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Heteroatomic Aroma Compounds

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Foreword

The ACS Symposium Series was first published in 1974 to provide a mechanism for publishing symposia quickly in book form. The purpose of the series is to publish timely, comprehensive books developed from ACS sponsored symposia based on current scientific research. Occasion-ally, books are developed from symposia sponsored by other organizations when the topic is of keen interest to the chemistry audience.

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 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

Although the flavor industry has been in existence for more than 150 years, flavor research in its current form is relatively new beginning with the advent of gas chromatography (early 1960s). As academic institutions have developed both teaching and research programs to service the flavor and related food industries, more information has become available in the public domain. Prior to academic involvement, very little information related to flavor science was published because this is traditionally a very secretive industry. As publications started to appear, the major venue for the publication of scientific information has been the proceedings of various international symposia. These have included symposia offered in Europe (e.g., Weurman, Wartburg and Greek symposia) as well as those offered through the flavor subdivision of the American Chemical Society (ACS) Division of Agricultural and Food Chemistry. This book is the result of a symposium sponsored by the ACS in Washington D.C. in the fall of 2000.

The editors (symposium organizers) invited leading scientists from academia and industry to present lectures and ultimately to publish this book, which is a comprehensive treatment of the topic of flavor chemistry. This book is organized into sections dealing with various heteroatomic molecules (e.g., nitrogen, sulfur, oxygen and miscellaneous atoms). This was done because many of the methodologies for study are based on the detection of a specific atom. For example, one may choose an analytical method that selectively extracts or detects sulfur compounds. Also, particular atoms are often associated with certain mechanisms of formation and sensory character. Therefore, an organization based on the occurrence of a specific atom seemed reasonable.

The first section of this book (six chapters) focuses on sulfur-containing volatiles. There is little question that sulfur chemicals are very often the key to a given aroma and unfortunately present the greatest problems in analysis and identification. The problems in analysis and identification are the result of the extremely low sensory thresholds of this group of compounds and the inherent instability or reactivity. Thus, the first chapter of this book written by Rouseff discusses analytical techniques to determine volatile sulfur compounds in foods. The application of aroma isolation techniques such as distillation, codistillation, solid phase microextraction, liquid–liquid extraction, and sublimation are discussed in terms of efficiencies and potential artifact formation. The author has presented a thoughtful discussion of the advantages, limitations, and potential problems associated with using these aroma isolation methodologies. Sample-analyte dependent concentration techniques such as adsorption on mercury-based solid sorbents, metal foils, and impregnated filters are also presented as well as separation approaches based on HPLC and GC. The chapter ends with a discussion of the limits of detection, sensitivity, selectivity, and cost of flame photometric, chemiluminescence, atomic emission, and pulsed flame photometric GC detectors.

The second chapter considers the sensory significance of sulfur-containing aroma compounds. Blank points out that although volatile sulfur compounds account for only about 10 percent of all volatile components identified in foods, these compounds are extremely important constituents of the flavor of many foods. Blank has chosen to demonstrate the role of sulfur volatiles in foods by selecting certain sulfur-containing aroma-impact compounds and discussing in detail the sensory relevance. Having presented a discussion of the sensory importance of sulfur-containing volatiles in food and how we approach the analysis of the volatiles, the discussion then turns to how these compounds are formed in foods via natural processes (plants and microorganisms) and during thermal processing. Spinnler et al. (Chapter 3) focus their discussion on the conversion of various sulfur precursors (e.g., sulfates, sulfites, amino acids, and peptides) into sulfur-containing volatiles. They place special attention on the importance of acyl CoAs in thioester synthesis. Mottram and Mottram (Chapter 4) then present an overview of how sulfur-containing aroma compounds are formed during the thermal processing of foods. They note that both heterocyclic sulfur compounds (e.g., thiophenes, thiophenones, dithiolanes, trithiolanes, trithianes, thiazoles, and thienothiophenes) and non-heterocyclic sulfur

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compounds (e.g., thiols, sulfides, and disulfides) and typical products of thermal reactions in food. They provide a discussion of how precursors such as cysteine, methionine, and thiamin are converted to aroma compounds during thermal processing. These first four chapters are review in nature and are intended to provide the reader with an appreciation of flavor research focused on sulfur compounds.

The final chapters in the sulfur section present original research on sulfur volatiles. The chapter by Mottram and Elmore focuses on the role of lipids in forming sulfur volatiles. They note that a number of 3-thiazolines, thiazoles, thiapyrans, and thiophenes with 2-alkyl substituents have been found in cooked beef and lamb. These compounds derive from the interaction of lipid autoxidation products, such as saturated and unsaturated aldehydes, with simple intermediates of the Maillard reaction, such as hydrogen sulfide, ammonia, and dicarbonyls. They point out that, although the aromas of these compounds are weak, they may influence flavor by modifying the formation of other compounds in the Maillard reaction or autoxidation of lipids. Thus the effect on food flavor may be indirect as opposed to making a direct contribution to aroma. The final sulfur chapter reports on original work by Lin et al. employing GC–Olfactometry (GC–O) to identify five sulfur-containing odorants in commercial not-from-concentrate (NFC) grapefruit juice. They found these sulfur compounds imparted both characterizing and supporting aroma attributes to the juice. Two compounds, 3-mercaptohexyl acetate and 3-mercaptohexan-1-ol were reported for the first time in grapefruit juice. They also reported on the effect of pasteurization on sulfur-containing aroma compounds.

The next section of this book presents a similarly organized discussion of nitrogen-containing aroma compounds in foods. Although nitrogen-containing aroma compounds perhaps are less important to food aroma on a global basis, there is little question that nitrogen compounds sensorially characterize the aroma of some foods (discussed in Chapter 9). Rajesh and Peppard start this section (Chapter 7) with a discussion of numerous techniques to isolate, identify, and quantify nitrogen-containing aroma compounds in foods. They have included a discussion of solvent extraction, adsorption, and ion exchange chromatography, molecular and steam distillation, static and dynamic headspace sampling, and solid phase microextraction for the isolation of nitrogen-containing volatiles. Analytical techniques used to characterize

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and/or quantify nitrogen heterocycles include multidimensional chromatography and GC–O, as well as GC with various types of nitrogen-specific detection or atomic emission detection. The most commonly employed techniques are described, and examples are cited for analyzing food and beverage flavors for nitrogen-containing aroma compounds.

Rizzi presents an overview of the biosynthesis of nitrogen-containing aroma compounds beginning with the incorporation of atmospheric N into ammonia and then ammonia into a host of other aroma precursors (Chapter 8). He discusses how some of these aroma compounds are formed in nature through a combination of enzyme and nonenzyme catalyzed reactions. The next chapter (by Demyttenaere et al., Chapter 9) focuses on the formation of nitrogen-containing aroma compounds during the thermal processing of foods. This overview presents the chemistry and flavor characteristics of cracker-like flavors, such as 6-acetyl-1,2,3,4-tetrahydro-pyridine and 2-acetyl-1-pyrroline, to illustrate how this group of aroma chemicals are formed in foods. These authors detail the mechanisms of formation, instability, and synthesis of these compounds. The final two chapters of this section present original research involving nitrogen-containing aroma compounds. Le Quéré et al. present their work on the formation and sensory character of a homologous series of 2-alkyl-2,4,5-trimethyl-2,5-dihydro-oxazoles in blue cheese (Chapter 10). Their work illustrates the application of GC-sniffing, GC/MS, GC/FTIR, NPD (nitrogen specific detector), ^{1}H-NMR and two-dimensional NMR in the characterization of these nitrogen-containing odorants. Rhlid et al. present their work on the formation and enhancement of an important nitrogen-containing volatile, 2-acetyl-2-thiazoline in roasted products (Chapter 11).

The final section of this book that is organized around a particular atom is the section on oxygen-containing odorants. This section again starts with a discussion of methods to determine oxygen-containing aroma compounds in foods, and then chapters are included that present overviews of their formation during thermal processing and biogenesis. The initial chapter written by Budin (Chapter 12) focuses on the methods used in the isolation and analysis of oxygen-containing aroma compounds. Like the other analytical chapters, his discussion includes some common methods of aroma isolation. However, he goes on to discuss some of the classical methods used in the analysis of this group of odorants. These classical methods include various derivatization methods such as reaction with 2,4-dinitrophenyl hydrazine, 1-pyruvylchloride-2,6-dinitrophenyl hydrazine, and cystamine. Unlike nitrogen- or sulfur-containing molecules, there are few truly selective techniques for the analysis of oxygen-containing molecules other than these classical methods.

Schieberle and Hofmann present an overview of the formation of oxygen-containing aroma compounds in foods during processing and storage (Chapter 13). They note that carbohydrates are the primary source of these odorants (lipid oxidation is a secondary source). They illustrate the mechanisms of formation of these odorants using 4-hydroxy-2,5-dimethyl-3(2*H*)-furanone and 3-hydroxy-4,5-dimethyl-2(5*H*)-furanone as examples. Peterson and Reineccius discuss the biosynthesis of several key oxygen-containing aroma compounds as examples of how these compounds are formed in nature (Chapter 14). The final chapter of this section presents an example of original research focused on oxygenated aroma compounds (i.e., free fatty acids in Parmesan cheese). Qian and Reineccius note that free fatty acids appear to be extremely important in characterizing this cheese (Chapter 15) for these compounds were found to have high odor activity values. As one would expect, the short chain fatty acids (C4 to C10) were found to be most important of the acids.

Although aroma compounds containing sulfur, nitrogen, and oxygen are generally considered to largely comprise the molecules of odor significance, some literature considers the sensory significance of selenium-containing and halogenated odorants. Kim and Reineccius present a discussion of methodologies used to study halogenated aroma compounds. This chapter (Chapter 16) includes numerous examples of methods used in the isolation and quantification of these compounds. Kittle and Deibler discuss the sensory significance of halogenated aroma compounds noting that these compounds are generally associated with off odors as opposed to desirable odors (Chapter 17). The chloroanisoles and chlorophenols are associated with medicinal, chemical, or musty aromas. The primary example of a food where these chemicals may make a positive contribution is in brominated aroma compounds found in some seafood. Wei and Ho report on the formation of selenium-containing aroma compounds during thermal processing of foods (Chapter 18). This study investigated the Maillard reaction of

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selenomethionine and glucose. Organoselenium, organoselenium–sulfur and pyrazine compounds were identified by GC–EI/MS and GC–NH3–CI/Ms and dimethyl diselenide was the most abundant selenium compound in the extracts. By use of a buffered model system, the authors found maximum yields of pyrazines and dimethyl diselenide at pH 7, 80 min. However, the formation of ethyl selenoacetate was favored at a lower pH, with a maximum yield at pH 3, 80 min. The addition of diallyl disulfide to the model system produced several organosulfur, organoselenium, and organoselenium–sulfur compounds of interest.

The final section of this book (last three chapters) considers new methods for the analysis of aroma compounds. Although not closely related to the focus of this symposium, symposia offer the advantage of drawing large audiences where cutting edge research can be disseminated in a timely fashion (by oral presentations). Thus, these presentations (i.e., chapters) are included in this book. The first chapter in this group (by Buettner and Schieberle, Chapter 19) details how real-time magnetic resonance imaging (MRI) can be applied to understand how aroma is released from a food and perceived by the olfactory receptors. They demonstrated that no significant release of odorants occurs in the mouth during chewing and that the majority of odorants is, in fact, released to the sensory receptors in the first breath after swallowing. This information is remarkable and considerably advances our knowledge of aroma release during eating and the potential role of temporal profile in this context. Engel et al. presented their work on evaluating taste active substances in tomato and camembert cheese (Chapter 20) using omission testing. Little has been published on taste substances or methodologies for their study. This was a very thoughtful study adding a very useful methodology to the field. The final chapter in this book describes a method to monitor flavor development in coffee during roasting (Hofmann et al., Chapter 21). This method is based on a chemosensor array developed at the Technische Universität of München. Through classical flavor chemistry, they found that 2-furfuryl alcohol was a good indicator of flavor development during roasting (i.e., the formation of the key roast and ground coffee component 2-furfurylmercaptan). The authors were able to design a sensor that could specifically detect the indicator odorant (the key odorant is present at too low

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concentrations to be monitored using existing methodologies in real time) and thereby be used to monitor flavor development in coffee during roasting.

In summary, this book provides detailed discussions of methods of analysis, mechanisms of formation in plants, during fermentation and during thermal processing; sensory properties of some key aroma compounds; and their role as off flavors all based on the presence of a given atom. An effort was made to get a comprehensive treatment of these topics that will serve as a reference book in this field. Although many books are now available in this general topic area, no other single book offers such a comprehensive discussion of the analysis, formation, and sensory significance of these aroma compounds.

The primary audiences for this book are those involved in flavor research in the flavor and food industriesas well as in government and academic research laboratories. Related fields that would benefit from this book include environmental (e.g., the control of off odors), cosmetics or fragrance areas. The fundamental discussions of how to analyze volatiles, how they are formed, and how they might contribute to desirable and undesirable aroma would prove most useful.

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Chapter 1

Analytical Methods to Determine Volatile Sulfur Compounds in Foods and Beverages

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Techniques to determine volatile sulfur compounds in foods including: distillation, co-distillation, SDE, liquid-liquid extraction and sublimation are discussed in terms of efficiencies and potential artifact formation. Advantages, limitations and potential problems using SPME as well as purge, and trap concentration techniques are discussed. Sample-analyte dependent concentration techniques such as, adsorption on mercury based solid sorbents, metal foils, and impregnated filters are presented. Separation techniques including HPLC and GC are compared in terms of separation power, sample capacity and detector sensitivity. Limits of detection, sensitivity, selectivity and cost of flame photometric, chemilluminescence, atomic emission and pulsed flame photometric GC detectors are discussed. Advantages and limitations of isotope dilution analysis are presented.

Introduction

Sulfur compounds are one of the most important chemical classes in food flavors because they are potent and occur in a wide variety of foods. Sulfur compounds can be found as sulfides, disulfides, thiol esters, thiocyanides, hemidithioacetals, sulfur-substituted furans, thiophenes, thiazoles, and other heterocyclic forms, which complicates isolation, concentration and quantitation. Humans are particularly sensitive to certain sulfur forms. For example, thiols are especially potent; some have the lowest aroma thresholds of any food odorant. Sulfur compounds make significant contributions to the characteristic aromas in bread, meat, potato, coffee, beer, tomato, grapefruit, passion fruit, grape, blackcurrant, pineapple, melon, truffles, and numerous vegetables (*1*). The aroma impact can either improve or degrade food flavor. For example, a portion of the pleasant roasted aroma of fresh baked bread is due to the presence methional (*2*) but the "light-struck" off flavor in beer is due to 3-methyl-2-butenethiol (*3*). Concentration often determines the sensory perception of many sulfur compounds. For example, 4-methyl-4-mercapto-2-pentanone has been reported as responsible for the "catty" off-aroma in beer (*4*), but in lower concentrations is responsible for the characteristic aroma of grapefruit juice (*5*).

Most of the literature on the occurrence and chemistry of sulfur compounds in foods is sparse and scattered. However, limited information can be found (*6-9*). Sulfur compounds are difficult to measure in foods because they are typically found in the presence of much higher levels of carbohydrates, proteins, lipids and a host of other volatiles. Isolation and concentration techniques must often be optimized for each food product. Even with extensive separation procedures, sulfur-containing fractions have a host of other chemical components that interfere with non-specific methods of analysis. Sulfur compounds are also highly reactive, easily oxidized, dimerized or otherwise altered from thermal processes, exposure to enzymes, or during the actual analysis. Thus, the ideal analytical measurement technique would be sensitive, selective, not induce artifacts, provide identification information and be of moderate cost. In this chapter, various separation and concentration techniques will be presented and compared. Analytical measurement techniques will also be discussed in terms of selectivity, sensitivity, accessibility and cost.

Isolation and Concentration Techniques

One factor that contributes to the difficulty in separating and analyzing sulfur compounds is their structural diversity. Solubility and volatility range from the polar thiocyanides and thiols to the relatively non-polar furan thiols and thiazoles. Thus, it is difficult to isolate all sulfur compounds with a single or

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even a combination of separation techniques. Various food matrices can also present unique challenges and many foods will require sample specific preparation approaches depending on the sulfur analyte of interest. A few of the more widely used techniques will be discussed in the following sections.

Distillation

Distillation offers one of the easiest procedures to separate volatile sulfur compounds from food matrices. However, since sulfur compounds are highly reactive, the possibility of thermal decomposition must always be considered. High vacuum distillation has been employed to reduce potential artifact formation. Werkhoff and coworkers (*10*) employed high vacuum distillation to isolate the volatiles in yellow passion fruits. After enrichment by preparative multidimensional capillary gas chromatography, they found 47 sulfur-containing volatiles. Thirty-five of these components were reported for the first time in passion fruit, and 23 of these sulfur compounds had not been previously reported as constituents in any food flavors. Unfortunately, high vacuum distillation is not a trivial sample preparation method, requiring extensive time (4-6 hr) and equipment to accomplish, and is not well suited for routine analysis.

Simultaneous distillation-extraction, SDE, has been used to isolate and concentrate sulfur volatiles. The Likens-Nickerson procedure has been used for the isolation of flavor volatiles in commercial yeast extracts (*11*). Using this procedure, 115 sulfur-containing flavor components were identified for the first time. Observed compounds included a wide range of sulfur structures such as: aliphatic sulfur-containing components, hemidithioacetals, sulfur-substituted furans, thiophenes, thiazoles, 3-thiazolines, 5- and 6-membered cyclic polysulfides, C3-C12-alkyl-substituted perhydro-1,3,5-dithiazines (thialdines), bicyclic perhydro-1,3,5-dithiazines as well as 2,4,6-trimethylperhydro-1,3,5-oxathiazine and 2,4,6-trimethylperhydro-1,3,5-thiadiazine. Others have (*12*) employed a pentane:ethyl ether solvent in the SDE (2 hr) to isolate thiols formed from the reduction of disulfides in the presence of proteins (albumin). Co-distillation has also been employed on certain products. For example, water can be added to, then distilled from olive oil to separate polar volatile compounds after back extraction with an immiscible organic solvent (*13*).

Liquid-Liquid Extraction

This classical separation technique works well for liquid samples of reasonable viscosity. The concern with single or even double extractions is the uneven extraction efficiencies. Since sulfur compounds exhibit such structural diversity, the physical and chemical properties will range widely and extraction

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efficiencies can span from 10 to 95%. If multiple internal standards are added to the sample (and if standards are similar to the compound of interest), uneven extraction efficiencies can be compensated for. Another compensation approach for uneven extraction efficiencies is to use deuterated standards of the sulfur compounds of interest (*14*). These compounds may be used as internal standards in isotope dilution assays. Another less complicated technique to compensate for uneven extraction efficiencies is to employ continuous liquid-liquid extraction for several hours. Even compounds with low extraction efficiencies should eventually be extracted during the course of the procedure. A final approach is to employ a series of extracting solvents of different polarity. Thus, a compound not well extracted by one solvent would be more effectively extracted by the second.

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Sublimation

Vacuum sublimation (*15*) has been used as a low temperature sample clean up technique for selected mid range thiols in citrus products (*5*). It is used in combination with liquid-liquid extraction, followed by column chromatography and the final vacuum sublimation step to produce a clean extract, essentially free of non-volatiles. However, this technique is unsuitable for highly volatile, low molecular weight sulfur compounds as they will be lost in the vacuum sublimation step.

Static Headspace -SPME

Solid Phase Micro Extraction, SPME, is a relatively rapid, inexpensive, solvent-less headspace sampling technique that has been widely employed to sample headspace volatiles. SPME combines sampling and pre-concentration in a single step. In headspace SPME, the coated fused silica fiber is introduced into the headspace above the sample for a specific time to allow headspace volatiles to be adsorbed on the fiber coating. The fiber is withdrawn and exposed in a heated GC injector to desorb the volatiles into the GC for subsequent separation. Two equilibria determine the amounts of headspace volatiles sampled. The first equilibrium is between the vapor phase analyte and the matrix analyte. The second is between the vapor phase analyte and the analyte in the SPME coating. Equilibrium time for less volatile compounds can be shortened significantly by agitation of both matrix phase and headspace, reduction of headspace volume, and increase in sampling temperature. It is worth noting that even with low molecular weight sulfur compounds, true equilibrium may not be achieved until 90 minutes (*16*). Therefore, most SPME sampling is carried out under non-equilibrium conditions were exposure time is highly critical. It has been reported (*17*) that some SPME fibers strongly discriminate against polar and very volatile compounds, but this problem is not as severe with newer carboxen-polydimethylsiloxane fibers (*18*).

SPME has been employed for the analysis of a wide range of sulfur compounds in foods. It has been used to sample the volatiles from yellow onions (*Allium cepa*) (*19*) produced enzymatically after rupturing the plant cells. Sulfides and disulfides in wines were determined using SPME and GC with flame photometric detection, FPD (*20*). These investigators also noted that the most volatile compounds were the least extracted by the coated fibers. They still reported recoveries >94% for the sulfides of interest with detection limits ranging between 3 μg/L and 50 ng/L. SPME has also been used for the determination of 1-(methylthio)-propane, 1-(methylthio)-1-propene, dimethyl sulfide and bis-(methylthio)-methane in black and white truffles (*17*) and sulfur dioxide along with 2-methyl-1-butanethiol and 3-methylthiophene in beer (*18*). Unfortunately, few of these studies considered the possibility of sulfur artifact formation.

