic, and/or product stability reasons. As depicted in Figure 2, 8-oxotheobromine formation in defatted cocoa powders that are fortified with ascorbate and  $\text{Fe}^{3+}$  increases substantially over time, reaching 10-fold the level of the control at a final assay concentration of 2 mM ascorbate.

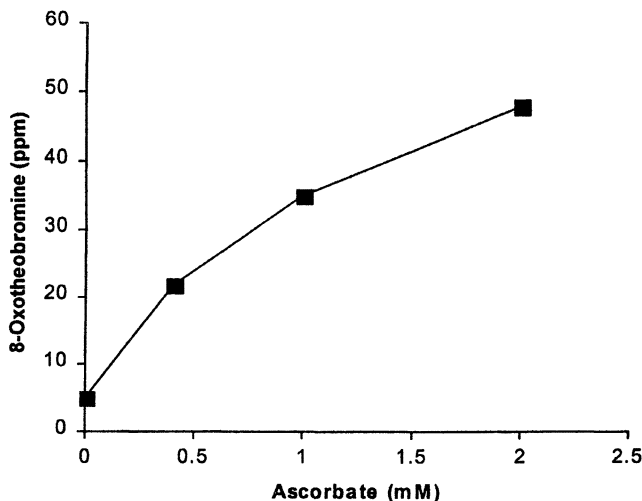


Figure 2. Catalysis of 8-oxotheobromine formation in cocoa powder in the presence of ascorbate. All incubations were at pH 6.8 containing  $\text{Fe}^{2+}$  ( $5.6 \mu\text{g}/\text{mL}$ ). Entries are averages of duplicate determinations. Quantification of 8-oxotheobromine was by LC-MS/MS using deuterated 8-oxotheobromine as internal standard.

The augmentative action of ascorbate is even more prominent in this *in situ* model after addition of chelated iron (as  $\text{Fe}^{3+}$ -EDTA), reaching  $> 150$  ppm of the methyluric acid analogue in the cocoa powder after 1h (Table 2). Cocoa powder incubated under slightly alkaline conditions even further increased the level of 8-oxotheobromine. This effect may be attributable to more facile autoxidation of cocoa polyphenolics, and thus more efficient redox cycling of the  $\text{Fe}^{3+}/\text{Fe}^{2+}$  chelates.

To further demonstrate the pro-oxidant impact of ascorbate in the presence of a metal catalyst in this particular model, an equivalent amount of pure theobromine ( $1.97 \text{ mM}$ ) at concentrations as present in the cocoa powder under study was

incubated in the presence of ascorbate/Fe<sup>3+</sup>-EDTA. Ascorbate-driven oxidation furnished 26 ppm 8-oxotheobromine at pH 6.8 after 1h at 37°C. This represents a 6.7 % turnover of the total theobromine present to the C-8 hydroxylated congener.

**Table 2. Influence of Ascorbate and pH on 8-Oxothobromine Formation in Cocoa Powder.**

<i>Conditions<sup>a</sup></i>	<i>8-Oxothobromine<sup>b</sup></i> (ppm)
Control <sup>c</sup>	5.3
Ascorbate (0.4 mM)	6.3
Ascorbate (1 mM)	8.2
Ascorbate (1 mM) + Fe <sup>3+</sup>	51.5
Ascorbate (1 mM) + Fe <sup>3+</sup> - EDTA	155.6
Ascorbate (1 mM) + Cu <sup>2+</sup> - EDTA	21
Effect of pH <sup>d</sup> :	
water <sup>e</sup>	157.7
potassium phosphate, pH 6.5	155.1
potassium phosphate, pH 6.8	155.6
potassium phosphate, pH 7.0	179.2
potassium phosphate, pH 7.8	190.9

<sup>a</sup> Defatted cocoa powder (16.8 mg) incubated for 1h at 37°C, pH 6.8, transition metals all at 5.6 µg/mL, as described in "Experimental Procedures". <sup>b</sup> Entries are averages of duplicate determinations and quantified by LC-MS/MS using deuterated 8-oxotheobromine as internal standard. <sup>c</sup> Only cocoa powder. <sup>d</sup> all incubations with 1 mM ascorbate. <sup>e</sup> Incubation of cocoa powder in distilled water, pH 7.5.