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SPME can be used to isolate sulfur compounds from samples that do not contain large concentrations of terpenes or hydrocarbons, which are preferentially adsorbed on the thin coating that provides limited sample capacity. In a thorough examination of the use of SPME to sample and concentrate low molecular weight thiols and sulfides, Haberhauer-Troyer and coworkers (*16*) observed several problems with SPME. They found problems with artifacts, fiber to fiber variation, low storage stability, and a profound dependence of extraction efficiency on relative humidity. The fiber-to-fiber sampling efficiency variation from identical fibers is shown in Figure 1. This study demonstrates that in order to maintain the highest sampling precision, the same fiber should be used throughout the course of the experiment. They reported day-to-day repeatability ranges of 4-11% for the Carboxen-PDMS fiber and considerably higher variations for the pure PDMS fiber. It is also worth noting that they observed unstable results when the fibers were used for more than 150 injections.

Dynamic Headspace - Purge and Trap

There are several problems in using the purge and trap technique for sulfur compounds. The first problem is determining the temperature at which the purge will be carried out. As shown in Figure 2, increasing temperature during purging will increase volatiles in the gas phase and produce a stronger analytical signal. It should be noted that the relative increase with temperature is somewhat compound specific, with sulfides producing greater purging yields than similar thiols. Since many sulfur compounds are present at the lower end of the detection limits of the analytical device, any technique that can increase the analytical signal is worth considering.

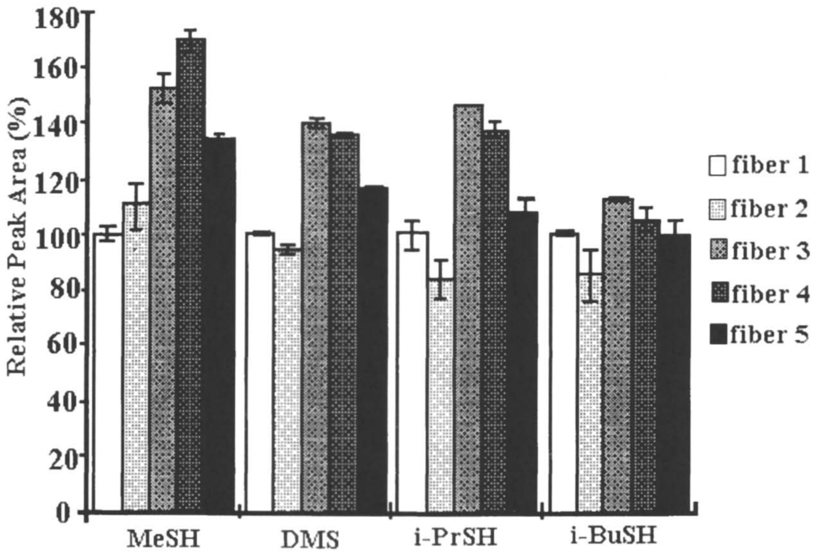

Figure 1. *Comparison of recoveries from a standard mixture of four sulfur compounds for five new Carboxen-PDMS fibers. Peak areas are normalized to that of fiber 1. (Reproduced with permission from reference 16. Copyright 1999 Elsevier Science.)*

However, Spanier and Drumm (*21*) demonstrated that increasing sample temperature during purging of cooked beef produced misleading chromatograms because artifacts were formed and the original sulfur compounds were converted into the form that was finally analyzed. This is shown in Figure 3, where a beef sample purged at 100°C, formed two entirely new compounds (artifacts) at retention times around 53 min. Other artifact peaks can be observed at lower retention times as well.

Carbon has been used as a trapping material because it is an effective collector of sulfur compounds. The prob0lem with carbon traps is that they often do not release sulfur compounds quantitatively. Incomplete desorption will produce low recoveries and can vary significantly depending on the type of sulfur compound being analyzed. The temperature range that will desorb sulfur compounds from the trap and yet minimize thermal artifacts is narrow and will vary with the trapping material and sulfur analyte. If too low a temperature is used, some sulfur compounds will be incompletely desorbed. Higher desorption temperatures will increase the amounts of materials desorbed but also increase the likelihood of artifacts being formed. Desorption temperatures required to completely volatilize higher molecular weight sulfides might also cause low molecular weight thiols to dimerize.

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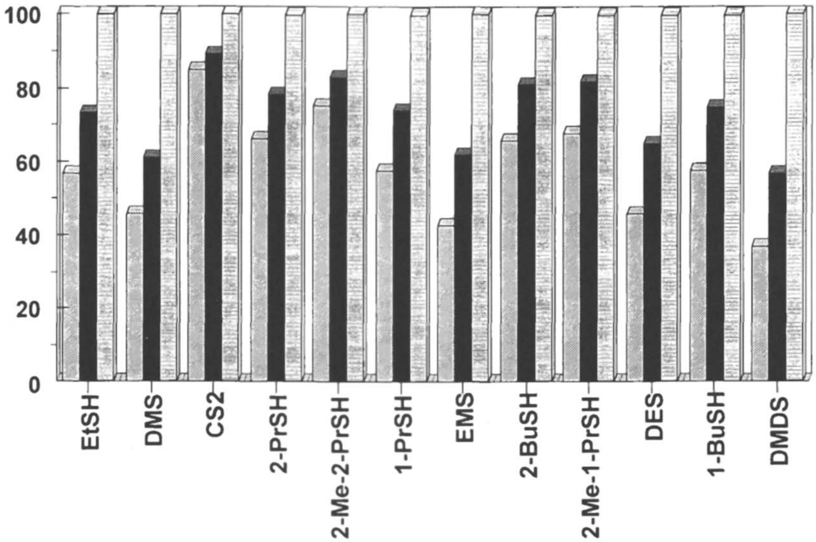

Figure 2. *Influence of sample temperature during sparging on amounts of sulfur compounds trapped. Light grey = 30°C, black = 50°C and dark grey= 80°C, EtSH = ethanethiol, 1-PrSH = 1-propanethiol, DMS = dimethyl sulfide, DES = diethyl sulfide, and DMDS = dimethyl disulfide. (Reproduced with permission from reference 22. Copyright 1995 Elsevier Science.)*

One potential solution is to employ a capillary cryogenic trap of inert material. Headspace volatiles are trapped using liquid nitrogen on a silanized capillary and then rapidly heated to re-volatilize the condensed components. Unfortunately cryogenic traps are sometimes less efficient with considerable less capacity than solid absorbents. Even with cryogenic traps, the possibility of artifacts must be considered. An example of possible artifact formation when cryogenic traps are employed is shown in Figure 4. The authors (*22*) attributed the relatively high amounts of dimethyl disulfide and low dimethyl sulfide to the thermal drying used in manufacture of their powdered coffee sample. Since the thermal process in making the coffee was more severe than that used in sample preparation, the explanation is reasonable. However, they did not address the possibility that any portion of the dimer could have been formed during sample preparation and subsequent analysis.

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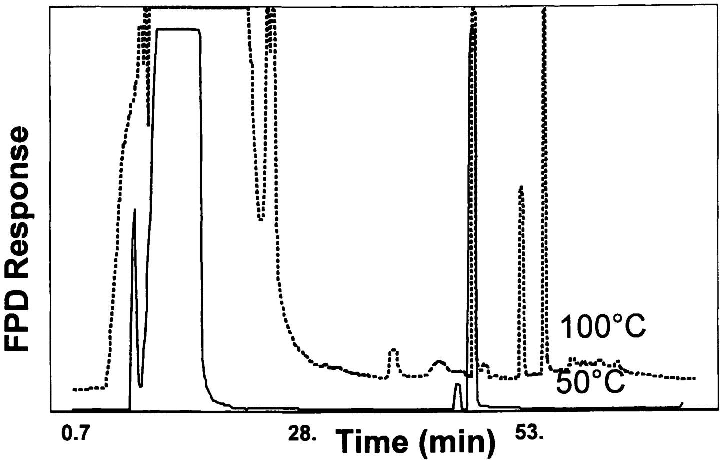

Figure 3. Beef sulfur volatiles obtained at two purging temperatures. (Reproduced with permission from reference 21. Copyright 1994 Elsevier Science.)

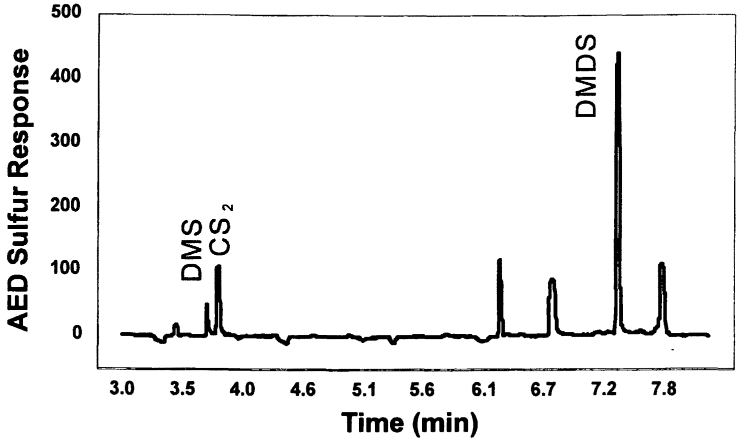

Figure 4. Sulfur volatiles collected from coffee powder after adding boiling water and purged at 85°C for 6 min and using a cryogenic trap. (Reproduced with permission from reference 22. Copyright 1995 Elsevier Science.)

Miscellaneous Techniques

One of the classic ways to separate and concentrate thiols is to add mercury or lead chloride to form a precipitate of the insoluble sulfur complex. Thiols can be regenerated using acid after the precipitate is separated from the sample (*23*). This procedure works satisfactorily for hydrogen sulfide and some of the lower molecular weight thiols. However, there is little evidence that this procedure works as well for thiols greater than C_3. Gold-coated glass beads can be used to concentrate low levels of hydrogen sulfide and a few low molecular weight sulfur compounds (*24*). As much as 10 L of headspace gas can be passed through the beads and the collected sulfur compounds eluted by heating. This procedure has been used to determine H_2S and other simple sulfur compounds in citrus juices (*25*). Later investigations (*26*) demonstrated that the extraction efficiencies for the best metal foils were low ranging from 28-45% and the collection capacity varied considerably from compound to compound. Collection capacity for compounds like dimethyl sulfide was limited to less than 3 ηg sulfur but the capacity for dimethyl disulfide was over 20 ηg sulfur. Therefore, results using these techniques should be considered semi-quantitative. Other sample enrichment techniques include chemically impregnated filters with silver or mercury salts (*27,28*), solid adsorbents such as Carbosieve B and Mol. Sieve (*29,30*) and cryogenic traps (*31,32*). However, most of these concentration techniques have capacity (break through) problems and uneven extraction efficiencies.

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More recent procedures have separated and concentrated thiols from liquid samples using a column material that has a selective affinity for thiols. The sample is first extracted with pentane: dichloromethane, and then passed through a column containing Bio-Rad Affi-Gel 501 (a crosslinked agarose gel containing phenylmercuric chloride groups to bind thiols). Potential interfering substances are eluted with pentane: dichloromethane (2:1) and finally elution of the thiols is accomplished with dithiothreitol. This technique has been used to analyze isomers of the aroma compound 8-mercapto-p-menthane-3-one in blackcurrant flavorings (*33*). However, this technique has not been successful in determining the similar 1-p-menthene-8-thiol from grapefruit juice (*34,35*).

Volatile thiols such as 2-methyl-3-furanthiol have been determined in wines by treating the aqueous distillate from a low temperature vacuum distillation with an organomercuric salt such as p- hydroxymercuribenzoate, p-HMB. Thiols were then released using an excess of cystine (*36*) and analyzed using GC-MS or GC-O. Another variation of this technique is to extract a wine sample with dichloromethane, and then purify the extract with p-HMB (*37*).

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Separation Techniques

Gas Chromatography (GC)

High-resolution capillary gas chromatography has been the technique of choice to separate the complex chemical mixtures that are typically found in foods and flavorings because of its enormous separation power. However, the injector temperatures commonly used (200-250°C) are more than sufficient to create sulfur thermal artifacts. A more acceptable alternative is to use a temperature programmable injector or cool-on-column injection.

It has been demonstrated that bis-(1-propenyl)-disulfide, a common component in *Allium* oils, rearranges at 85°C to form 2-mercapto-3,4-dimethyl-2,3-dihydrothiophene which can in turn undergo further rearrangements (*38*). It was found that initial mild injector/oven temperatures and slow temperature gradients were necessary to minimize thiosulfinate decomposition. The effect of temperature gradient on volatile decomposition is shown in Figure 5. It should be noted in comparing chromatogram A with chromatogram B, that peak 31, cis-zweibeleane, is greatly diminished, peak 9 is completely missing and a small new peak (18) dimethyl thiosulfonate is formed. It was also reported that if MS is employed, transfer line temperature should be at 100°C or lower to minimize thermal decomposition. Other concerns are discussed in the later section on artifact formation.

Even though capillary GC has enormous resolution capabilities, it is not uncommon for several components to co-elute with other components even with a relatively simple (150-component) mixture. To resolve this potential problem, many researchers employ a fractionation step after the initial isolation and preliminary concentration of volatiles. These simplified fractions are then evaluated using capillary GC. Fractionation techniques include open column chromatography using silica columns, preparative HPLC, preparative TLC and flash chromatography. When these fractions are concentrated, they can often be concentrated to a greater extent than would be possible with the entire sample. This approach is highly attractive for the analysis of sulfur compounds as higher concentrations make identification easier by providing better quality mass spectra. There is also the added bonus of reducing the possibility of creating artifacts in the concentration process because each fraction contains fewer matrix components as potential reactants.

Multidimensional capillary GC is being increasingly used to fractionate complex food flavors. Even though they are expensive, multidimensional systems can be automated to run 24 hr a day as opposed to the batch process described above. The first GC typically uses a wide bore (high capacity column) from which cuts are transferred to a second column, which typically contains a dissimilar stationary phase. A mass spectrometer or sulfur specific detector is usually employed after the second column. Using this procedure, simplified volatile fractions can usually be evaluated at higher concentrations with greatly reduced possibilities of co-elution.

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High Performance Liquid Chromatography (HPLC)

This technique has considerably less separation power than high-resolution capillary GC. In addition, HPLC detectors generally lack similar sensitivity and selectivity. However, HPLC separation takes place entirely at room temperature, which can be a critical advantage when separating and identifying thermally unstable sulfur compounds. Since some sulfur compounds are highly reactive and sulfur artifacts can be formed in hot GC injectors (*39*), genuine care must be taken to be certain the compounds detected using GC were initially present in the sample and are not artifacts. Unfortunately, lack of sensitivity, resolution, and selectivity of HPLC has limited sulfur applications to common situations in which sulfur compounds are present in relatively high (mg) amounts. Block and coworkers (*40*) examined the sulfur compounds in onions and garlic using both normal and reverse phase HPLC. Figure 6 shows the normal phase chromatogram of a white onion extract. It is worth comparing Figures 5 and 6 because they are both extracts from white onion. The HPLC chromatogram is actually more complex than the GC-MS chromatograms. Using HPLC, the authors were able to identify several sulfur compounds for the first

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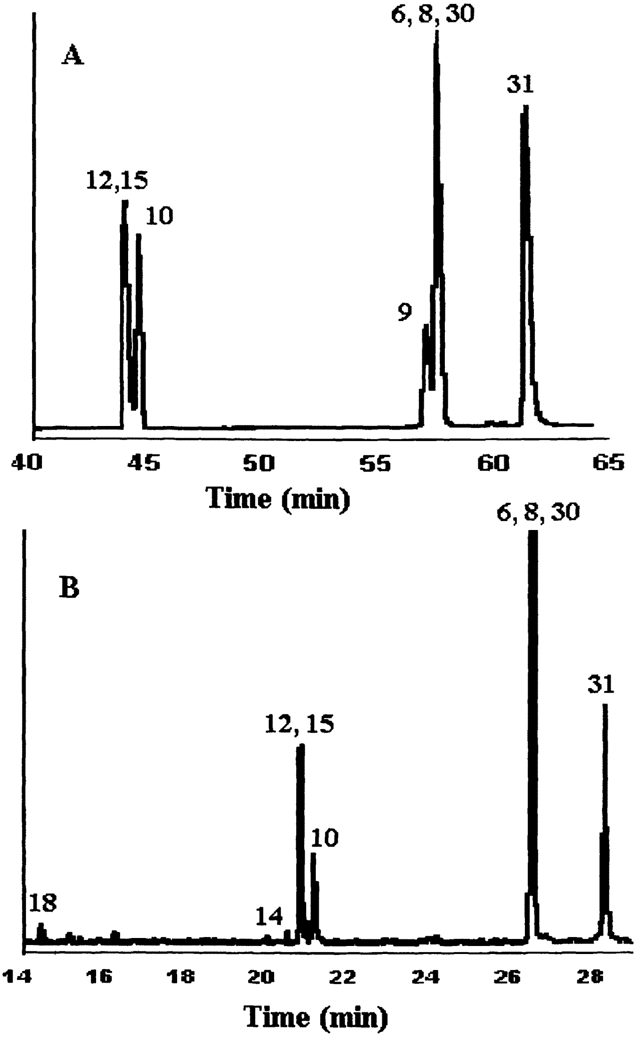

Figure 5. *Comparison of identical white onion extracts separated on the same DB-1 column using two temperature programs. In program A, initial temp was 0°C held for 5 min., then increased at 2°C/min to 200°C. Temperature program B was initially at 0°C then increased to 200°C at 5°C/min. Y-axes correspond to TIC responses. (Reproduced with permission from reference 38. Copyright 1992 American Chemical Society.)*

time. The newly identified compounds were not observed even under optimal GC-MS conditions. Still with all its limitations, HPLC is probably the best technique to determine if the sulfur species is actually present in a plant extract or food. The major limitation is its lack of sensitivity. Unfortunately, aroma active levels of most sulfur compounds in foods are many orders of magnitude lower than minimum detection limits for HPLC with UV detection.

It would appear that the application of HPLC-MS would be a highly suitable tool for the determination of highly reactive compounds. The separation would take place at ambient temperature (thus avoiding thermal artifacts) plus greater detector sensitivity and the additional ability of identification (using fragmentation patterns) would seem highly attractive. Even though HPLC has been employed as a preparative separation and followed by GC-MS (*41-43*), the only example of directly coupled HPLC-MS of sulfur compounds found was an exploratory study of onion and garlic volatiles (*40*).

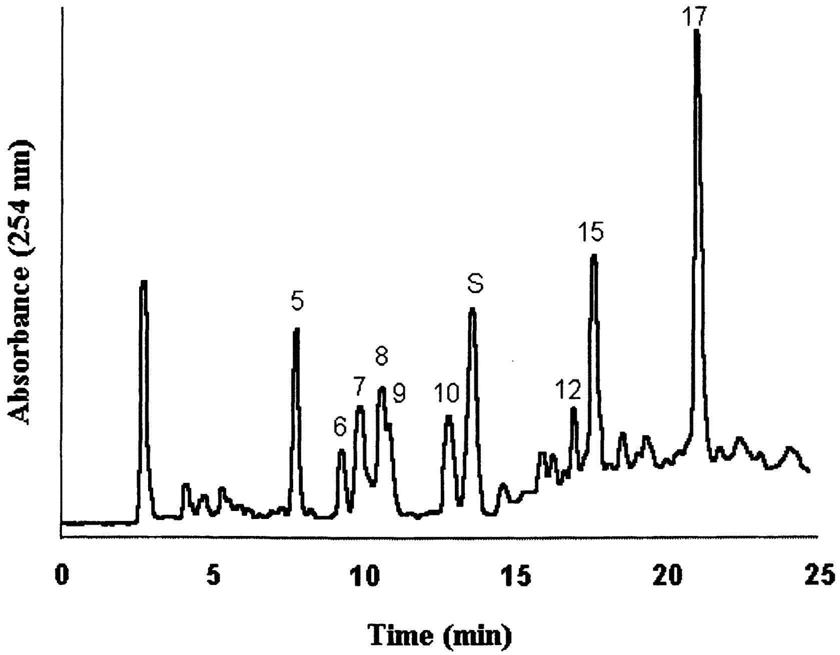

Figure 6. *HPLC of thiosulfinates in white onion (Allium cepa) on a silica column using an isopropanol: hexane gradient. (Reproduced with permission from reference 40. Copyright 1992 American Chemical Society.)*

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Detection and Quantitation

Artifact formation

As discussed in the GC section, artifacts can be a serious problem in the accurate analysis of sulfur compounds (*39,44*). Sulfur artifacts can be formed during sampling and concentration if the sample is exposed to elevated temperatures. Examples of elevated temperature exposure would include distillation, or solvent-distillation extraction, SDE, or during headspace analysis (either static or dynamic) where samples are warmed to increase volatile concentrations. The hot (200-250°C) GC injector port can be a major source of sulfur decomposition. Mindful of possible artifact formation, Kubec and coworkers (*45*) examined dimethyl thiosulfinate peak areas using three injector temperatures, 140, 180 and 220 C. They found that 93% of the thiosulfinate survived the 180°C injector but only 18 % survived the 220°C injector. They choose to use the 180°C setting for their experiments because of fears of incomplete sample volatilization at 140°C.

After thiosulfinates, thiols are probably the next most reactive form of sulfur and occur in a wide range of food products. Thiols are known to dimerize even under refrigerated storage temperature (*46*). Other workers (*16*) have found that up to 24% of dimethyl sulfide is oxidized to dimethyl disulfide as a result of being desorbed from a SPME fiber in an injector equipped with a narrow bore SPME injector liner. Fortunately, higher thiols were not oxidized to a significant degree. With these facts in mind, one should consider the possibility of artifact formation whenever disulfides are identified. Werkhoff and coworkers (*10*) identified a number of new disulfides in yellow passion fruit. They considered the possibility that these compounds could have been formed during extraction or concentration, but concluded that the low temperature sample concentration and careful GC analysis did not produce the observed disulfides. In addition, since most disulfide artifacts are symmetric and most of the observed disulfides were asymmetric, they felt the observed disulfides were real.

Flame Photometry

The flame photometric detector was one of the first detectors to offer the selectivity and sensitivity to detect low concentration sulfur compounds in food aromas. It utilizes the energy from a hydrogen/air flame to produce an excited molecular species, S_2. The energized molecule loses energy in the form of a broadband emission spectrum at a maximum emission wavelength of approximately 394 nm. Since the emitted light is due to bimolecular sulfur, the emitted light is proportional to the square of the sulfur concentration and is not

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linear with concentration. Excited molecular species containing other elements such as phosphorus can be formed under these conditions; therefore, an interference filter is placed in front of the photomultiplier tube to minimize emissions from P containing compounds (lambda max. 510-526 nm). There are several limitations to this technique, which include quenching due to hydrocarbon species and a nonlinear response. An example of this technique is shown in Figure 7 for the analysis of thiol esters in passion fruit (*47*). It should be noted that because of the exponential response, the larger peaks are completely off scale before the smaller peaks can be seen. This can be corrected in part by using the square root of the emission output.

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Chemiluminescence

The sulfur chemiluminescence detector, SCD, is based on three unique gas phase chemistries of which the final step is a photochemical reaction. The three chemical processes are shown below:

$$\text{Sulfur compounds} + H_2/\text{air} = SO + \text{other products} \qquad (1)$$

$$SO + O_3 = SO_2^* + O_2 \qquad (2)$$

$$SO_2^* = SO_2 + h\nu \qquad (3)$$

The first step involves the conversion of all sulfur species to a single species (sulfur monoxide) at a hot ceramic surface in a hydrogen rich atmosphere. The sulfur monoxide is transferred to a gas phase reaction chamber under partial vacuum where it is reacted with ozone. The product of this reaction is an excited sulfur dioxide molecule, which returns to the ground state by giving off a photon of light. A photomultiplier tube then measures the amount of light produced. Usually a sharp cut off filter is placed in front of the photomultiplier tube to eliminate interferences. Since the photochemical reaction takes place under vacuum, quenching is rarely a problem. One of the real advantages of this detector is that the response from all sulfur compounds is the same, at least in terms of molar concentrations, because a common species is formed. It is thus possible to determine the concentrations of know species without having to run individual calibration curves.

This detector was evaluated as a replacement for a FPD for the determination of sulfur compounds in beer (*48*). It was found to have better selectivity, sensitivity, and linearity and it was not susceptible to hydrocarbon-quenching effects, as was an FPD. Instrument modifications needed to adapt this detector were minimal. Other investigators employed an in-bottle purge and

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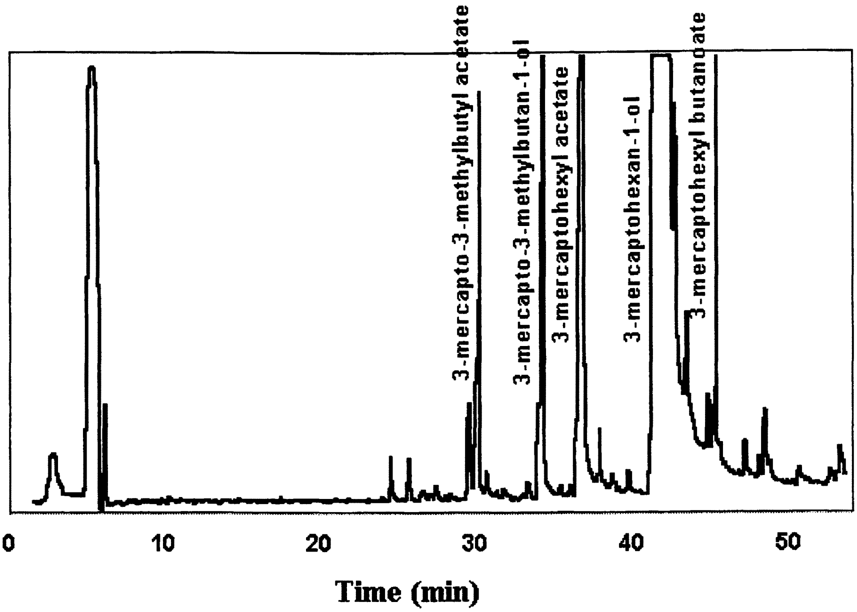

Figure 7. Detection of various thioesters in yellow passion fruit using a flame photometric detector. (Reproduced with permission from reference 47. Copyright 2000 American Chemical Society.)

trap sampling technique (coupled to a capillary GC with a sulfur chemiluminescence detector) to detect, identify, and quantify sulfur compounds in beer. They found sulfury notes identified during sensory panel assessments of beer did not correlate with measured dimethyl sulfide concentrations. Yet other sulfur peaks were observed that did correlate with sensory sulfury notes and they suggested the use of this technique for quality control (*49*).

Since low-level sulfur compounds influence the aroma of coffee, the SCD has been used to detect sulfur components in roasted and brewed coffee headspace samples (*50*). Dimethyl sulfide, carbon sulfide, thiophene, dimethyldisulfide, 2-furfuralthiol and 4-methylthiazole were quantified with detection limits that ranged from 0.002 ηg (carbon disulfide) to 0.028 ηg (2-furfuralthiol).

Pulsed Flame Photometry Detection (PFPD)

Pulsed flame photometry is a recently developed technique (*51,52*) that differs from FPD in that it does not employ a continuous flame. Hydrogen and air are added to the GC column effluent at a low flow rate that cannot sustain a

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continuous flame. Organic compounds from the column effluent are combusted in the ignited flame when the mixture reaches a hot electric igniter. However, the flame rapidly starves and self extinguishes. This combustion-extinction cycle is repeated every 2-4 seconds. The combustion of hydrocarbon molecules in the flame produces such molecular radicals as CH, C_2 and OH whose flame emissions are very short lived (0-2 ms). Heteroatomic compounds will produce molecular radicals such as S_2, HPO and HNO that emit in the cooler region of the flame and consequently, emissions occur at longer times (3-25 ms). PFPD utilizes this time difference by employing gated amplifiers to record light only during certain time intervals. Elemental selectivity is also enhanced by controlling flame temperature, and by optical filters placed in front of the photomultiplier detector.

Although the combination of time, flame temperature and optical filters can be used to detect as many as 28 different elements (*53*), this detector is still most sensitive for sulfur and phosphorus. An example of the use of gated amplifiers to achieve separation between phosphorus and sulfur compounds is shown in Figure 8. It can be seen that the emission due to phosphorus is longer than the hydrocarbon emissions but shorter than the sulfur emission. The gated amplifier is off during the first few milliseconds after combustion because this portion of the flame emission is due primarily to hydrocarbons. The detector is turned on between 3-8 ms to measure and integrate the light emission, which are due primarily to phosphorus. Finally, another detector is turned on between 8-25 ms to measure sulfur emission. It should be noted that the early portion of the sulfur signal is not measured in order to minimize possible interferences from phosphorus.

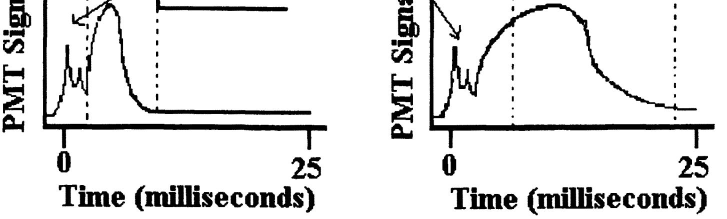

Figure 8. Comparison of the use of gated amplifiers to obtain selectivity between phosphorus and sulfur compounds. (Reproduced from reference 54.)

This technique offers many advantages in terms of sensitivity, selectivity, stability and cost. By employing two gated amplifiers, it is also possible to determine two elements simultaneously. However, gas flows are extremely critical. Quenching and non-linear responses can still be a problem. Some PFPD detectors offer a square root output option that is approximately linear within limited concentration ranges.

Atomic Emission Detection (AED)

Atomic emission detection is based on the principle of exciting the ground state atom into an excited state and then observing the line spectra emission. Unfortunately in the case of sulfur atoms, the typical hydrogen flame lacks sufficient energy to form atomic sulfur and excite it to the lowest excited state. Microwave-induced plasma has sufficient energy to excite sulfur as well as a host of other elements to excited atomic states. The emitted light from the excited atoms is separated using a diffraction grating and quantified using a photomutiplier tube or photodiode array. If a photodiode array is used, several wavelengths corresponding to several elements can be monitored simultaneously. However, the use of a plasma excitation source along with multi-wavelength optical detection equipment has caused AED to be one of the most expensive GC detectors. This disadvantage is partially offset by superior detection limits and multi-element capability. This detector also has one of the largest linear responses of any detector. A large linear range might be considered more of an advantage in environmental analyses where large concentration ranges might be expected. However, most sulfur compounds in foods are usually found at the lower range of detection limits. One of the more interesting applications of AED is the study of sulfur containing odorants in human breath (*55*). Shown in Figure 9 are the chromatograms of the sulfur compounds in the headspace of raw garlic compared with a similar gas sample taken from the exhaled breath from a human that has consumed the garlic.

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Comparison of Detection Techniques

An excellent comparison between AED, FPD and SCD detection was presented by Mistry and coworkers (*56*). These authors examined the linear range, minimum detection limits and selectivity of flame photometric, chemiluminescence and atomic emission detectors using a single set of sulfur reference standards. They found that the AED gave 6-8 times greater dynamic range and the lowest detection limits (as low as 1 pg) compared to the other two detectors. However, the SCD showed the least interferences from non-sulfur

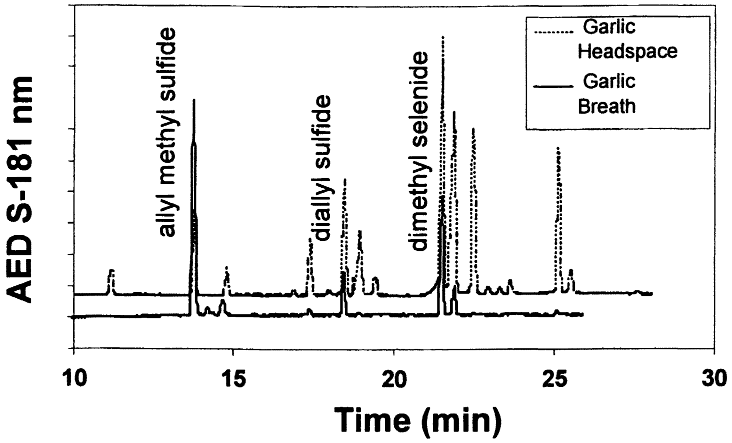

Figure 9. *Comparison of sulfur containing volatiles in garlic headspace with breath sulfur compounds from a human who had recently consumed garlic using plasma induced atomic emission detection. (Reproduced with permission from reference 55. Copyright 1996 American Chemical Society.)*

Table I. A Comparison of Detection Limits for Three Types of Sulfur Specific GC Detectors

Compound	Minimum Detection Level (pg)		
	FDP	SCD	AED
Butyl sulfide	199	20	2
Butyl disulfide	99	10	1
Thiophenol	205	2000	102
Phenyl sulfone	200	100	1

Source: Reference 56.

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compounds. Identical GC conditions were used for each detector. Minimum detection limits for selected compounds used in this study are listed in Table I.

Even though AED detection offers superior detection limits and simultaneous detection of 2-4 elements, this capability comes at a steep price and is by far the most expensive detector. It is unfortunate that a PFPD detector was not considered in this study. It would probably have shown detection limits below that of the FDP and very similar to those of AED. Both AED and PFPD claim minimum detection limits of 1 pg of S. Since detection limits are one of the main factors in the successful determination of sulfur aroma compounds, PFPD or AED would be suitable. AED detection has the advantage of simultaneous multi-element detection but the cost of this detector is out of the reach of most researchers. In trying to optimize performance (especially sensitivity) and cost, most researchers choose either the PFPD or SCD detector to determine sulfur based aroma compounds.

Isotope Dilution Analysis

Isotope dilution analysis is an excellent technique to accurately quantify volatiles in complex mixtures, particularly if the compounds are unstable or exist at low concentrations. Isotope dilution analysis was initially developed for the determination of glucose in plant tissue (*57*). Schieberle and Grosch were the first to utilize this technique to quantify flavor components (*58*) and determined several of the key aroma impact compounds in bread crust. This technique can provide accurate quantitative information about specific volatiles as they exist in the original matrix. However, stable isotopes of the analytes of interest must be synthesized or purchased. The isotope is then essentially the perfect internal standard, which will compensate for incomplete extraction, losses during sample preparations, etc. Deuterium is the isotope most commonly used because it is less expensive than other isotopes. Care must be taken to be certain the deuterium is synthesized into a non-exchangeable position. Isotope dilution has been used to determine potent thiols in coffee as shown in Figure 10. Recently Buettner and Schieberle used deuterium labeled thiols, and multidimensional GC with CI-MS to quantify of the major sulfur-containing, aroma impact compounds in grapefruit juice (*59*). The limitations of this technique are the cost or availability of stable isotopes and the detection limits of most GC-MS detectors require that the sample be considerably concentrated, which may promote artifact formation. An excellent critique of this method has been recently published (*60*).

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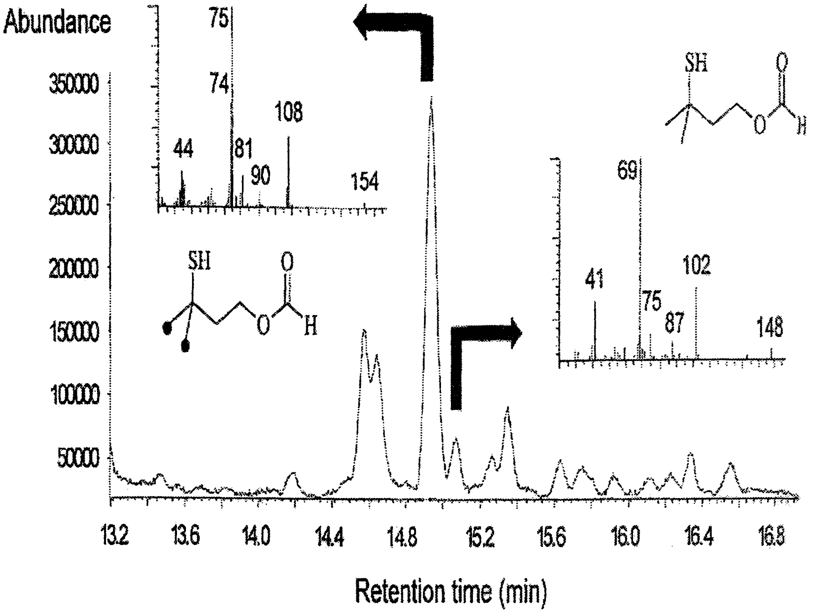

Figure 10. Isotope dilution analysis of 3-mercapto-3-methylbutyl formate in a roast and ground coffee aroma extract enriched with deuterated 3-mercapto-3-methylbutyl formate. (Reproduced with permission from reference 60. Copyright 1998 American Chemical Society.)

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Chapter 2

Sensory Relevance of Volatile Organic Sulfur Compounds in Food

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Volatile organic sulfur compounds account for only about 10 percent of all volatile components identified in edible products. However, these compounds are important constituents of the flavor of many foods, beverages and natural isolates. Typically these compounds occur in low concentrations and contribute significantly to characteristic aroma notes due to low odor thresholds. Selected character-impact sulfur compounds found in natural and processed foods are discussed in detail and the sensory relevance is estimated on the basis of odor quality, threshold values, and sensory studies.

Volatile organic sulfur compounds are important constituents of food flavors (*1*). The TNO compilation of volatile compounds consists of about 700 sulfur-containing molecules, which correspond to approximately 10% of the total number of volatiles listed (*2*). Sulfides represent the major chemical class with more than 440 compounds of which the disulfide group alone accounts for about 100 different molecules. Further well-known chemical classes are thiazoles (~100), thiophenes (~100), and thiols (~60).

The most frequently listed sulfur-containing volatile molecule is dimethyl disulfide reported to occur in more than 110 food products (*2*). Further compounds are dimethyl sulfide (~90), benzothiazole (~80), dimethyl trisulfide (~70), methional (~60), methylmercaptan (~60), hydrogen sulfide (~50), thiazole (~30), 2-acetylthiazole (~30), 2-methylthiophene (~30), and others. However, frequent occurrence does not necessarily mean high sensory relevance. This can be estimated by various other means, such as aroma quality, intensity, and threshold values. These data, in combination with the concentration, allow a fairly good estimation of sensory contribution.

This article focuses on the sensory relevance of sulfur-containing compounds, in particular thiols, using examples from various types of natural and processed food products. The few examples chosen represent only a small part of the vast literature on odor-active sulfur compounds. A comprehensive overview on analysis, formation, and functional properties of S-compounds is given in the ACS Symposium Series 564 (*3*). The particularity of the chemistry involved in the formation of S-containing compounds is discussed by Block in an excellent review (*4*).

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Experimental Procedures

It is not the purpose of this paper to discuss in great detail the various analytical and sensory approaches suitable to assess the role of individual compounds to the overall aroma of a food. Recent review articles are recommended for more information (*5-9*). However, key elements of some of the analytical and sensory approaches will be discussed to better understand sensory relevance.

Isolation of Thiols

A first and very important step in the characterization of flavor is to obtain an aroma extract with the authentic note of the food. The majority of sulfur compounds such as sulfides, thiazoles, and thiophenes are chemically stable and can easily be extracted. Thiols, however, are reactive species and susceptible to oxidation, dimerization, and reaction with carbonyls. Therefore, they deserve special attention to assure that losses during the entire sample preparation procedure are reduced to a minimum.

Trapping thiols with *p*-hydroxymercuric acid (pHMB) has been shown to be an efficient method to isolate traces of sensory-relevant thiols (Figure 1). Darriet et al. (*10*) identified 4-mercapto-4-methyl-2-pentanone (**1**) as a character impact compound of Sauvignon wines after having smelled it by the GC-sniffing technique in an aroma extract. The selective enrichment of thiols is based on the reaction of thiols with pHMB at pH 8.5 and room temperature. The derivative is water-soluble and can be separated from lipophilic materials. As the combination of thiols with pHMB is reversible, it is then released by an excess of cysteine or glutathione for identification by GC-MS. As shown in Figure 2, odorant **1** was identified in Sauvignon blanc wines where it occurs in trace amounts.

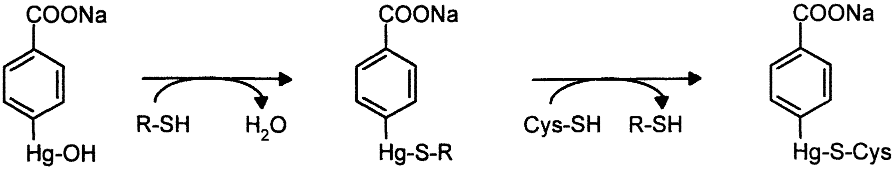

Figure 1. Enrichment of thiols using p-*hydroxymercuric acid as a selective trapping reagent.*

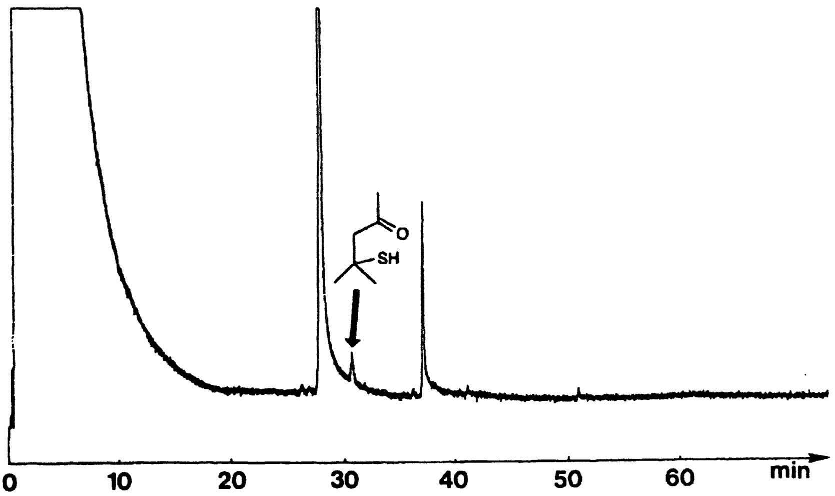

Figure 2. Chromatogram of a Sauvignon blanc wine extract after treatment with p-hydroxymercuric acid and an excess of cysteine to isolate 4-mercapto-4-methyl-2-pentanone (1). (Reproduced with permission from reference 10. Copyright 1995 John Wiley & Sons.)