## Discussion

The addition of ascorbic acid to foods is generally regarded as a health-beneficial and product stabilizing factor. However, numerous reports have highlighted the paradox action of this water soluble vitamin in foods and model systems under aerobic conditions when exposed to trace levels of catalytically active metals (20,23,24). As shown here, fortification of cocoa powders at pH 6.8 with exogenous Fe-EDTA and ascorbate drastically increased levels of 8-oxotheobromine (nearly 30-fold). Ascorbate alone did not have much effect, even though the particular defatted



cocoa powder under study contained 15 ppm natural iron load, reported to reach up to 200 ppm in certain powders (25).

The major factor contributing to the pro-oxidative reactions observed in cocoa powder solutions is the availability of catalytically active iron. Addition of ferric iron salt alone showed only a minor increase in 8-oxotheobromine, which can be explained in part by decreased solubility and more site specific interactions with cocoa constituents, and thus less accessibility of iron to the target molecule. However, fortification of cocoa powder solution with relatively low levels of  $\text{Fe}^{3+}$ -EDTA (0.56  $\mu\text{g/mL}$   $\text{Fe}^{3+}$ ) had a significantly positive impact on C-8 hydroxylation, resulting in an 8-fold increase of 8-oxotheobromine vs the control.

Thus, natural cocoa constituents, such as for example flavonoids, are able to mediate the reduction of the  $\text{Fe}^{3+}$ -EDTA chelate to the active ferrous state. The formation of hydrogen peroxide in the presence of catalytically active metals via polyphenol-driven autoxidation furnishes the reagents required for Fenton chemistry, procuring the highly deleterious hydroxyl radical. Determination of a major product of hydroxyl radical attack upon theobromine, i.e. 8-oxotheobromine - reflects the *in situ* pro-oxidative effect of various food additives and preservatives, as demonstrated here for iron and ascorbic acid.

The employment of inherent stable and non-volatile chemical markers that reflect oxidation in methylxanthine-rich foods and beverages - either in the raw materials, intermediate, or finished products - are important tools in quality control and shelf-life assessment. A recent report on cocoa bean quality criteria has identified endogenous amyl alcohols and their ratios to other volatile constituents as indicative of flavour quality and age of the beans (26). As already demonstrated in coffee with the trimethyluric acid analogue of caffeine (15), pro-oxidative reactions in cocoa powders are indeed positively correlated to the formation of 8-oxotheobromine.

Future work will focus on the levels of this chemical marker in cocoa beans of various geographical origin, and the impact of various parameters such as bean storage, degree of fermentation, roasting, and processing.

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## Chapter 40

# Capillary Electrophoresis of Some Caffeinated Soft Drinks

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Capillary electrophoresis (CE) is a family of techniques which shows tremendous promise for the analysis of beverages. The very high resolving power, short analysis time, low sample requirement, sensitivity and ease of automation associated with CE make it ideal for the analysis of aqueous samples, such as soft drinks. Capillary zone electrophoresis (CZE), using carbonate buffer at pH 9.5 and detection at 200 and 280 nm, was applied to different cola samples. Caramel, caffeine, acesulfame K, aspartame, saccharin and sodium benzoate all migrated within 12 minutes. Seven of the non-diet and 12 of the diet colas contained high-sulfur, high-nitrogen Class IV caramel at concentrations estimated to be between 0.57 and 0.82 g solids per litre.

Capillary electrophoresis (CE) comprises a group of separation techniques that have attracted considerable interest over the last ten years for the analysis of aqueous samples. Separations are based, for example, on the charge:mass ratio in free solution (capillary zone electrophoresis, CZE), interactions between micelles and solutes (micellar electrokinetic chromatography, MEKC), isoelectric point (capillary isoelectrofocusing, cIEF), size (dynamic sieving electrophoresis, DSE) or partitioning between stationary and mobile phases (capillary electrochromatography, CEC). The simplest and most commonly used method is CZE. The advantages of CE over HPLC include very high separation efficiency, typically 4 000 000 theoretical plates per meter, compared to around 100 000 plates per meter for an HPLC column (1), nanoliter-size injection, separation in an aqueous medium and short run time. Detection at 200 nm, often with increased sensitivity, is possible because no organic solvent in present is the buffer, allowing detection of analytes with weak chromophores.