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Screening of Potent Odorants

A frequently used technique to screen odorants from odorless volatile compounds is based on gas chromatography in combination with a sniffing port at which an odorant can be detected by smelling the effluent of the column (*11*). A more sophisticated approach of GC-Olfactometry (GC-O) includes sniffing of serial dilutions until no odor-active region is perceived. The two most applied methods are CHARM analysis (*12*) and Aroma Extract Dilution Analysis (AEDA) (*13*), which have been recently reviewed in detail (*5-8*).

The usefulness of GC-O is illustrated on two examples. Although the meat-like and roasty smelling compound 2-methyl-3-furanthiol (MFT, **2**) has been known since 1976 (*14*), it was only when GC-O became a generally accepted technique in flavor research that this odorant was frequently reported in food products such as tuna fish (*15*), cooked beef (*16*), boiled chicken (*17*), and roasted coffee (*18*). In some of these products, GC-O, performed on capillary columns of different polarity, was the only means of identification (*16,18*) as mass spectra could not be obtained due to inadequate concentrations for analytical detection.

1-*p*-Menthen-8-thiol (**3**), a flavor impact constituent of grapefruit juice, was first reported by Demole and coworkers in 1982 (*19*). This is one of the most powerful flavor compounds found in nature with a detection threshold of 0.0001 μg/kg water for the racemic mixture. Recently, the black currant-like smelling odorant 4-mercapto-4-methyl-2-pentanone (**1**) was additionally reported as a key constituent of grapefruit juice (*20*). Again, identification was based on GC-O data, as the concentration for GC-MS was too low. Furthermore, methional (**4**) was found as another S-containing odorant in grapefruit juice. The chemical structures of these compounds are presented in Figure 3.

These data suggest GC-O to be a particularly powerful tool for screening and identification of odor-active thiols, which have threshold values in the low and sub-ppb range, provided the compounds are known and the reference compounds available. The sensory relevance of such odorants is due to the low odor thresholds.

Quantification of Potent Odorants

As already mentioned, detection and identification of odorants with very low threshold values is rather difficult, particularly of thiols that are unstable and susceptible to chemical reactions. Therefore, the ability to obtain reliable quantitative data is a major challenge in flavor research. Isotope Dilution Assay (IDA) is a method used to overcome these limitations (*7,21,22*). It is based on the use of stable isotopomers of the analytes as internal standards. The potent odorants MFT (**2**), 2-furfurylthiol (FFT, **5**), 3-mercpato-2-pentanone (3M2P, **6**),

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and 2-mercapto-3-pentanone (2M3P, 7) were simultaneously quantified in boiled beef using the deuterated analogs d-MFT, d-FFT, and d-3M2P as internal standards (*23*) (Figure 4).

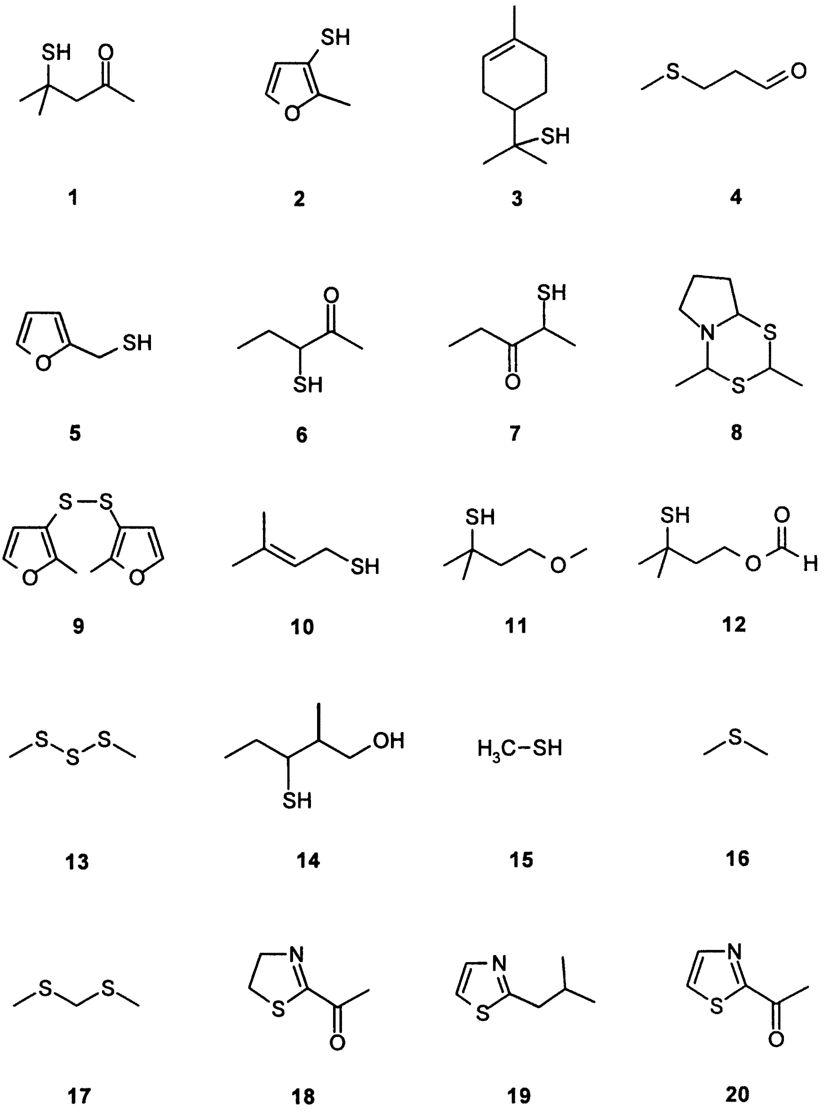

Figure 3. Examples of sulfur-containing compounds that have low sensory thresholds and often play a key role in many food flavors (1,24) (See Table I for corresponding names and threshold values).

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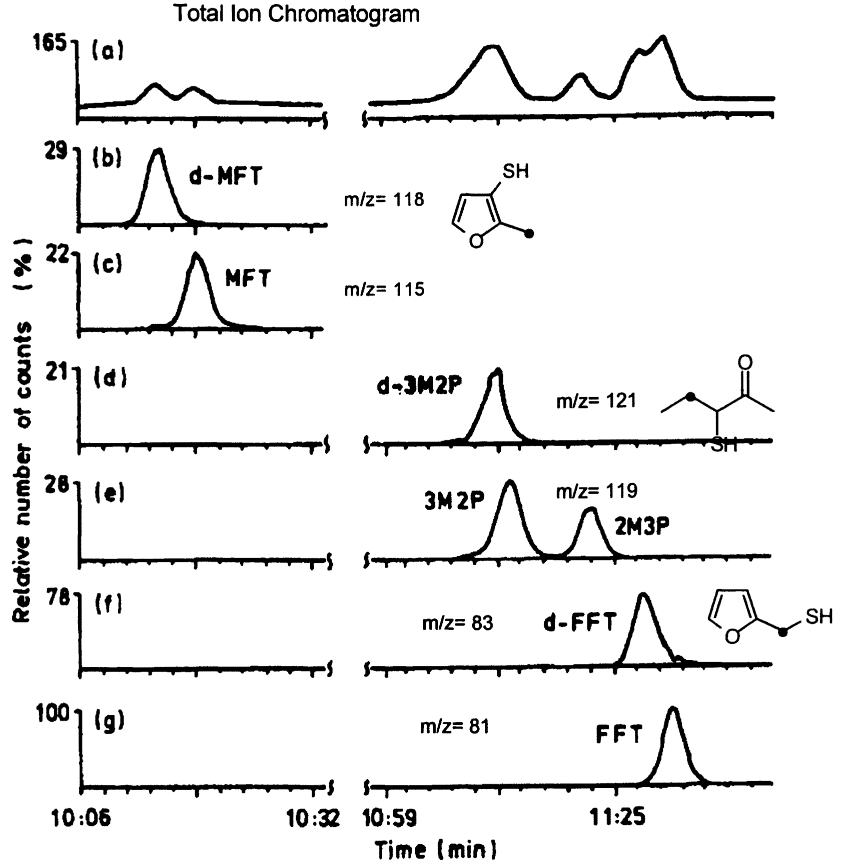

Figure 4. Quantification of 2-methyl-3-furanthiol (MFT), 2-furfurylthiol (FFT), 3-mercapto-2-pentanone (3M2P) and 2-mercapto-3-pentanone (2M3P) by isotope dilution assays using the corresponding deuterated isotopomers d-MFT, d-FFT, and d-3M2P as internal standards. (a)=total ion chromatogram; b-g=selected ion chromatograms for (b) m/z 118 for d-MFT, (c)= m/z 115 for MFT, (d)= m/z 121 for d-3M2P, (e) for m/z 119 for3M2P and 2M3P, (f)= m/z 83 for d-FFT and (g)= m/z 81 for FFT. (Adapted from reference 23. Copyright 1998 American Chemical Society).

Odor Thresholds

Apart from odor quality, the threshold value of a compound is another important characteristic for estimating sensory relevance. Threshold values can be determined orthonasally (odor) or retronasally (aroma) using various solvents, such as air, water, alcoholic solutions, oil, emulsions, starch, and deodorized food. Perception thresholds are obtained if the odorant is different from the solvent. In the case of recognition thresholds, the characteristic aroma is still recognizable. In general, sulfur-containing compounds, such as odorants **1-21** (Figure 3), have low sensory thresholds and, therefore, often play a key role in many food flavors. Depending on the matrix used for evaluation, the threshold values may significantly differ. Some examples are listed in Table I. Odor thresholds must be carefully selected from literature, and ideally threshold values are confirmed by a trained sensory panel employed in sensory studies.

Table I. Odor Thresholds of Sulfur-Containing Compounds

Odorant	*Odor Quality*	*Air [ng/L]*	*Water [μg/kg]*
Pyrrolidino[1,2-*e*]-4*H*-2,4-dimethyl-1,3,5-dithiazine (**8**)	Roasty, shellfish		$1x10^{-11}$
bis-(2-Methyl-3-furyl)-disulfide (**9**)	Sulfury, meaty	0.001	$2x10^{-5}$
(*R*)-1-*p*-Menthen-8-thiol (**3**)	Sulfury, grape fruit		$2x10^{-5}$
4-Mercapto-4-methyl-2-pentanone (**1**)	Sulfury, black currant		$1x10^{-4}$ 0.001[a]
3-Methyl-2-buten-1-thiol (**10**)	Sulfury, foxy	0.02	$3x10^{-4}$
4-Methoxy-2-methyl-2-butanethiol (**11**)	Sulfury, catty	0.0002	0.001 0.02[b]
3-Mercapto-3-methylbutyl formate (**12**)	Sulfury, catty	0.0003	0.003
2-Methyl-3-furanthiol (**2**)	Sulfury, meaty	0.002	0.007
2-Furfurylthiol (**5**)	Sulfury, coffee	0.02	0.01, 0.4[b]
Dimethyl trisulfide (**13**)	Cooked cabbage	0.1	0.01 0.2[a], 2.5[b]
(2*R*,3*S*)-3-Mercapto-2-methyl-1-pentanol (**14**)	Sulfury, onion	0.0001	0.03
Methional (**4**)	Cooked potato	0.2	0.2, 0.2[b]
Methanethiol (**15**)	Sulfury, cabbage		0.2, 0.1[b]
Dimethyl sulfide (**16**)	Sulfury, tomato		0.3 10[a], 1.2[b]
bis-(Methylthio)-methane (**17**)	Sulfury, truffle		0.3, 3[b]
3-Mercapto-2-pentanone (**6**)	Sulfury, catty	0.1	0.7
2-Acetyl-2-thiazoline (**18**)	Roasty, popcorn	0.02	2
2-Isobutylthiazole (**19**)	Green ,tomato		3
2-Acetylthiazole (**20**)	Sulfury, roasty	3	10
Hydrogen sulfide	Sulfury, egg		10

a Threshold determined in an aqueous ethanol solution (10%, v/v).

b Threshold determined in deodorized oil.

SOURCE: Adapted from references 1 and 24.

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The Odor Activity Value (OAV) Concept

By definition, OAV is the ratio of the concentration of an odorant to the threshold value (*12*) and is a synonym to aroma value (*25*) and odor unit (*26*). It is evident that reliable OAVs can only be calculated if correct data are obtained for the threshold and concentration of the odorant. The threshold should preferably be determined in the food itself or in a matrix closely representing the food product, e.g. in oil for margarine aroma, in water or better in an aqueous 10% ethanolic solution for wine aroma, and in starch for bread aroma.

In their work on tomato aroma, Buttery and coworkers calculated the OAVs in order to estimate the sensory relevance of volatile constituents of fresh tomato (*27*). They concluded that only a few compounds occur in concentrations well above the odor thresholds, *i.e.* only few volatile compounds have OAV>1 as shown in Table II.

2-Isobutylthiazole (**19**) with an OAV=12 belongs to that group of compounds. Interestingly, despite the relatively low OAV, this odorant is known to be a key constituent of tomato flavorings. In 1970 Kazeniac and Hall (*28*) had already reported that 2-isobutylthiazole, in amounts of 25-50 μg/kg, improves the fresh tomato note and mouthfeel of canned tomatoes.

Table II. Some Key Volatile Constituents of Fresh Tomato Aroma

Odorant	*Concentration [μg/kg]*	*Odor Threshold [μg/kg water]*	*OAV*
(*Z*)-3-Hexenal	12000	0.25	50000
Hexanal	3100	4.5	630
β-Ionone	4	0.007	630
1-Penten-3-one	520	1	520
(*E*)-β-Damascenone	1	0.002	500
3-Methylbutanal	27	0.2	130
(*E*)-2-Hexenal	270	17	16
2-Isobutylthiazole (**19**)	36	3	12

SOURCE: Adapted from reference 27. Copyright 1989 American Chemical Society.

Sensory Studies

Although the relative importance of individual odorants can be estimated on the basis of OAVs, the interactions of odorants with each other or the food matrix remain unconsidered. Flavor recombination studies, albeit time-consuming, offer an attractive way to better estimate the role of odorants in a complex food aroma. As shown for fresh basil aroma (*29*), six odorants with

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OAV>1000 were found to contribute to the overall aroma (Table III). Additional odorants with OAVs of 5 to 500 were wine lactone, methyl cinnamate, estragol, α-pinene, and decanal. A model mixture of the 11 odorants revealed the typical fresh basil aroma. The similarity of the model to fresh basil was described as very good, i.e. 3.0 on a scale from 0 to 3.

Table III. Concentrations and Odor Activity Values (OAVs) of Important Odorants of Fresh Basil Leaves

Odorant	*Concentration [mg/kg]*	*OAV*
(*Z*)-3-Hexenal	12.4	413000
1,8-Cineol	64.0	246000
4-Mercapto-4-methyl-2-pentanone (**1**)	0.01	83300
Linalool	60.2	40000
4-Allyl-1,2-dimethoxybenzene	495	9900
Eugenol	89.0	8900

SOURCE: Adapted with permission from reference 29. Copyright 1997 Eigenverlag Universität Potsdam.

Omission tests were performed to better understand the relative importance of individual odorants. The absence of eugenol or (*Z*)-3-hexenal diminished strongly the similarity to the complete mixture, indicating the essential roles of these compounds to fresh basil aroma (Table IV). Also the absence of α-pinene, 4-mercapto-4-methyl-2-pentanone, linalool or 1,8-cineol led to defininte decreases in similarity.

Table IV. Omission Tests with the Basil Model Mixture

Compound removed from the model aroma	*Similarity*[a]
Eugenol	0.7
(*Z*)-3-Hexenal	1.0
α-Pinene	1.3
4-Mercapto-4-methyl-2-pentanone (**1**)	1.6
Linalool	1.8
1,8-Cineol	1.9

[a] The similarity was scored on a scale from 0 to 3: 1= weak, 2= middle, 3= strong.
SOURCE: Adapted with permission from reference 29. Copyright 1997 Eigenverlag Universität Potsdam.

Selected Food Aromas

Many S-compounds have been reported in the literature to have the same basic organoleptic properties as a food and are called character-impact compounds (Table V, Figures 3 and 5). These compounds are considered as essential constituents in flavor compositions, particularly if the compounds have low odor thresholds.

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Table V. Character-Impact Sulfur Compounds in Foods

Sulfur Compound	*Occurrence*	*Reference*
4-Mercapto-4-methyl-2-pentanone (**1**)	Wine (Sauvignon), grape fruit	10, 30
2-Methyl-3-furanthiol (**2**)	Beef meat, cooked	14, 16
(*R*)-1-*p*-Menthen-8-thiol (**3**)	Grape fruit	19, 30
Methional (**4**)	Potato chips	31
2-Furfurylthiol (**5**)	Coffee, roasted	32, 33
Pyrrolidino[1,2-*e*]-4*H*-2,4-dimethyl-1,3,5-dithiazine (**8**)	Shellfish	34
4-Methoxy-2-methyl-2-butanethiol (**11**)	Black currant, olive oil (Spain)	35, 36
(2*R*,3*S*)-3-Mercapto-2-methyl-1-pentanol (**14**)	Onion (fresh)	37
Dimethyl sulfide (**16**)	Tomato, paste	38
bis-(Methylthio)-methane (**17**)	Truffle (white)	39
2-Acetyl-2-thiazoline (**18**)	Beef meat, roasted	40
2-Isobutylthiazole (**19**)	Tomato, fresh	41
2-Phenyl isothiocyanate (**22**)	Horseradish	42
2-Methyl-4-propyl-1,3-oxathiane (**23**)	Passion fruit	43
Ethyl 3-mercaptopropanoate (**24**)	Grape (Concorde)	44
Ethyl 3(methylthio)-propanoate (**25**)	Pineapple	45
8-Mercapto-*p*-menthan-3-one (**26**)	Buchu leaf oil	46
Propanthial *S*-oxide (**27**)	Onion, fresh	4
Propyl propanethiosulfonate (**28**)	Onion, fresh	1, 4, 47
Dipropyl disulfide (**29**)	Onion, boiled	1, 4, 47
trans-1-Propenyl propyl disulfide (**30**)	Onion, boiled	1, 4
2-(Propyldithio)-3,4-dimethylthiophene (**31**)	Onion, fried	1, 4
3,4-Dimethyl-2,3-dihydrothiophenethiol (**32**)	Onion, fried	1, 4, 48
bis-(2-Propenyl)-disulfide (**33**)	Garlic, fresh	1, 4, 49

SOURCE: Adapted from references 1 and 3.

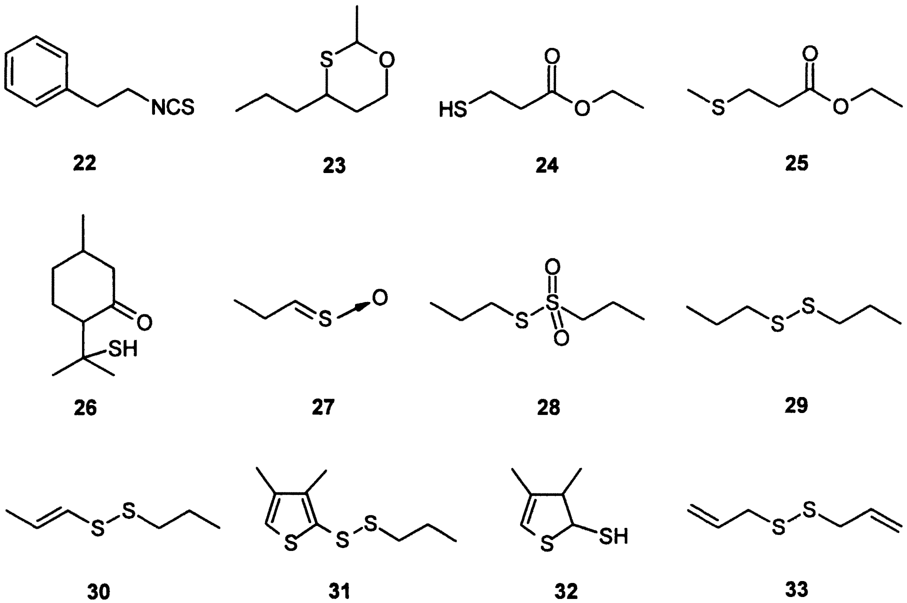

Figure 5. Examples of important S-containing character-impact compounds in foods. (See also Table V for corresponding names and odor descriptors).

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The *Allium* species are known to be rich in S-containing volatile constituents. Propanthial *S*-oxide (**27**), the onion lachrymatory factor, and propyl propanethiosulfonate (**28**) are characteristic compounds of raw onions (*1,4*). Volatile compounds contributing to the aroma of cooked onions are for example dipropyl disulfide (**29**) and *trans*-1-propenyl propyl disulfide (**30**) with odor thresholds of 3.2 and 2 µg/L water, respectively (*1*). 2-(Propyldithio)-3,4-dimethylthiophene (**31**) and 3,4-dimethyl-2,3-dihydrothiophenethiol (**32**) have odors reminiscent of fried onions (*1,48*).

In an extensive study, Pickenhagen and coworkers recently identified a new chemical class of raw onion constituents, i.e. 3-mercapto-2-methyl-1-pentanol (**14**) (*37*). The *anti* enantiomers show particularly low threshold values of 0.03-0.04 µg/L water. The *syn* isomers are less odor-active and have higher threshold values of 12-30 µg/L water (*50*).

Surprisingly, little data has been published on the volatile S-compounds listed in Table V that demonstrate sensory relevance (*1*). Reliable threshold values, concentrations, and sensory studies are rarely available. This may be due to the instability of S-containing compounds, thus making identification and sensory characterization difficult.

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The sensory relevance of sulfur-containing compounds will be illustrated with examples from natural and processed foods, such as olive oil, wine, cooked meat, and roasted coffee. These are only a few examples showing the importance of S-odorants to food flavors.

Olive Oil

The aroma composition of olive oils from different provinces has been studied (*36,51*). Surprisingly, one of the key odorants in Spanish olive oil was identified as 4-methoxy-2-methyl-2-butanethiol (**11**) (Table VI), which was absent in olive oils from Italy and Morocco (*36*). The odor was described as catty, black currant-like and with a high potency. This odorant having a high OAV amongst 21 compounds was thought to be a character-impact constituent of Spanish olive oil (*51*).

Table VI. Some Key Odorants Found in Spanish Olive Oil

Odorant	*Concentration [μg/kg]*	*Odor Threshold [μg/kg oil]*	*OAV*
Acetaldehyde	410	0.22	1865
4-Methoxy-2-methyl-2-butanethiol (**11**)	4.3	0.017	255
(*S*)-Ethyl 2-methylbutyrate	14	0.26	55
3-Methylbutanal	70	2.2	32
2-Methylbutanal	102	5.4	19
trans-4,5-Epoxy-(*E*)-2-decenal	22	1.3	17
Acetic acid	1840	124	15
Ethyl 3-methylbutyrate	5.3	0.62	8.6
Propanal	75	9.4	8.0

SOURCE: Adapted from reference 51.

A model mixture composed of 21 odorants revealed an overall aroma, which was very close to that of the authentic Spanish olive oil sample, i.e. a similarity was determined to be 2.7 ± 0.3 on a scale from 0 to 3. As shown in Figure 6, the model aroma and olive oil have very similar sensory profiles. The fruity note in the model was slightly less pronounced, and the pungent note was a bit more intense.

In further sensory experiments, individual odorants were omitted from the model aroma to study relative importance to the overall aroma. The absence of acetaldehyde and propanal (S1), acetic acid (S2), 3-/2-methylbutanal (S3), or ethyl 2-/3-methylbutyrate (S4) only slightly changed the similarity, which dropped from 2.7 to 2.3. However, when odorant **11** was removed from the model aroma (S5), the similarity was reduced to 0.9, thus indicating that this odorant is an essential volatile constituent of Spanish olive oil (Figure 7).

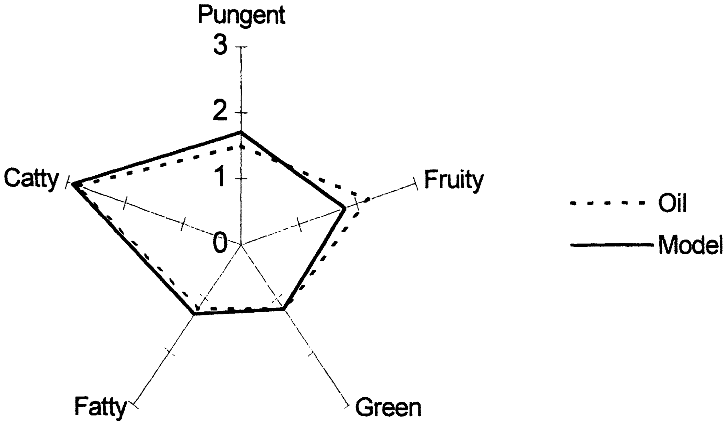

Figure 6. Sensory profiling of the model aroma consisting of 21 odorants as compared to Spanish olive oil. (Adapted from reference 51.)

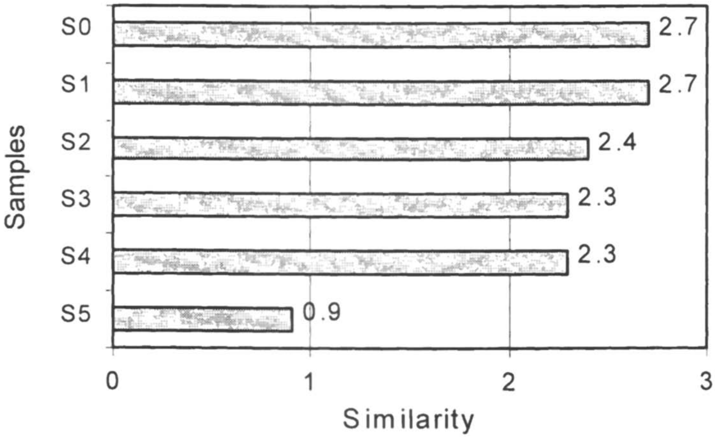

Figure 7. Effect of odorants on the aroma profile of the model mixtures S0 (complete model aroma, 21 odorants), S1 (no acetaldehyde and propanal), S2 (no acetic acid), S3 (no 3-/2-methylbutanal), S4 (no ethyl 2-/3-methylbutyrate), and S5 (no 4-methoxy-2-methyl-2-butanethiol). (Adapted from reference 51.)

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Wine

The important role of S-compounds, particularly thiols, has been shown in different wine varieties. 4-Mercapto-4-methyl-2-pentanone (black currant-like, **1**) was identified in Sauvignon Blanc using GC-O (*10*). As shown in Figures 8 and 9, further thiols were reported in Sauvignon Blanc (*52,53*), i.e. 4-mercapto-4-methyl-2-pentanol (citrus peel-like, **34**), 3-mercapto-3-methyl-1-butanol (broth-like, **35**), 3-mercaptohexyl acetate (passion fruit-like, **36**), and 3-mercapto-1-hexanol (grapefruit-like, **37**).

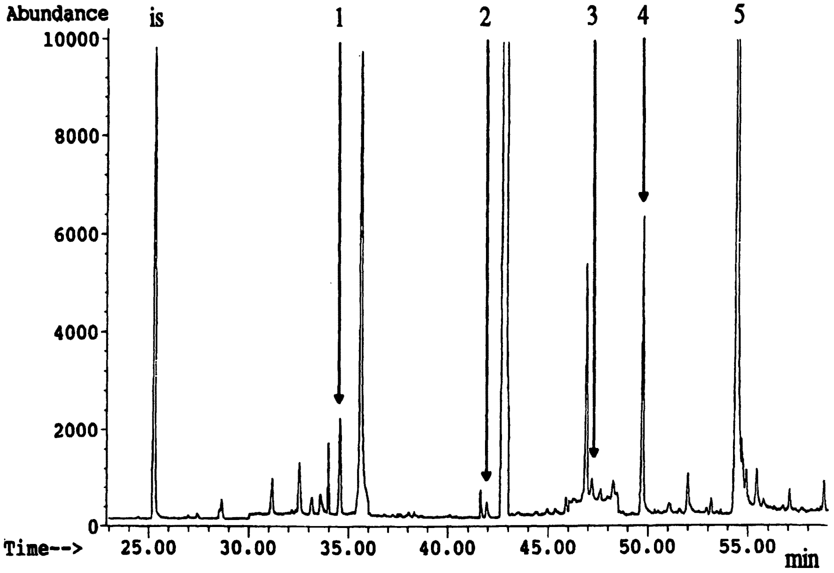
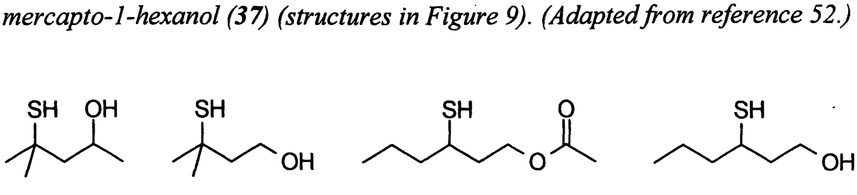


Figure 8. Volatile S-containing constituents of Sauvignon blanc wine: 1, 4-mercapto-4-methyl-2-pentanone (1); 2, 4-mercapto-4-methyl-2-pentanol (34); 3, 3-mercapto-3-methyl-1-butanol (35); 4, 3-mercaptohexyl acetate (36); 5, 3-mercapto-1-hexanol (37) (structures in Figure 9). (Adapted from reference 52.)



34 **35** **36** **37**

Figure 9. Structures of volatile S-containing constituents of Sauvignon blanc wine. (See also Figure 3. for structure of 4-mercapto-4-methyl-2-pentanone, 1).

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A first indication for the sensory relevance of these compounds was obtained by determining the perception odor thresholds in water and in an aqueous ethanol solution (12 %, v/v). As shown in Table VII, the thresholds differ by several orders of magnitudes.

Table VII. Perception Odor Thresholds of Selected Wine Constituents

Odorant	*Water [μg/kg]*	*Aq. EtOH (12%) [μg/kg]*
4-Mercapto-2-methyl-2-pentanone	0.0001	0.0011
4-Mercapto-4-methyl-2-pentanol	0.02	0.055
3-Mercapto-3-methyl-1-butanol	1.3	1.5
3-Mercaptohexyl acetate	0.0023	0.0043
3-Mercapto-1-hexanol	0.017	0.06

SOURCE: Adapted from references 10, 52, and 53.

The sensory relevance was estimated by calculating the odor activity values OAV, i.e. the ratio of concentration to odor threshold (Figure 10). 4-Mercapto-2-methyl-2-pentanone (**1**) showed the highest OAV followed by 3-mercapto-1-hexanol (**37**). Aroma contribution is due to relatively low odor thresholds and high concentrations, respectively, resulting in OAV of about 15 to 20. In agreement with these results, Sauvignon blanc wines with no or low amounts of odorant **1** were described as not or weakly typical (<9 ng/L), those containing higher amounts as typical (18 ng/L) or very characteristic (34 ng/L) (*54*).

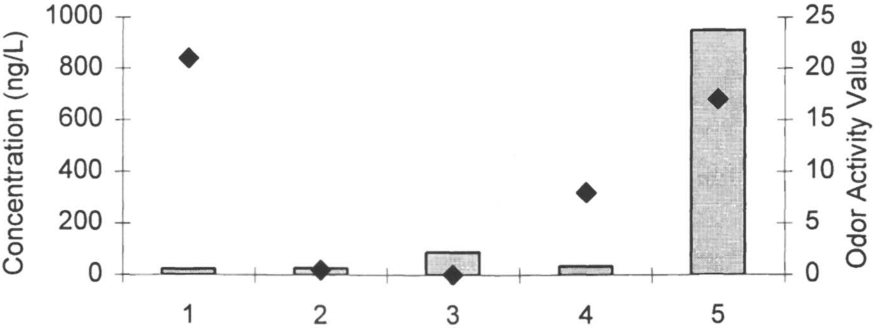

Figure 10. Sensory relevance (♦) of volatile S-constituents of Sauvignon blanc wine as related to concentration (bars): 1, *4-mercapto-4-methyl-2-pentanone (1);* 2, *4-mercapto-4-methyl-2-pentanol (34);* 3, *3-mercapto-3-methyl-1-butanol (35);* 4, *3-mercaptohexyl acetate (36);* 5, *3-mercapto-1-hexanol (37).* (Data are from reference 52.)

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In the Scheurebe wine, four S-containing odorants were identified, i.e. 4-mercapto-4-methyl-2-pentanone (**1**), dimethyl sulfide (**16**), dimethyl trisulfide (**13**), and 3-(methylthio)-1-propanol (*55*). However, only odorant **1** was found to significantly contribute to the overall aroma; moreover, as shown in Figure 11, the OAV was the highest amongst all volatile compounds studied. The threshold values were determined in an aqueous ethanol solution (10%, v/v).

These results suggest odorant **1** is a character impact compound of Scheurebe wines (*56*). This was confirmed in omission experiments. A mixture of 42 odorants quantified in Scheurebe revealed the authentic aroma profile of this wine: similarity was determined to be 3 on a scale from 0 to 3. When odorant **1** was omitted from the model mixture, the similarity dropped to 0.5, thus indicating the significant contribution of this single odorant to the overall aroma.

In Bordeaux red wines, along with 3-mercapto-1-hexanol (**37**) and the respective acetate (**36**), the broth-like smelling odorant 3-mercapto-2-methyl-1-propanol was identified (*57*). Only the (*R*)-isomer is present in these wines. The threshold values of (*R*) and (*S*) enantiomers in water are 5 and 40 μg/L and in an aqueous ethanol solution (10%, v/v) 25 and 120 μg/L, respectively (*58*). The concentrations in young red wines were found to be much higher (25-70 μg/L) as compared to aged red wines (1-4 μg/L) and white wines (1-2 μg/L).

Cooked Meat

The role of S-compounds in meat flavors is well known. The diversity of S-containing volatile compounds in meat flavors is due to thermally induced degradation of sulfur sources, such as cysteine and thiamin, which react with sugar degradation products in the course of the Maillard reaction to generate various chemical classes such as thiols, sulfides, and S-heterocycles. Surprisingly, only a few S-compounds play a major role in the aroma of meat flavors.

In a comprehensive study on stewed beef and pork, Guth and Grosch identified the odorants contributing to the aroma of the juices (*59,60*). They have established a list of odorants obtained by GC-O analysis of the headspace and a solvent extract. Quantification experiments were performed using the IDA technique based on internal standards labeled with stable isotopes. As shown in Table VIII, three S-compounds play a certain role in the aroma profile of stewed meat juices, *i.e.* methanethiol (**15**), methional (**4**) and 2-furfurylthiol (**5**).

To check if all of the important odorants were identified, model aromas consisting of 15 odorants were sensorially evaluated and the sensory profiles compared with those of the authentic meat juices. As shown in Figure 12 for stewed beef, the model aroma was very close to the beef juice. The similarity was found to be 3 on a scale from 0 to 3. In the case of stewed pork with a similarity of 2.5, sensory profiling indicated that the model aroma was too tallowy and roasty, but not fatty enough.

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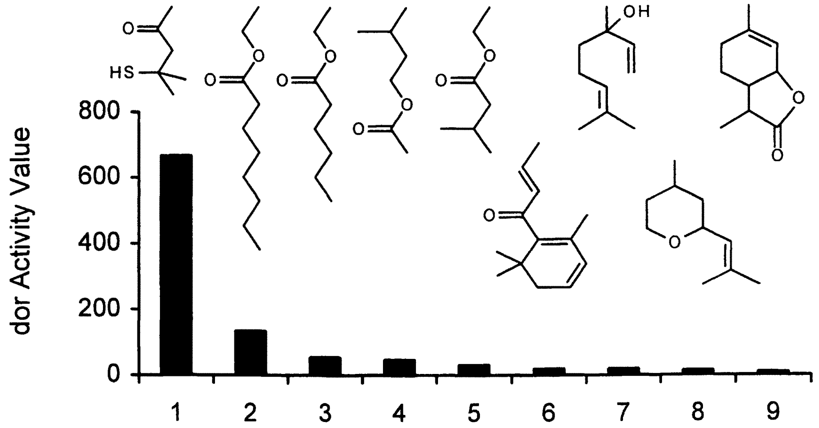

Figure 11. Estimation of the sensory relevance of odorants identified in Scheurebe wine: 1, 4-mercapto-4-methyl-2-pentanone (1); 2, ethyl octanoate; 3, ethyl hexanoate; 4, 3-methylbutyl acetate; 5, ethyl isobutyrate; 6, (E)-β-damascenone; 7, linalool; 8, cis-rose oxide; 9, wine lactone. (Data are from 56.)

Table VIII. Some Key Odorants Found in Stewed Meat Juices

Compound	*Concentration [μg/kg]*		*OAV*	
	Beef	*Pork*	*Beef*	*Pork*
Methanethiol (**15**)	300	500	1560	2500
12-Methyltridecanal	52	< 0.5	520	< 1
4-Hydroxy-2,5-dimethyl-3(2*H*)-furanone (Furaneol)	8000	2700	320	108
Acetaldehyde	6400	1500	256	60
Methional (**4**)	13	23	65	115
(*E*,*E*)-2,4-Decadienal	12	10	60	50
2-Furfurylthiol (**5**)	0.5	0.6	50	60
3-Methylbutanal	10	21	25	53
Sotolone	5	3	17	10
Hexanal	72	15	7	2
Acetic acid	200000	270000	6	8

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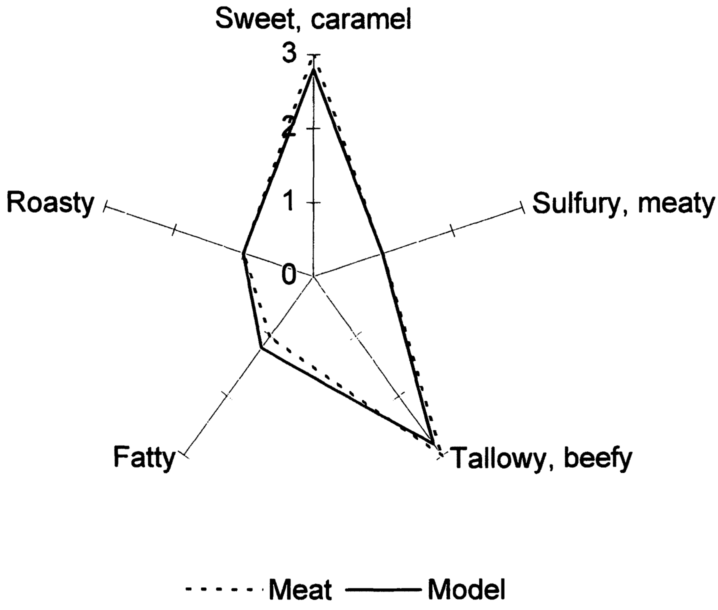

Figure 12. Odor profile of stewed beef juice (solid line) and the corresponding model aroma (dashed line). The juice matrix for sensory evaluation of the model aroma was an emulsion composed of water, coconut oil, gelatin, K_2HPO_4, lactic and glutamic acid. The pH was adjusted to 5.7 with NaOH (1 mol/L). (Data are from reference 60.)

The role of individual odorants in the stewed beef model aroma was studied by omission experiments. As shown in Figure 13, the aroma profile did not change much if 2-furfurylthiol was removed from the model aroma (S1). However, omission of 12-methyltridecanal (S5), 4-hydroxy-2,5-dimethyl-3(2*H*)-furanone (S6), or methanethiol (S7) led to a significant decrease in similarity, thus indicating the essential role of these individual odorants to the overall aroma of stewed beef juice.

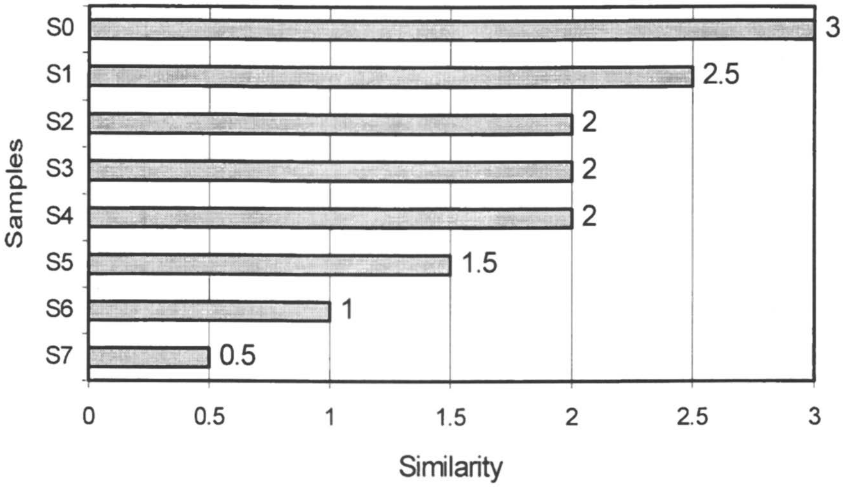

Figure 13. Effect of odorants on the aroma profile of the model mixtures S0 (complete model aroma, 15 odorants), S1 (no 2-furfurylthiol), S2 (no acetaldehyde), S3 (no sotolone), S4 (no acetic acid), S5 (no 12-methyltridecanal), S6 (no Furaneol), S7 (no methanethiol). (Data are from reference 59.)