According to the legal definition, soft drinks include fruit drinks (but not fruit juices), soda water, tonic water and artificially carbonated water, whether flavored or unflavored, ginger beer and herbal beverages (2). They are aqueous

solutions or dispersions of a selection of components, including flavorings, sugars, artificial sweeteners, other compounds contributing to taste (e.g., caffeine, acidulants), preservatives and coloring materials (e.g., azo dyes, carotenoids and caramel).

Most of the major ingredients of soft drinks can be determined easily by HPLC (3), but some components, notably caramels, present an analytical challenge. The simultaneous determination of components present in soft drinks by HPLC is sometimes possible (4). For example, a reversed-phase method has been used to separate acesulfame-K, aspartame, saccharin, sorbic acid, benzoic acid and caffeine in less than 15 minutes (5). HPLC methods for the separation of synthetic food colors have not so far included the simultaneous analysis of other components such as sweeteners and preservatives (3).

CE offers the possibility of separating most, if not all, of the components of an individual soft drink within one run. Several CE methods have been published for the simultaneous analysis of sweeteners and preservatives (6-10), while different, but related, methods have been published for the determination of preservatives and synthetic colors (11,12). Most previous studies on the CE separation of soft drink components have used borate buffer at pH 9.4-10.0 (7,10-12) and, e.g., acesulfame K, aspartame, benzoic acid, methyl, ethyl and propyl 4-hydroxybenzoates, saccharin and sorbic acid can all be separated within 9 minutes (7). Good separation, within 12 minutes, of a wider range of sweeteners (acesulfame K, alitame, aspartame, dulcin and saccharin), as well as caffeine, benzoic acid and sorbic acid, has been achieved by MEKC, involving pH 8.6 borate/phosphate buffer containing sodium deoxycholate (8).

Recently, we described a CZE method for the identification and quantitation of Class IV caramels in soft drinks (13). This paper reports the separation of other major declared ingredients of colas and related drinks, using the same conditions of analysis.

## Experimental

Class IV caramels were obtained from two British manufacturers. Quinoline yellow and brilliant blue were obtained from Pointings Ltd (Prudhoe, UK). Sucrose was from a local supermarket, acesulfame K was from Hoechst, Germany, and aspartame, caffeine, saccharin and sodium benzoate (all the purest grade available) were from Sigma (Poole, UK). A total of 55 soft drinks was obtained from both supermarkets and local shops. They comprised colas colored with caramel, other caramel-containing drinks, and drinks sold as 'green cola', i.e., colored with quinoline yellow and brilliant blue, instead of caramel. The colas colored with caramel could be placed into eight categories (A-H), according to the major declared ingredients they contained. Four further categories were used for the other caramel-containing drinks (I, J) and for the green colas (K, L), as shown in Table I.

Solutions of caramel were prepared in water. All samples were 0.2  $\mu\text{m}$  filtered before analysis.

Table I Categorization of colas

Category	Major Declared Ingredients									
	Caramel	Caffeine	Sugar	Aspartame	Acesulfame K	Saccharin	Sodium benzoate	Quinoline yellow	Brilliant blue	
A	x	x	x							
B	x	x		x			x			
C	x	x		x	x		x			
D	x			x			x			
E	x	x	x				x			
F	x	x		x			x			
G	x	x	x			x	x			
H	x		x			x	x			
I			x			x	x			
J	x	x		x			x	x	x	
K		x	x			x	x	x	x	

The CE method has been described (13). Briefly, samples were separated by CZE using 50 mM carbonate buffer at pH 9.5 and a 48.5 cm (40 cm to the detector), 50  $\mu\text{m}$  i.d., x3 bubble capillary. Detection was at 200 and 280 nm. The instrument was a Hewlett-Packard HP<sup>3D</sup>CE equipped with a diode array detector and an HP<sup>3D</sup>CE ChemStation.

## Results and Discussion

Our preliminary studies compared four buffers over the pH range 9.5 to 11.0, i.e., borate, phosphate, glycine and carbonate (13). Borate and phosphate resulted in longer migration times for the colored caramel peak, due to complexation with buffer components. Carbonate gave clearer e-grams than glycine with all the major declared ingredients, apart from caramel, being separated within 8 minutes, and a total run time for samples containing caramel of 12 minutes.

E-grams of the caramel standard and of a cola sample from category A, with detection at 200 nm, are given in Figure 1. The two traces are strikingly similar, the main difference being the presence of caffeine at about 3.2 min in the cola. The other major declared ingredients were also separated using the described method. Examples of e-grams with monitoring at 200 nm for samples from categories B, C and J are shown in Figure 2. Saccharin, acesulfame K and sodium benzoate all absorb strongly at 200 nm and migrate with the caramel peak (when present), reducing the accuracy of quantitation. Monitoring at 280 nm (Figure 3) reduced the size of the peaks due to the artificial sweeteners and preservatives, thus improving the accuracy of quantitation of the caramel. More work is needed to establish the optimum conditions for separation of all these components (which ideally should be completely resolved) in caramel-containing soft drinks. When complete resolution is not possible, quantitation of individual compounds may be improved by using a wavelength at which co-migrating components have minimal absorbance.

Using the same CE conditions of analysis, standard aspartame solutions gave a good peak shape, but aspartame gave a poor peak shape for drink samples, including colas (see Figure 2c, for an example). Standard aspartame runs well by CZE in other buffers, phosphate-borate mixture at pH 9 (14) or glycine at pH 9 (15). Aspartame is stable at pH values as low as 3 (4), but it has been shown that 55% of the aspartame content of a pH 2.55 diet beverage are converted to identifiable degradation products after storage for 50 weeks at 20°C (14). Also, diketopiperazine (DKP), at a level of 4-5% of the total amount of aspartame, has been reported in Diet Seven-Up® soft drink (14). In the current study, any aspartame degradation products may have co-migrated with aspartame under the chosen conditions of analysis. This requires further investigation.

Brilliant blue and quinoline yellow were present in the category J sample. Brilliant blue gave a broad peak that co-migrated with aspartame and which could be a further reason for the poor peak shape for aspartame in the category J sample. Quinoline yellow gave two main peaks, at around 8 and 9 minutes. The buffer strength used is not ideal for these synthetic dyes which give sharper peaks at lower buffer strengths, e.g., 15 mM (16).