The interaction of odorants was studied in additional sensory experiments (Table IX) by varying the composition of the model aroma (*59*). Model A was composed of the six odorants that were found to be essential in the omission experiments (Figure 13). Interestingly, the aroma of this model was very different from that of the complete model D (S0 in Figure 13). The sulfury, cabbage-like smell of methanethiol (**15**) was too intense. The predominance of **15** was reduced in model B by the addition of the S-containing odorants **4** and **5**, as well as (*E*,*E*)-2,4-decadienal and 3-methylbutanal. Also, by inclusion of these compounds the model aroma better represented the aroma of stewed beef.

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The aroma profile was further improved by adding diacetyl, resulting in a similarity score of 2.5 in model C. Finally, the round and characteristic aroma of stewed beef juice was obtained by addition of butyric acid (model D). These results also show that odorants with relatively low OAVs can significantly contribute to the overall aroma of a food product.

Table IX. Aroma Recombination Studies on Stewed Beef Meat Juice

Odorant in the model	*OAV*	*Model Aroma*			
		A	*B*	*C*	*D*
Methanethiol (**15**)	1560	+	+	+	+
12-Methyltridecanal	520	+	+	+	+
4-Hydroxy-2,5-dimethyl-3(2*H*)-furanone	320	+	+	+	+
Acetaldehyde	256	+	+	+	+
Sotolone	17	+	+	+	+
Acetic acid	6	+	+	+	+
Methional (**4**)	65	-	+	+	+
(*E*,*E*)-2,4-Decadienal	60	-	+	+	+
2-Furfurylthiol (**5**)	50	-	+	+	+
3-Methylbutanal	25	-	+	+	+
Diacetyl	3	-	-	+	+
Butyric acid	3	-	-	-	+
	Similarity	0	2	2.5	3

SOURCE: Adapted from reference 59.

A similar study was performed to determine the key odorants of boiled beef meat (*9*,*23*). The model aroma of a boiled beef sample with a relatively high fat content consisted of 16 volatile compounds (Table X). Omission experiments revealed that nine odorants contributed significantly to the aroma of boiled beef, including the S-containing compounds **4**, **5**, **6**, and **15**. They can be considered as the key odorants of boiled beef.

In this sample, 12-methyltridecanal did not significantly contribute to the aroma profile in the presence of octanal, nonanal, and (*E*,*E*)-2,4-decadienal, which were responsible for the fatty note. However, in cooked lean beef meat 12-methyltridecanal is a key odorant (*59*) because this compound originates from membranes of muscle tissue and not from the fat depot.

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Table X. Concentrations and Odor Activity Values (OAV) of Potent Odorants of Boiled Beef and Results of Omission Experiments

Odorant (omitted from the model) [a]	*Concentration [µg/kg]*	*OAV* [b]	*Number* [c]
2-Furfurylthiol (**5**)	29	2900	10***
4-Hydroxy-2,5-dimethyl-3(2*H*)-furanone	9075	908	10***
3-Mercapto-2-pentanone (**6**)	69	99	9***
Methanethiol (**15**)	311	1555	9***
Octanal	382	546	8**
2-Methyl-3-furanthiol (**2**)	24	3429	8**
Nonanal	1262	1262	8**
(*E*,*E*)-2,4-Decadienal	27	135	8**
Methional (**4**)	36	180	7*
12-Methyltridecanal	962	9620	6
Dimethyl sulfide (**16**)	105	350	5
(*Z*)-2-Nonenal	6.2	310	4
Acetaldehyde	1817	182	4
1-Octen-3-one	9.4	188	3
(*E*)-2-Nonenal	32	128	3
Methylpropanal	117	167	1

a Odorant omitted from the model aroma containing the compounds listed in the table. The base of the model consisted of 10% sunflower oil in a phosphate buffer of pH 5.7.

b OAVs were calculated on the basis of odor threshold values in water.

c Number of 11 assessors detecting the reduced model in triangle tests with significance * ($P < 0.05$), ** ($P < 0{,}01$), and *** ($P < 0.001$).

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Coffee

Coffee aroma is another example that demonstrates the role of S-containing odorants. The following compounds have been reported as characteristic volatile constituents of roast and ground coffee aroma (*18,61*): 2-methyl-3-furanthiol (**2**), methional (**4**), 2-furfurylthiol (**5**), 3-methyl-2-buten-1-thiol (**10**), 3-mercapto-3-methylbutyl formate (**12**), dimethyl trisulfide (**13**), and methanethiol (**15**). The amounts of these odorants in coffee aroma were found to be well above threshold concentrations (*62*).

Omission tests revealed 2-furfurylthiol (**5**) as an outstanding S-containing odorant of a typical roast and ground coffee aroma (*33*). The absence (Exp.1, Table XI) lowered the sulfurous/roasty note so that 15 of 20 assessors detected a difference between the complete and reduced model aroma. The important role of odorant **5** established in the omission experiments confirmed the assumption of Reichstein and Staudinger that 2-furfurylthiol is a key compound of coffee aroma (*32*). However, its aroma impact was much lower in coffee beverages due to lower yields of this compound achieved by the brewing process (*63*).

The omission of other S-containing odorants, individually (Exp. 2-4) or in combination (Exp. 5), was detected by only 10 assessors. This detection level was below the confidence limit of 95 %, thus suggesting that these compounds are not likely essential odorants of coffee aroma. On the contrary, certain pyrazines, phenols, cyclic enolones, diones, and Strecker aldehydes are important constituents of a well-balanced coffee aroma (*33*).

Table XI. Aroma of the Roasted Arabica Coffee Model as Affected by the Absence of S-containing Compound

Exp	*Compound(s) omitted*[a]	*Number*[b]
1	2-Furfurylthiol (**5**)	15[c]
2	2-Methyl-3-furanthiol (**2**)	10
3	3-Mercapto-3-methylbutyl formate (**12**)	10
4	3-Methyl-2-buten-1-thiol (**10**)	10
5	Odorants **2**, **10**, **12**, methional (**4**), dimethyl trisulfide (**13**), methanethiol (**15**)	10

a Odorant(s) omitted from the model aroma containing 27 compounds. The base of the model consisted of sunflower oil and water (1:20, v/v).

b Number of correct answers in triangle tests (maximum answers: 20).

c Significant aroma difference ($P < 0.05$) between the complete and reduced model.

SOURCE: Adapted from reference 33.

Structure-Odor Activity Relationships

Certain thiols (Figure 14) have particular structural features that elicit a catty type odor, which often is also described as black currant-like, foxy, grape fruit-like, buchu leaves-like, etc. The most typical catty note is shown by the odorants **1**, **26**, **39**, and **41** (*64*). The character impact odorant of tomcat's urine, however is thought to be 3-mercapto-3-methyl-1-butanol (**40**) (*65*).

Most of the odorants shown below have similar, but not identical odor characteristics. Compounds **3**, **26**, and **37** have tropical/fruity undertones, odorants **14**, **35**, **42**, and **43** elicit savory/meaty notes, whereas **10** and **24** show a more foxy/skunky character, particularly at higher concentrations. Some of these odorants can be considered as character impact compounds, e.g. **3** for grapefruit (*19*), **11** for black currant (*35*), **14** for onions (*37*), and **26** for buchu leaves (*46*).

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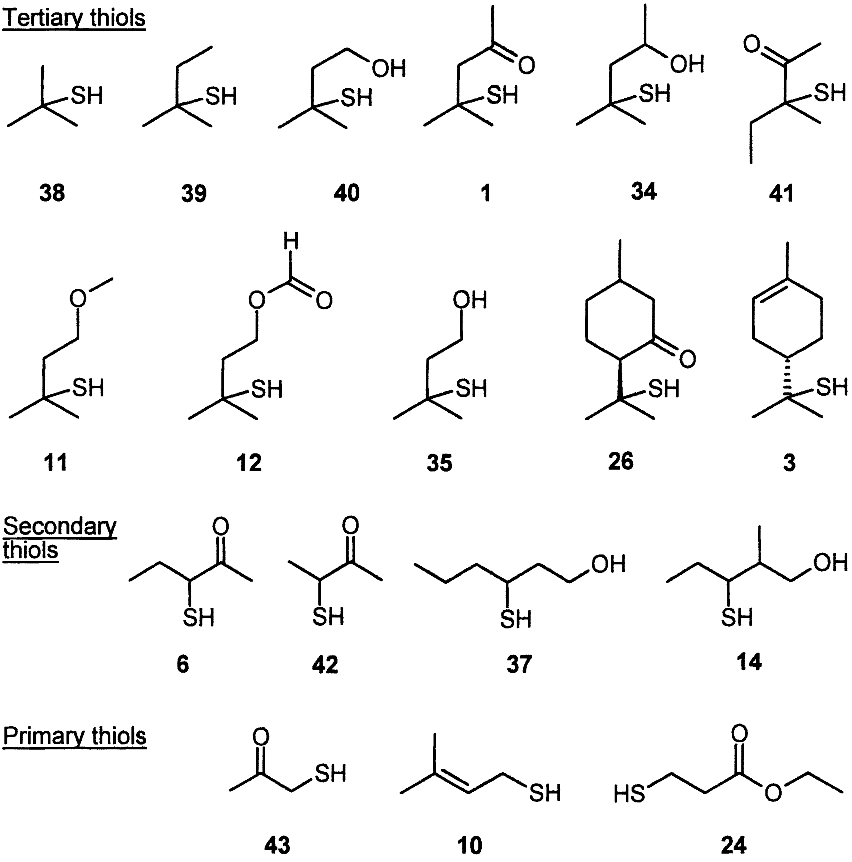
Tertiary thiols
SH
SH
OH
SH
O
SH
OH
SH
O
SH
38
39
40
1
34
41
O
SH
H
O
O
SH
OH
SH
O
SH
SH
11
12
35
26
3
Secondary thiols
O
SH
O
SH
OH
SH
OH
SH
6
42
37
14
Primary thiols
O
SH
SH
O
HS
O
43
10
24

Figure 14. Examples of thiols that have particular structural features which elicit common odor-activity relationships.

According to Polak et al. (*64*), the tertiary mercapto amyl structure is the most characteristic feature shared by the majority of the compounds with the catty odor. The superimposibility of the carbon skeleton and the presence of a tertiary SH group appear to be sufficient for odor similarity. Exemptions are compounds **6** and **10**, which show typical catty notes, but are secondary and primary thiols, respectively.

The presence of a carbonyl group is not essential for the catty note. It makes little difference in odor quality whether the SH group is in the α (**41**) or β (**1**) position to the keto function. The estimated distances between SH and C=O of

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about 2-4 Å do not differ markedly either in the eclipsed or staggered conformers. The α- and β- mercapto ketones might therefore both be present in conformations in which the functional groups overlap and accommodate the same receptor sites.

The most potent representatives of this group are compounds **1**, **3**, **10**, **11**, **12**, and **39**. Odor thresholds also depend strongly on structure. As shown in Table XII, tertiary thiols have lower threshold values as compared to primary and secondary thiols (*66*). Derivatization of the thiol group usually leads to an increase in threshold values.

Table XII. Odor Thresholds of S-Containing Compounds in Relation to Structure

Compound	*Structural Feature*	*Odor Threshold [µg/L beer]*
1-Butanethiol	primary SH	0.7
2-Butanethiol	secondary SH	0.6
2-Methyl-1-propanethiol	primary SH	2.5
2-Methyl-2-propanethiol (**38**)	tertiary SH	0.08
3-Methyl-2-butanethiol	secondary SH	0.2
2-Methyl-2-butanethiol (**39**)	tertiary SH	0.00007

SOURCE: Adapted from reference 66.

Off-Flavors

Sulfur containing odorants not only contribute to the characteristic aroma of a food, but can also lead to off-flavors. Food-born off-flavors occur by different causes. For example, the loss of key odorants or formation of intrinsic or new potent odorants can result during processing and/or storage, which will alter the characteristic aroma balance of the food.

Many S-compounds contribute importantly to the characteristic aroma of a food. However, in case of component overdose the overall aroma turns to off-notes. This has been reported for the foxy smelling ethyl-3-mercaptopropanoate (**24**) in Concord grape (*44*). In coffee, too high concentrations of 3-mercapto-2-buten-1-thiol (**10**) or 3-mercapto-3-methylbutyl formate (**12**) will also alter the typical coffee aroma.

Another source of off-flavors is the generation of S-compounds during processing or storage. Well-known examples are 3-mercapto-2-buten-1-thiol (**10**) in beer (*67*) or 4-mercapto-4-methyl-2-pentanone (**1**) in packaged meat-based foods (*68*). Compound **10** is formed by light-induced radical reactions of

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beer constituents (Figure 15). Isohumulones decompose to 3-methyl-2-butenyl radicals whereas SH radicals stem from decomposition of S-containing amino acids and proteins in riboflavin-photosensitized reactions (*69*).

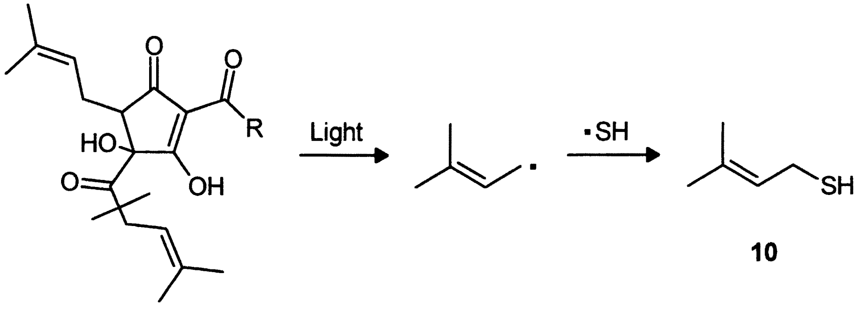

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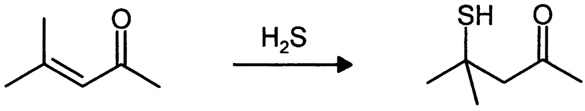
Figure 15. Formation of 3-mercapto-2-buten-1-thiol (10) responsible for the sunstruck off-flavor of beer. (Adapted from reference 69.)

As shown in Figure 16, odorant **1** can be formed from mesityl oxide and hydrogen sulfide originating from a packaging material and cooked meat, respectively (*70*). A well-known example for the formation of this off-flavor in chilled cooked meat stored in plastic bags is cooked ham (*71*).



1

Figure 16. Formation of 4-mercapto-4-methyl-2-pentanone (1) from mesityl oxide and hydrogen sulfide, responsible for the catty off-note in certain cooked meat products (Adapted from reference 70.)

The fishy off-flavor formed in dry spinach upon storage was shown to come from two compounds present within a certain concentration range, *i.e.* (*Z*)-1,5-octadien-3-one and methional (*72*), having odor thresholds of 0.001 μg/L and 0.2 μg/L water, respectively. An aqueous solution containing 0.16 μg/L of the octadienone and 10 μg/L of methional showed a pronounced fishy odor. The

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geranium-like and potato-like notes of the individual odorants were less intense. These odorants may cause fishy off-notes in many different food products as they are readily formed by lipid oxidation of unsaturated fatty acids and *Strecker* degradation of methionine, respectively. However, they can also contribute to the natural fishy odor of cooked seafood as recently demonstrated for boiled cod (*73*).

Conclusions

While the occurrence of sulfur compounds is of general interest, the sensory properties of these compounds can be critical to the flavor of a food. Recent analytical methods based on sensory directed chemical analysis in combination with aroma recombination studies and omission tests offer an attractive approach to evaluate the sensory relevance of S-compounds to food flavor. Sensory experiments are very important and should be seen as an integrated part of flavor research. As shown in this paper, S-containing organic volatile compounds play an important role in many food flavors and can be essential for a characteristic note. However, a lack of these odorants or an overdose can lead to the formation of off-flavors.

Acknowledgement

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Chapter 3

Generation of Sulfur Flavor Compounds by Microbial Pathways

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The biogeneration of sulfur compounds is an excellent model for illustrating the complexity of flavor production by microbial pathways. This paper will concentrate on the precursors of sulfur-containing flavor compounds. We will focus are discussion on sulfur compounds more commonly found in nature; such as, sulfates and sulfites, amino acids and peptides, and finally we will discuss the importance of acyl CoAs in thioester synthesis.

The fermentation of crops is one of the oldest systems used by mankind to preserve foods and beverages. The use of microorganisms has also led to the diversification of products obtained yielding wines, beers and cheeses. In the industrial context of food products, flavor control is essential for producing attractive fermented foods and beverages. Not only is the understanding of

In Heteroatomic Aroma Compounds; Reineccius, G., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2002.

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microbial actions essential for this objective, but knowledge of the pathways involved may lead to the production of new commercially available natural flavor molecules.

In the traditional cottage industry of fermented foods, flavor control was obtained by trial and error. For many producers, this is still true for beer, wine, cheese, soy sauce and many other fermented foods. A more deterministic procedure is necessary for industrial processes in order to successfully develop new products.

The generation of flavor is a complex process in fermented foods because of the diversity of precursors, pathways, and flavor compounds produced. Flavor control and variation is also challenging due to the limited number of parameters that can be changed by a food technologist without adversely affecting other qualities of the food product.

There are four major factors involved when attempting to control microbial flavor generation: 1) The microorganisms and the enzymatic systems involved must be identified, 2) exchanges between microbial cells and the medium need to be characterized as this is important for precursor uptake and the flavor compounds sought, 3) direct precursors pathways must be identified, and 4) to avoid taints and to generate a typical flavor, the optimal environment should be known.

Sulfates and Sulfites

The understanding of sulfate assimilation (Figure 1) is the key to understanding the accumulation of volatile sulfur compounds by yeast in the brewing process. The enzyme sulfate permease is responsible for sulfate uptake. Two different enzymes with this function have been isolated from *Neurospora crassa,* in which sulfate uptake tightly controlled *(1).* Proceeding sulfate uptake, the second step is the incorporation of sulfate into adenosine phosphosulfate (APS). This step is tightly controlled at two levels. First, the reaction is controlled at the transcription level and secondly, the reaction can be controlled at the activity level where the enzyme is allosteric (it is inhibited when reaction products, such as phosphoadenosyl phosphosulfate (PAPS) accumulate).

The phosphorylation of APS to PAPS is also tightly controlled at the transcription level where PAPS is then reduced to sulfites. This step is much less tightly controlled, giving rise to the transient accumulation of SO_2 in the course of beer fermentation. This SO_2 combines primarily with aldehydes which can limit acetaldehyde reduction *(3).* The only advantage of SO_2 accumulation in beer is the relative stabilization of the beer. The reduction of SO_2 to H_2S is

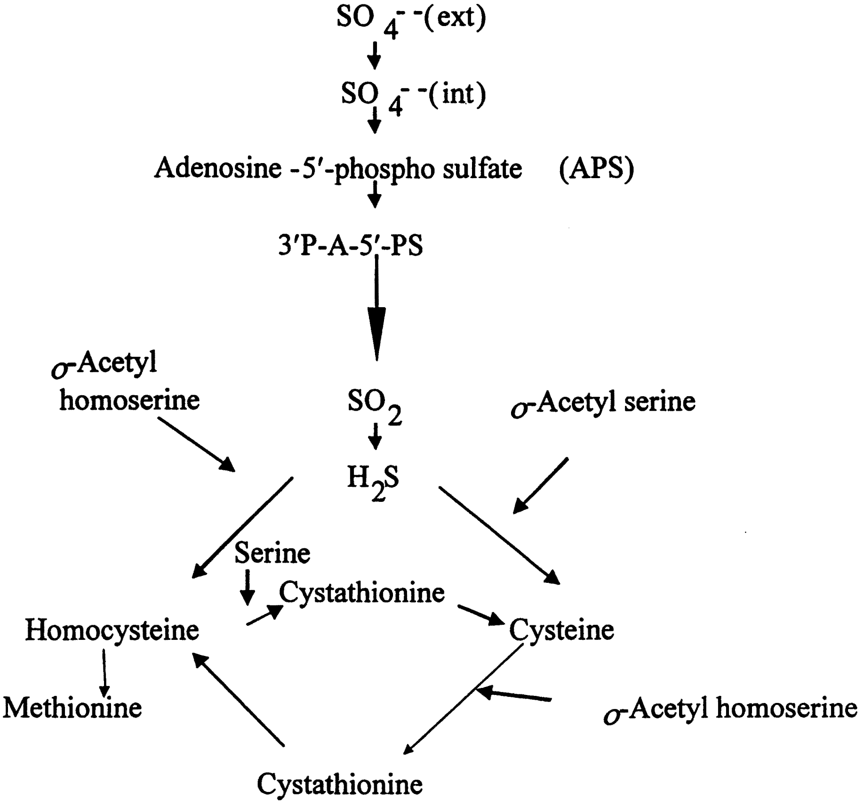
SO 4-- (ext)
SO 4-- (int)
Adenosine -5'-phospho sulfate (APS)
3'P-A-5'-PS
σ-Acetyl homoserine
SO2
σ-Acetyl serine
H2S
Serine
Cystathionine
Homocysteine
Cysteine
Methionine
σ-Acetyl homoserine
Cystathionine

Figure 1. Reduction of sulfate by Saccharomyces cerevisiae (2).