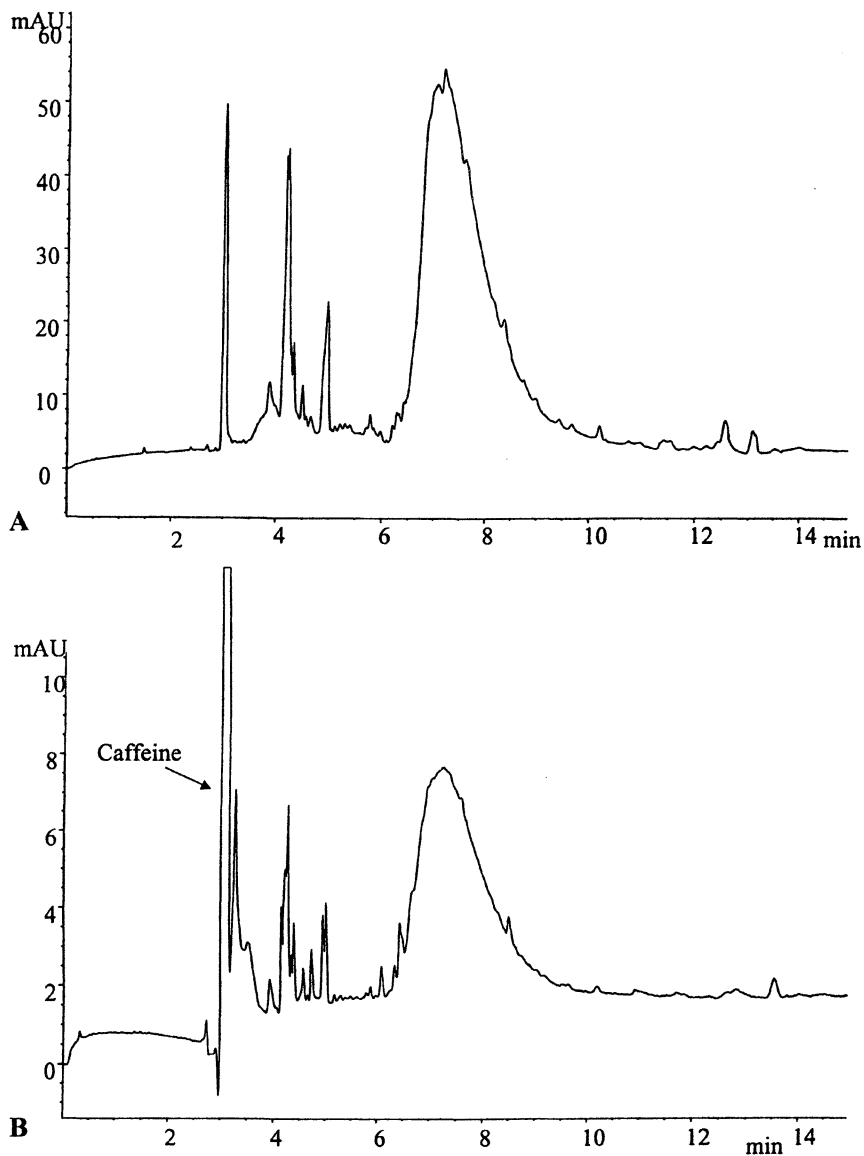


Figure 1. E-grams of (a) the caramel standard and (b) a category A cola (see Table I), run in 50 mM carbonate buffer at pH 9.5 with detection at 200 nm.

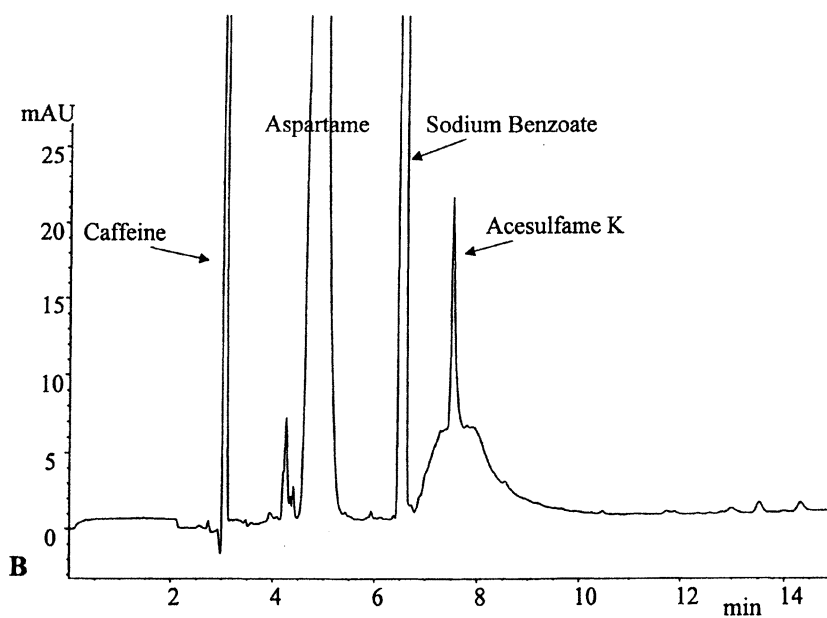
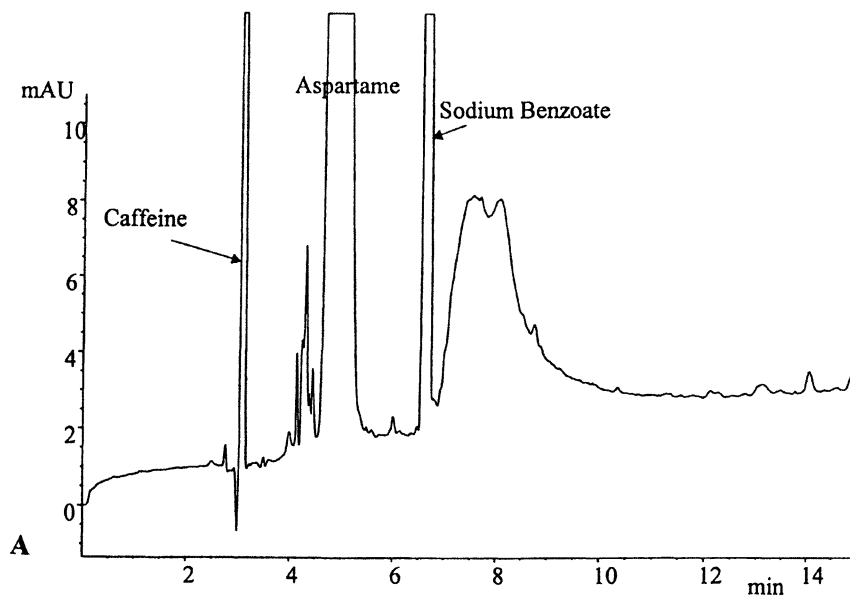