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poorly regulated and H_2S can also accumulate, but amounts of H_2S formed depend on the yeast strain used.

Nitrogen depletion in wine fermentation may favor these accumulations *(4)*. This may occur with certain grape varieties for example in Riesling, Chardonnay, and Syrah varieties *(5)*. The concentration of H_2S decreases with storage time. Finally, H_2S is incorporated into hydroxy-amino acids, such as acetyl homoserine, yielding homocysteine and L-methionine by methylation. Also, acetyl serine may be methylated to yield cysteine.

A cystathionine synthesis cycle controls equilibria between the synthesis of both sulfur amino acids. Cystathionine can be converted to cysteine and α-aminobutyrate with γ-cystathionine lyase or to homoserine and α-amino propionate with β-cystathionine lyase. This enzyme will be further discussed later in this text.

Amino Acids and Peptides

Amino acids and peptides are also efficient precursors of many volatile compounds. This is the case for L-cysteine, L-methionine and glutathione. We will focus on these three most common precursors.

Cysteine

Cysteine can be degraded via three different pathways. Cysteine can be decarboxylated to yield cysteamine *(6)*, deaminated to yield α-keto-3-thiopropionic acid and cysteine (as wells as yields α-keto-3-thiopropionic acid) is easily degraded to H_2S.

In addition, cysteine reacts readily with carbonyl groups and, as in *Boletus edulis* or toasted cheese, can yield 3,5-dimethyl-1,2,4-trithiolane *(7)*. The same mechanism may lead to the synthesis of 1,2,4-trithiolane or to lenthionine, responsible for the typical flavor of shiitake mushrooms (*Lentinus edodes*) *(8)*.

H_2S is a significant byproduct of cysteine degradation. In alcoholic beverages, H_2S from cysteine is produced in lager beers but much less in ales *(9)*. H_2S has an olfactory detection threshold at the ppb level and can reduce the flavor quality in wine and beer, and even impart the smell of rotten eggs. H_2S can react with CO_2 and ethanol to form carbonyl sulfide and ethyl sulfide. In addition, two carbonyl sulfide units can react to form CS_2 and CO_2, which occurs in lager beers. Since H_2S is present only in lager beers, ethyl sulfide is present only in this type of beer (0.34 to 0.4 μg/l). Ethyl sulfide can then be oxidized to diethyl disulfide *(10,11)*.

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Amyl mercaptan, detected in 1982 by Haboucha *(12)*, can be produced from amyl alcohol by the same mechanism (Figure 2). In beer, isohumulenes in hops are sensitive to photooxidation and can release 2-methyl and 2-butene radicals. These radicals can react with HS• radicals to yield 3-methyl-2-buten-1-thiol (MBT). This compound, in alcoholic solutions, has a very low threshold of olfactory detection (1µg/l). However, below the concentration that gives the characteristic taint, MBT may contribute positively to a typical beer flavor.

L-Methionine

L-Methionine can first be degraded via the Ehrlich-Neubauer pathway (Figure 3). A phosphopyridoxal-dependant transaminase yields α-keto-γ-methylthiobutyric acid (KMBA). This compound is first decarboxylated to methional which can then be reduced by alcohol dehydrogenase to methionol, or in some cases, to methanethiol and propionaldehyde. KMBA can also be oxidized to 3-methylthio-propionic acid.

Methionol and methionyl acetate are the major L-methionine breakdown products in *S. cerevisiae.* These two compounds are found in wines *(13)* but the functional roles are unclear. The bacterial species *Erwinia carotovora* can produce 0.9 mmol/l of methionol mixed with about 3 mmol/l of its acetate ester with a yield of 62% in 30 hours. This mixture has a typical mashed potato flavor *(14)*.

The most important aroma compound in the Ehrlich-Neubauer pathway is methional. It participates in the flavor of most fermented foods and is important for the typical taste of cheeses, especially cheddar *(15)*. Methional can also cause defects in beer, for example, it gives a worty flavor to alcohol–free beer *(16)*. Using GC-sniffing methods, these authors identified an intense flavor in the wort extract at a specific retention time. This intense flavor zone is also found in low alcohol beers but disappears in extracts of lager beers. This was explained by the process that in cold contact fermentation used to make low alcohol beer, methional is not reduced to methionol because the aldehyde function can be blocked by sulfites and also because of interactions with flavonoids *(17)*.

Methional can be reduced to methanethiol. This molecule plays a key role in the biosynthesis of many important sulfur compounds. Several compounds may be precursors of methanethiol, such as, methional and S-methylcysteine, but the main pathway is via methionine degradation by lyase activities.

There are several possibilities for S-terminal degradation (Figure 4). A first possibility involves deamination by a deaminase to produce ammonia. This pathway does not seem to be very common. At the moment, two main pathways for S-terminal degradation have been reported. One was discovered in *E. coli* in

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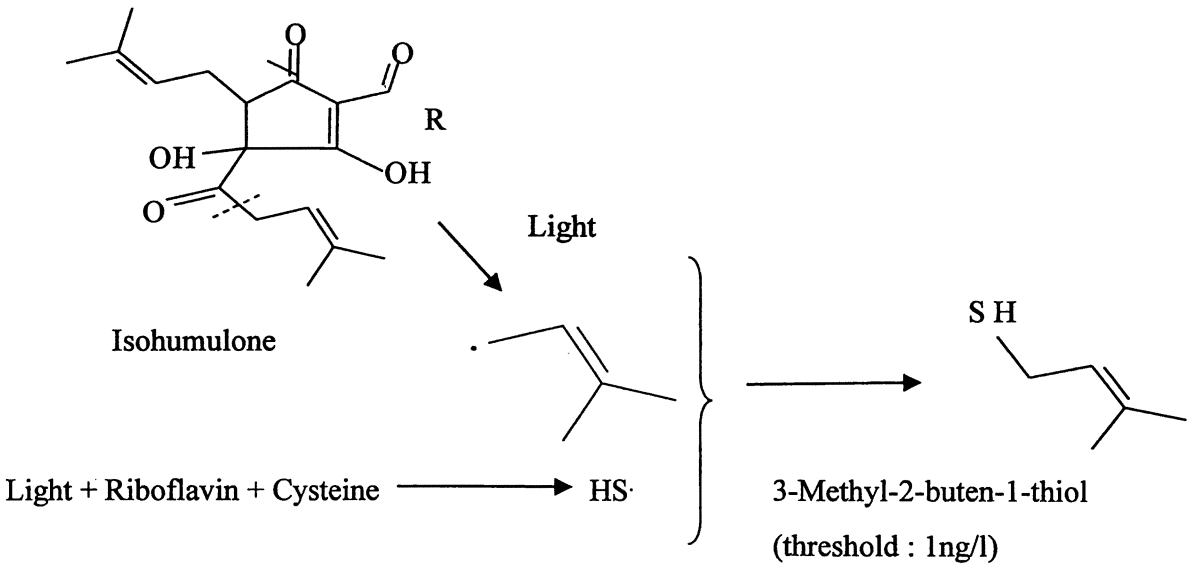
O
O
R
OH
OH
O
Light
Isohumulone
S H
Light + Riboflavin + Cysteine
HS·
3-Methyl-2-buten-1-thiol
(threshold : 1ng/l)

Figure 2. Formation of sunstruck flavor in beer (11).

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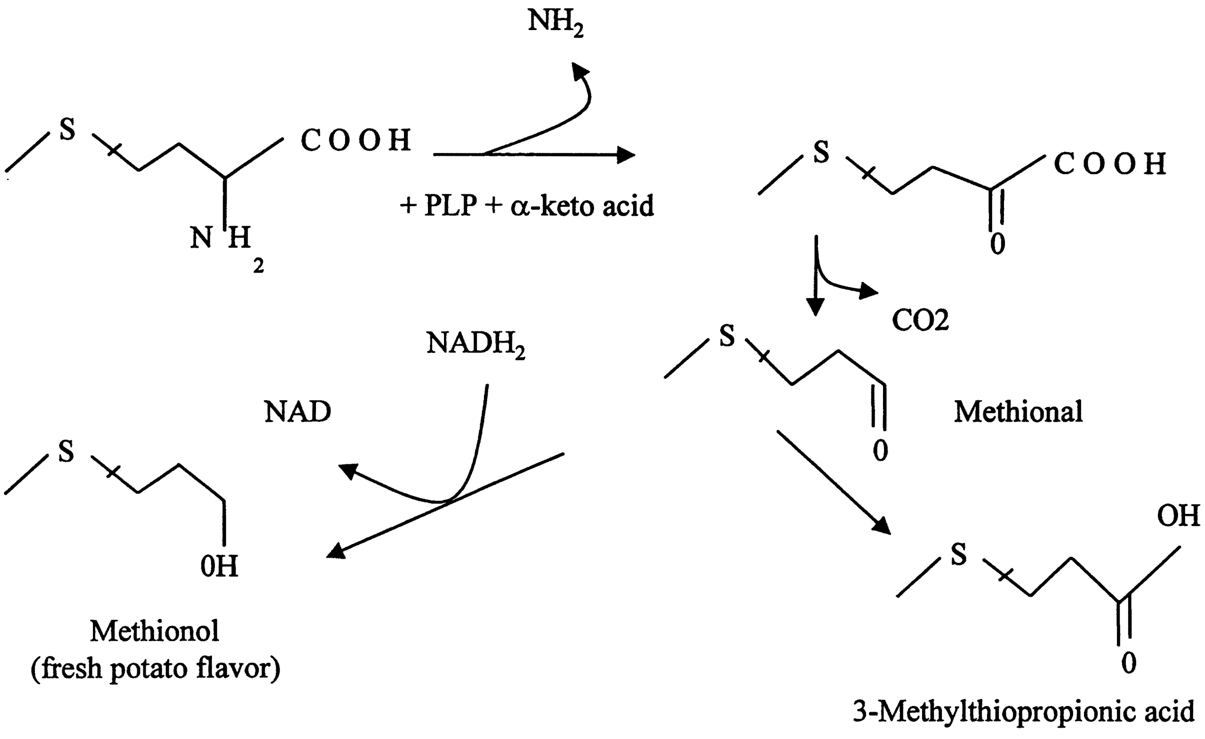
S
COOH
NH2
NH2
+ PLP + α-keto acid
S
COOH
0
CO2
S
0
Methional
NADH2
NAD
S
0H
Methionol
(fresh potato flavor)
OH
S
0
3-Methylthiopropionic acid

Figure 3. Scheme of the Ehrlich-Neubauer pathway.

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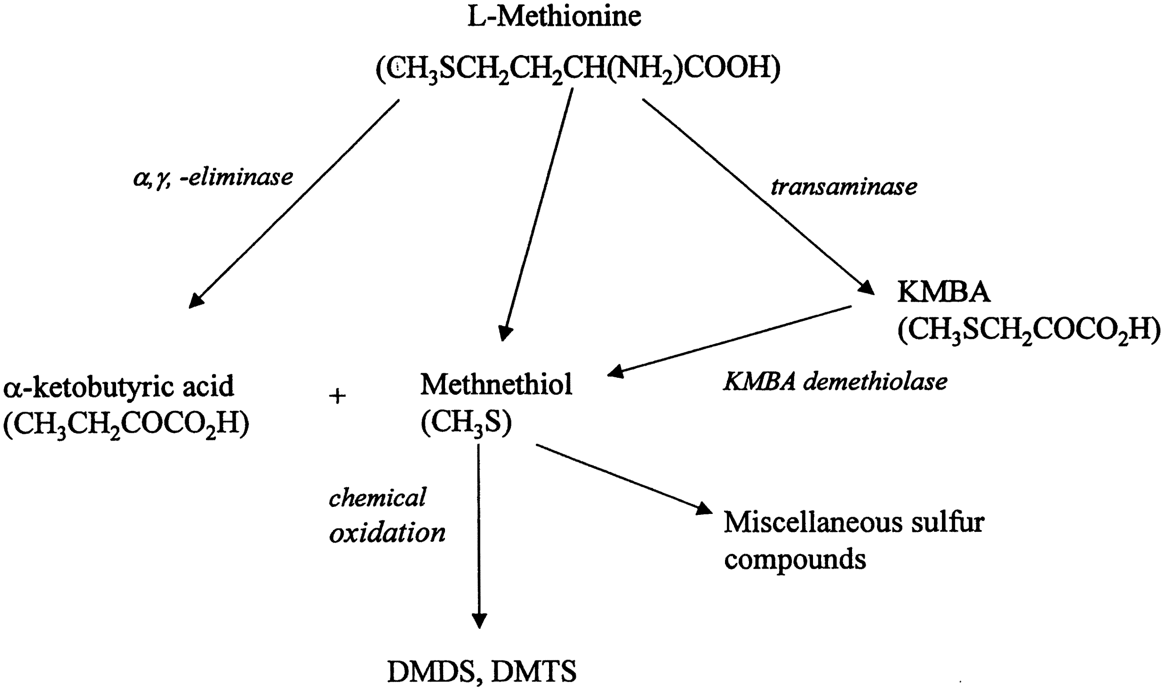
L-Methionine
$(CH_3SCH_2CH_2CH(NH_2)COOH)$
α,γ, -eliminase
transaminase
KMBA
$(CH_3SCH_2COCO_2H)$
α-ketobutyric acid
$(CH_3CH_2COCO_2H)$
+
Methnethiol
(CH_3S)
KMBA demethiolase
chemical
oxidation
Miscellaneous sulfur
compounds
DMDS, DMTS

Figure 4. Methionine degredation via γ-lyases.

1938 by Ornitake *(18)* and is also found in *Brevibacterium linens,* an efficient methanethiol producer used by cheese technologists *(19, 20).* This microbial pathway degrades L-methionine by a simultaneous α and γ elimination, producing α-ketobutyric acid, methanethiol and ammonia (this system is also used by many other organisms) *(21).* The gene encoding for this protein was first sequenced from the DNA of *Pseudomonas putida (22).* It was shown that the C-S bound in the β or γ position from the carboxylic function of different amino acids can be used as substrate by this enzyme *(23).*

The second route, largely used by microorganisms, is a two-step process *(24).* The first step produces KMBA by the transamination of L-methionine. In many cases this is the limiting step. KMBA is then demethiolated. This pathway is used by many cheese microorganisms. Methanethiol can then produce a much broader range of compounds.

Spontaneous oxidation of methanethiol yields DMDS, DMTS and dimethyl tetrasulfide, the latter having the lowest detection threshold in water (0.06 ppb). Methanethiol combines with acids or acyl CoA to produce methyl thioesters and with aldehydes to yield thioacetals. These pathways will be illustrated with examples taken from cheese technology because cheeses are a popular field of research for microbiologists and biochemists.

The development of cheese flavor occurs as the successive microflora in the cheese evolve. For example, typical Brie flavor is due to the successive growth of lactic acid bacteria, yeast (*Geotrichum candidum)* and *Penicillium camemberti.* In the case of Munster cheese, the successive flora will be lactic acid bacteria, yeast, *Geotrichum* and coryneform bacteria.

The job of the cheese technologist is to prepare the correct microbial formulation to obtain the desired flavors. A technologist does not act directly on flavor formulation, but rather on the catalysts that will generate flavors from proteins and fats. Many edible microorganisms can produce methanethiol. This is the case for: *Coryneform bacteria (25, 26, 27)*, *B. linens*, *B. acetylicum (19, 23)*, *Micrococcus* sp., *Staphylococcus equorum* and *S. lentus (26, 28) Pediococcus (29)*, *Propionibacterium (30)*, *Lactococci (27, 31, 32, 33)*, *Lactobacilli (23)*, and *G. candidum (34).*

Ammonia elimination is a key step determining the rate of production of cheese flavor and has been clearly shown in lactic acid bacteria. When an amino acceptor, such as α-ketoglutarate (α-KG) is added to a model medium or a cheese curd, the rate of flavor production increases *(35).* This is true when *Lactococci*, *Lactobacilli*, *Streptococci*, *Propionibacteria* or yeasts are used.

It has been shown that two major amino transferases are used in *Lactococcus lactis*; a branched chain amino transferase (BCAT) *(36)* and an aryl amino transferase (ARAT) *(37)*, and both enzymes are active towards L-methionine. Interesting results have recently been obtained using genetically modified strains of *Lactococcus lactis.* The genes coding for ARAT and BCAT

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were recently cloned and sequenced by the group of Gripon *(37, 38)*. The genes were deleted to produce a BCAT⁻ strain *(38)*, an ARAT⁻ strain, *(39)* and a strain lacking both genes *(40)*.About half the L-methionine is degraded by each of these amino transferases. The double mutant cannot catabolize L-methionine (Figure 5).

There exists an alternative to the addition of α-KG to cheese curd to improve ripening kinetics. This involves generating the compound from glutamic acid, which is very common in cheese, by use of a genetically modified lactic acid bacteria containing an exogen gene that codes for glutamate dehydrogenase. This possibility was recently published by Rijnen et *al.* *(41)* and is efficient on L-leucine and L-phenylalanine but apparently not on L-methionine.

The second step of L-methionine degradation involves a lyase. The lyase used by *B. linens* yields methanethiol directly from L-methionine, but cannot attack KMBA. Two other lyases have been detected in *Lactococci*. This microorganism uses a cystathionine-β-lyase in L-methionine production. This enzyme can also generate methanethiol from L-methionine, but the activity is 10 times lower than with its normal substrate, cystathionine *(31)*. It was recently shown in *Lactococcus lactis* that the MetC gene encodes for the cystathionine β-lyase, but this enzyme is not the only lyase involved in methionine demethiolation *(42)*. Also in *Lactococci* a cystathionine-γ-lyase has been shown to be involved in cysteine synthesis, and also shows reduced activity with L-methionine compared to its normal substrate *(33, 43)*. Recent results obtained with *Geotrichum* and other microorganisms suggest the participation of another undetermined enzymatic system that uses KMBA as a major substrate *(44)*.

Concerning soft cheese technology, yeasts are the microorganisms that proliferate after lactic acid bacteria. They are resistant to low pH and can consume lactate. Most yeast can produce MTL and its oxidation product, DMDS *(44)*. Figure 6 shows the capabilities of resting yeast cells to produce DMDS from L-methionine. In the case of *Kluyveromyces lactis,* for example, the quantity of DMDS produced is 4,000 times its sensory threshold in oil.

Other compounds can be produced by resting cells. In resting cells, MTL and DMTS are directly correlated with DMDS production. This is not the case for DMS. We have shown that *G. candidum* probably produces DMS by a pathway different than that of MTL, DMDS and DMTS *(45)*. The production of methylthioacetate (MTA) is less common. It was shown that among yeasts tested, *Geotrichum candidum* is the only species that can produce this compound (Figure 6).