Figure 2. E-grams of cola samples from (a) category B, (b) category C and (c) category J (see Table I), run in 50 mM carbonate buffer at pH 9.5 with detection at 200 nm.



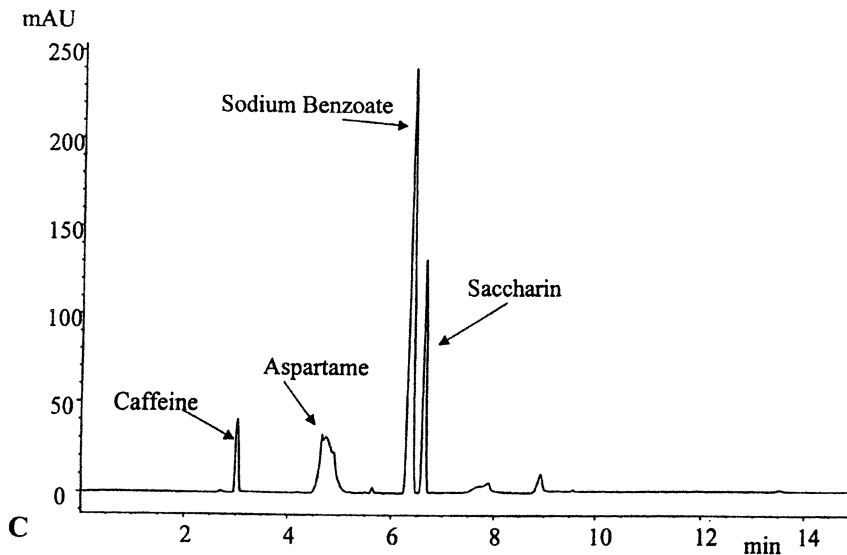


Figure 2. Continued.

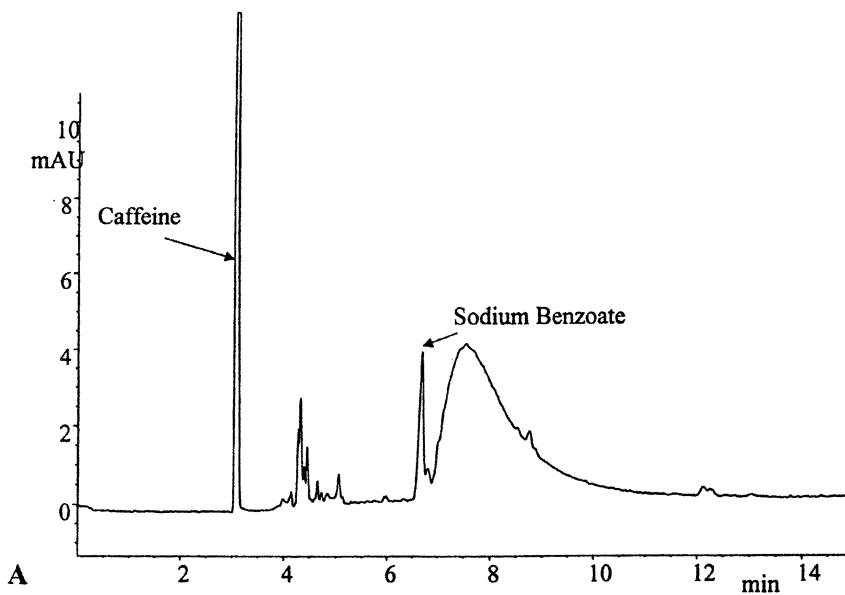
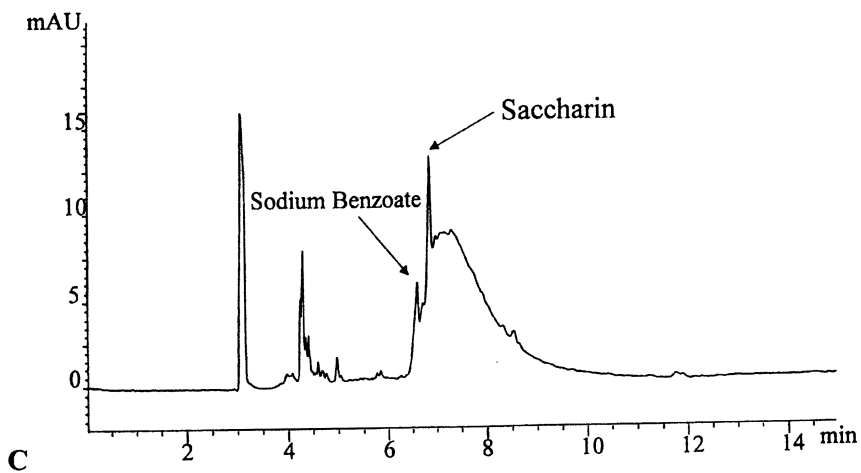
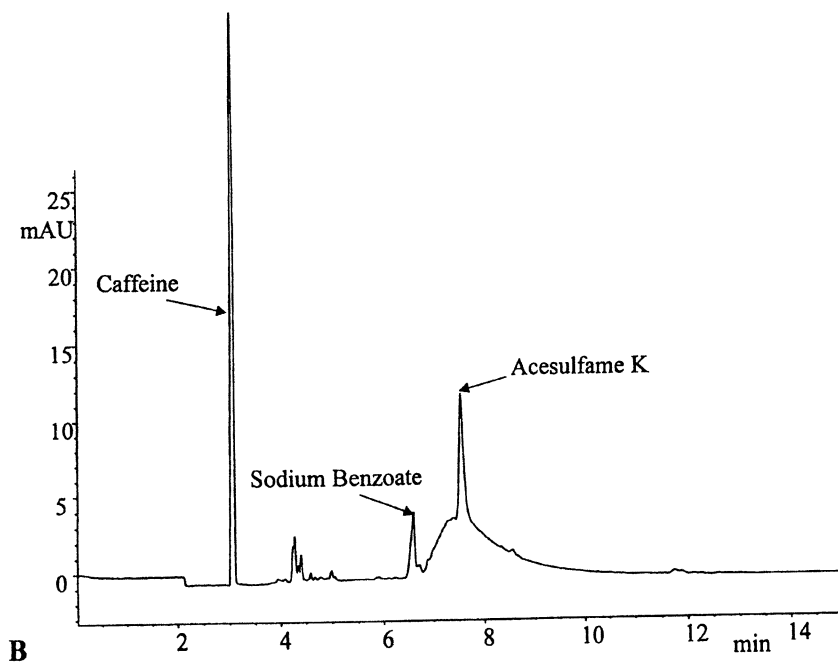


Figure 3. E-grams of cola samples from (a) category B, (b) category C and (c) category H (see Table I), run in 50 mM carbonate buffer at pH 9.5 with detection at 280 nm.

Figure 3. *Continued.*

## Conclusion

A reliable, robust and rapid CZE method, based on carbonate buffer, for the determination of caramel in soft drinks shows considerable potential for the simultaneous quantitation of several other major components of soft drinks. Such a procedure should permit the determination of all components of interest within a 12 minute run, giving benefits in sample throughput and running costs.

## Acknowledgments

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