An interesting point concerning *Geotrichum candidum* is that even though it grows very early in the cheese ripening process, it can dramatically change the typical flavor of a cheese produced. Molimard *et al.* *(46)* produced Camembert cheeses with different *Penicillium/Geotrichum* combinations and used sensory

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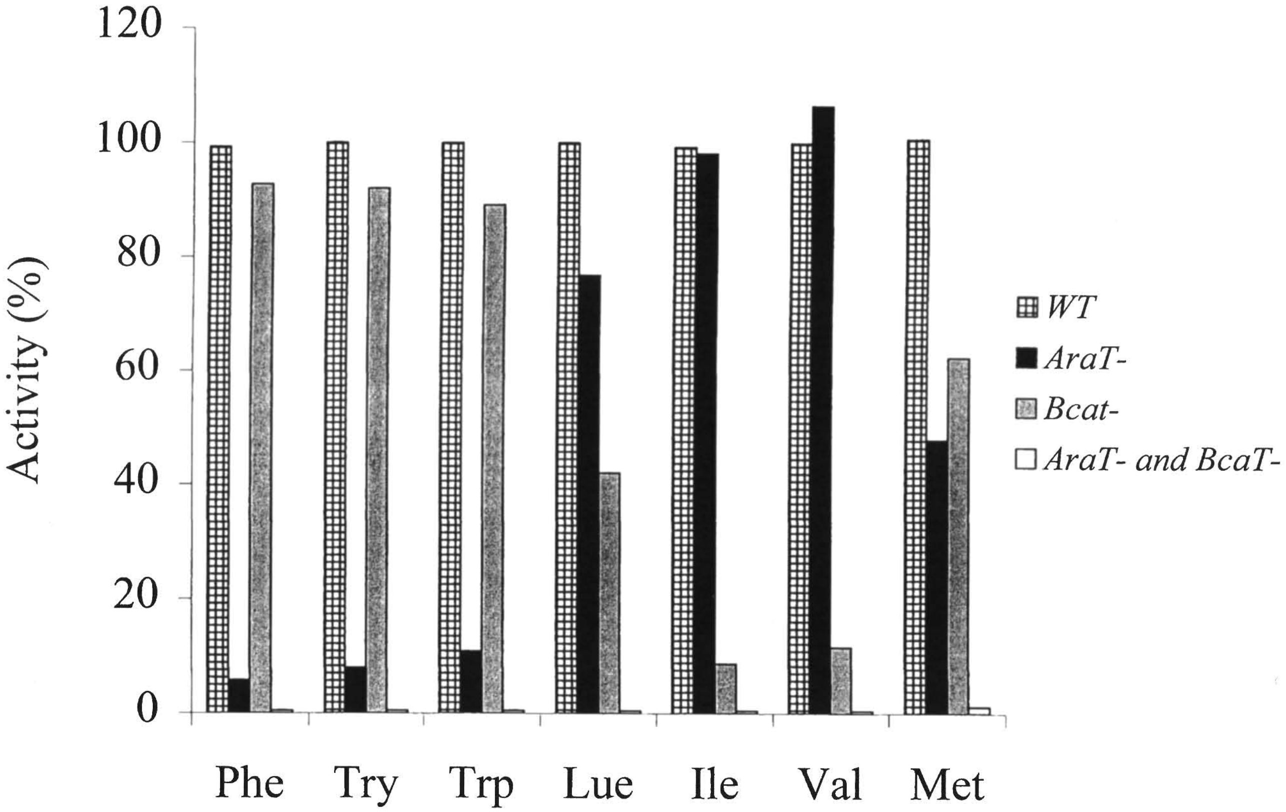

Figure 5. Role of AraT$^-$ and BcaT$^-$ in the transamination of amino acids in vitro.

analysis to characterize them. It was clearly shown that specific strains of *Geotrichum* could change the typical flavor cheeses compared to that produced with only *Penicillium*. For example, one strain developed creamy flavors with the four strains of *Penicillium camemberti* and another could develop cabbage flavor, and a stable-like flavor that was reminiscent of raw milk Camembert cheeses. This work shows that there is a large variety of expression of L-methionine degradation by strains of *Geotrichum.*

A variety of volatile sulfur compounds was observed in *Geotrichum candidum*, especially MTL and MTL the derivatives DMDS, and DMTS (Figure 7) *(34)*. The strategy for developing a rapid screening method is not easy because the expression of a metabolic pathway in pure culture is required, and the pathway of interest must be active in the technological conditions used (complex medium, presence of several cheese ripening microorganisms). Also, cheese ripening bacteria other than *B. linens* can produce sulfur compounds, which is the case for *Arthrobacter* species *(44)*.

Methylthio esters, and particularly methylthio acetate (MTA), are very common in fermented foods. In lager beer the concentration of MTA is between 4 and 31 ppb but is practically undetectable in ales *(47)*. This indicates the important role of redox potential for the production of sulfur compounds. Methyl thioesters are present in wines and cheeses and ethyl thioesters are present in wines and beers, but at concentrations 10 times lower than the methyl thioesters *(48)*

Since relatively few publications have reported the sensorial or physical properties of methyl thioesters, a new method was developed to compare the flavor properties of these compounds. A combined approach associated with GC sniffing methods to obtain sensory characteristics *(49)*, and HPLC methods to determine the degree of lipophilicity was used *(50)*. A library of thioesters was first synthesized. The odors of these compounds were evaluated by sniffing a sequence of serial dilutions by GC-sniffing methods. The contribution of each thioester to flavor (flavor efficiency) was compared by developing the concept of BEGC loads (best estimation of lower amount detected by sniffing) at the GC sniffport. This method does not determine threshold values since the concentrations of the compounds are not precisely known at the GC sniffport; however, it can be used to compare the sensitivity of the nose for an array of compounds at about the same concentration. This approach offers several advantages in that it is rapid, limits problems of impurities, qualifies the odors of the compounds, and provides quantitative values to compare using olfactory potency by BEGC loads and retention properties in food matrices by lipophilicity .

Screening of flavor producing capabilities in *Geotrichum candidum* demonstrated that only four out of 10 strains could produce thioesters. The major thioesters produced were methylthio acetate (MTA), methylthio

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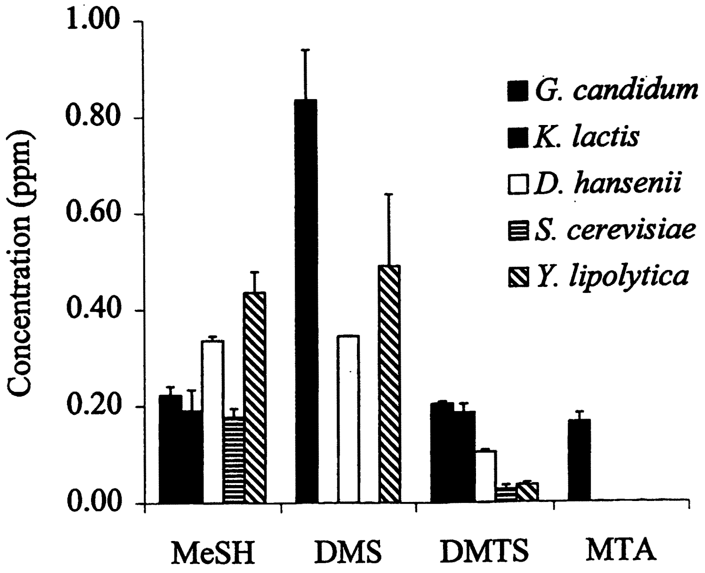

Figure 6. Production of sulfur compounds in resting cells (methanethiol(MTL), dimethyldisulfide (DMS), dimethyldisulfide (DMDS), dimethyltrisulfide (DMTS), methylthio acetate (MTA)).

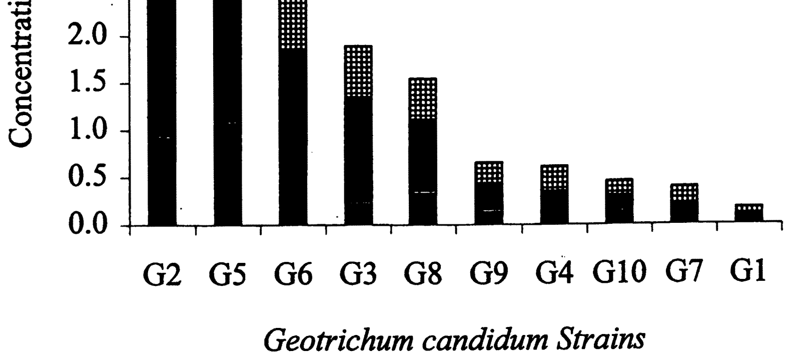

Figure 7. Sulfur compounds formed by oxidation of methanethiol (MTL), dimethydisulfide (DMDS) and trimethyltrisulfide (DMTS)(34).

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isovalerate (MTIV), methylthio isobutyrate (MTIB), methylthio propionate (MTP) and methylthio hexanoate (MTH) *(34)*. These compounds were produced at the end of the growth phase and are thus, typically primary metabolites, like esters. This suggests that synthesis could be related more to activated acids than to the direct acid concentrations. Other microorganisms can produce thioesters, such as, *Arthrobacter sp.*, *B. linens*, *C. glutamicum* and *Micrococcus luteu* (Figure 8) *(24)*. Cell free extracts (CFE) of *G. candidum* could not transform acids into thioesters directly. Acyl CoAs were efficient precursors and the chemical substitution of CoA by methanethiol was very efficient. Consequently, the possible role of an enzyme was unclear.

To test the role of the enzyme, an experiment was conducted that involved combining acetyl CoA and methanethiol, acetyl CoA, methanethiol and CFE and acetyl CoA, methanethiol and thermally degraded CFE. The results of this study showed that the synthesis of MTA was significantly improved by CFE but not by denatured CFE *(51)*. Temperature was the main factor influencing the rate of production. The rate of the chemical reaction increased up to 56°C. A significantly higher rate was observed with CFE between 25 and 45°C. The pH range of 7.0-7.5 was optimal for the reaction. At these pH values, the biological reaction was twice as efficient as the chemical reaction alone. The stability of thioesters was dramatically reduced above pH 7.5. For other thioesters, MTP, MTB, MTH, MTIB and MTIV, the addition of cell free extracts did not change the rate of substitution of acetyl CoA by the S-methyl group in the thioester. It would thus, seem that the specificity of the enzyme in *G. candidum* is limited to acetyl CoA. For the formation of other thioesters, apparently it is strictly a chemical substitution *(51)*. The conversion of L-methionine to flavors is summarized in Figure 9.

MTL can also combine with formaldehyde or other carbonyl compounds to produce thioacetals (2,4-dithiapentane and 2,4,5-trithiahexane) that are very commonly found in cheeses. For these compounds, there are no data on the possible role of enzymes in the production. Another possible precursor to consider is S-methyl methionine. S-methyl methionine is an efficient substrate for DMS production. It has been reported *(52)* that when the concentration of S-methyl methionine is increased, the varietal cheese flavor decreased and the maturation flavor increased. At concentrations greater than 100 ppb, it gives an unpleasant green olive flavor. It is present in cheeses and may have a role in the complexity of the flavor of cheese.

DMS is present in lager beer at 16-21 µg/l. S-methyl methionine is produced during the germination of barley. During kilning, it is degraded to DMS and DMSO. The DMS concentration in wort is usually more than 100 ppb and decreases during the fermentation process *(53)*. The DMSO present in wort is biologically reduced by yeast to DMS *(54)*, but dimethylsulfone does not seem to be reduced.

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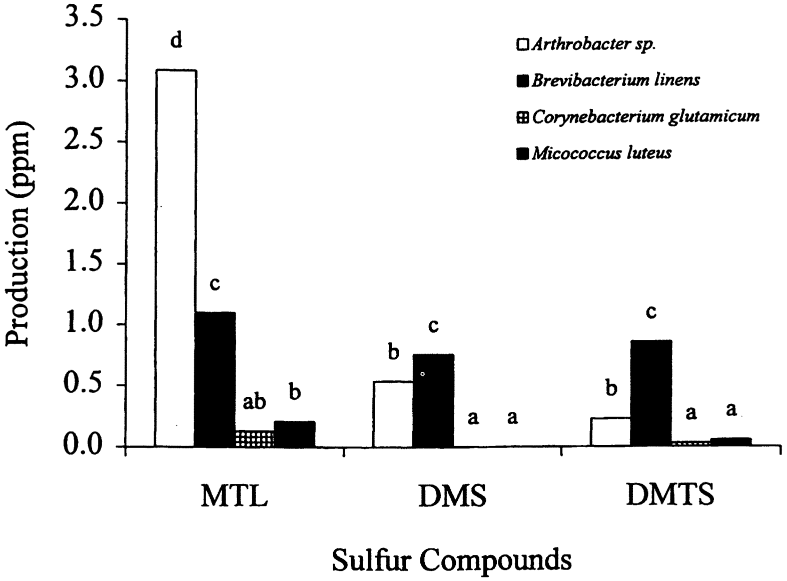

Figure 8. Efficiency of diverse ripening bacteria to produce sulfur flavor compounds. (Columns with like letters do not significantly differ in value, α=0.05).

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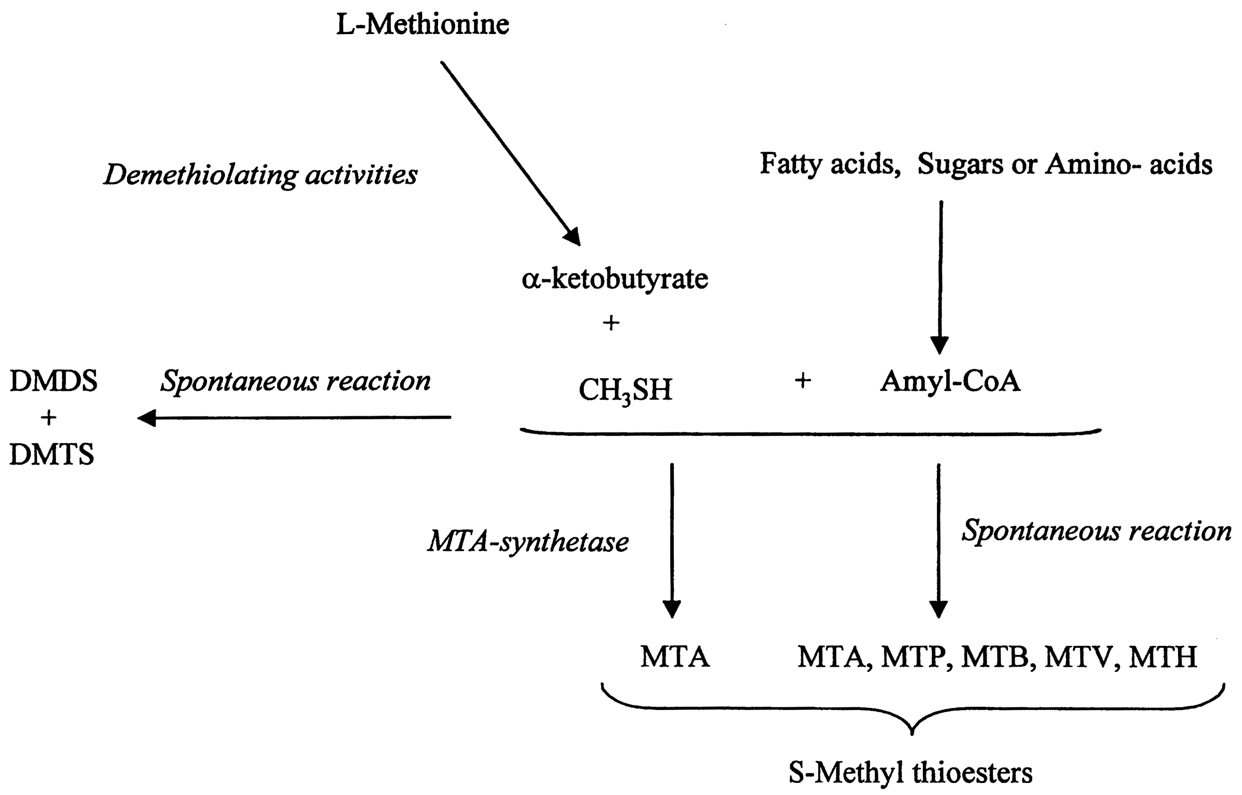
L-Methionine
Demethiolating activities
Fatty acids, Sugars or Amino- acids
α-ketobutyrate
+
DMDS
+
DMTS
Spontaneous reaction
CH₃SH
+
Amyl-CoA
MTA-synthetase
Spontaneous reaction
MTA
MTA, MTP, MTB, MTV, MTH
S-Methyl thioesters

Figure 9. Conversion of methionine and acyl CoA to thioesters.

Conclusions

Fungi and bacteria can produce a variety of sulfur flavor compounds. They are traditionally used to diversify foods and beverages produced by fermentation. Better control of the complex generation of sulfur compounds, especially in traditional fermented foods, is necessary because these compounds are very important for the typical flavor of fermented foods. Some of the pathways that generate these compounds remain unknown, such as, the synthesis of DMS and thioacetals. A better understanding of these biological systems could be used to produce natural flavor molecules or to produce precursors for natural flavor syntheses.

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Chapter 4

An Overview of the Contribution of Sulfur-Containing Compounds to the Aroma in Heated Foods

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Many of the most potent odorants found in foods contain sulfur. The exceptionally low odor threshold values of many of these compounds mean that only trace quantities are necessary to make a significant contribution to flavor. Heterocyclic sulfur compounds, including thiophenes, thiophenones, dithiolanes, trithiolanes, trithianes, thiazoles, and thienothiophenes, are typical products of thermal reactions in food. Non-heterocyclic sulfur compounds, such as thiols, sulfides and disulfides are also important to the aroma of heated foods. The main source of sulfur in the reactions that lead to these compounds is the amino acid, cysteine. Cysteine degradation, in the Maillard reaction or through hydrolysis, produces hydrogen sulfide, which is a vital reactant for the production of sulfur-containing aroma compounds. Other important sulfur-containing precursors are methionine and thiamin.

Volatile compounds containing sulfur make very important contributions to the aromas associated with various different foods, and often define the characteristic flavor of the food. Volatile sulfur compounds are also responsible

In Heteroatomic Aroma Compounds; Reineccius, G., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2002.

for many of the highly objectionable odors associated with the degradation of biological material of plant and animal origin by the action of putrefactive microorganisms. Thus, the chemistry of volatile sulfur compounds is important in both flavor and environmental sciences.

Sulfur compounds contribute to the desirable aromas of both cooked and uncooked foods. Although the majority of aroma volatiles in fruits are esters, aldehydes, and terpenoid hydrocarbons, small quantities of specific sulfur-compounds provide characteristic aromas to some fruits. For example, 1-p-menthene-8-thiol is a character impact compound in grapefruit *(1)*, 4-methoxy-2-methyl-2-mercaptobutane and 8-mercapto-p-methan-3-one are important in blackcurrant aroma *(2)*, and certain thioesters may be important in melon flavor *(3)*. These compounds are secondary metabolites of cell biosyntheses.

Compounds containing sulfur play a more important role in the flavor of vegetables, especially members of the Brassica and Allium families *(4)*. The flavors of onions, garlic and leeks are dominated by aliphatic thiols and sulfides. However, they are only produced when the cells are ruptured, releasing alliinase enzymes which act on S-oxide amino acids present in the allium bulbs. In brassicas, enzyme action on glucosinolates produces thiocyanates, isothiocyanates and nitriles, which contribute to the aroma of these vegetables. Thiols and sulfides may also be produced, mainly during heating, through the hydrolysis of sulfoxides, thioglucosides and sulfur-containing amino acids. Methional, which plays an important role in the flavor of cooked potatoes, is formed from the amino acid methionine.

For the most part, sulfur-containing aroma compounds in fruits and vegetables are the result of biochemical and enzymatic pathways. However, many of the important sulfur-containing aroma compounds in food are derived from reactions occurring during the thermal processing of food. Many hundreds of such aroma compounds have been isolated from heated foods and the aim of this review is to examine some of these compounds, their role in the characteristic aromas of cooked foods, and the pathways by which they are formed.

Classes of Volatile Sulfur Compounds Found in Heated Foods

Although aliphatic thiols, sulfides, and disulfides are found in the volatiles of heated foods, the majority of the compounds produced as a result of thermal treatment contain heterocyclic sulfur. Some of the important classes are shown in Figure 1 and include heterocycles with 1, 2 or 3 sulfur atoms, as well as compounds containing sulfur in conjunction with nitrogen or oxygen. Over 250 different sulfur-containing volatiles have been reported in heated foods, with the

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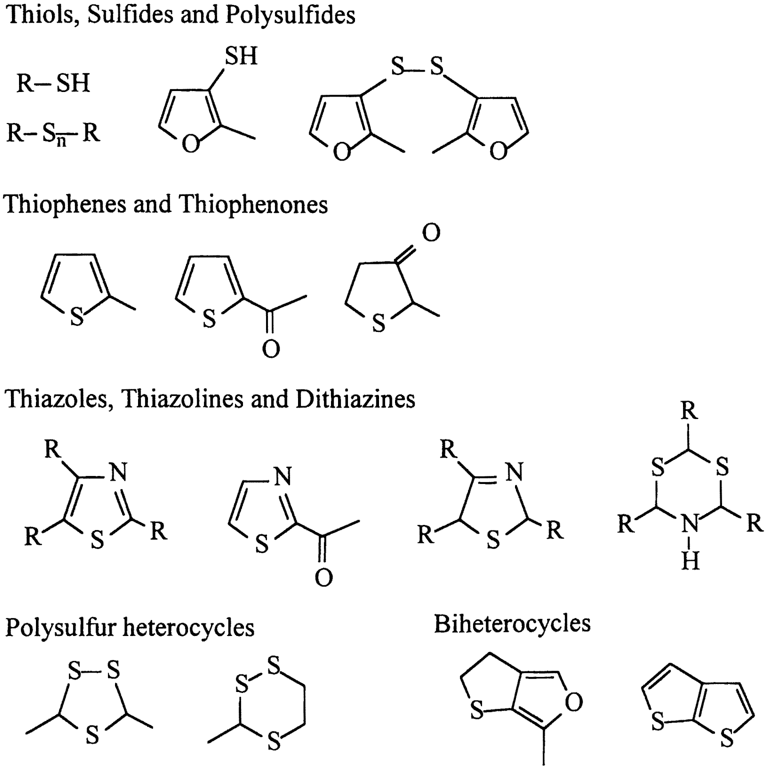
Thiols, Sulfides and Polysulfides
R–SH
R–S$_n$–R
SH
O
S–S
O
O
Thiophenes and Thiophenones
S
S
O
O
S
Thiazoles, Thiazolines and Dithiazines
R
N
R
S
R
N
S
O
R
N
R
S
R
R
S
S
R
N
R
H
Polysulfur heterocycles
S–S
S
S
S
S
Biheterocycles
S
O
S
S

Figure 1. Some classes of sulfur-containing aroma compounds found in heated foods.

largest numbers in coffee and meat. The numbers of sulfur-containing and nitrogen-containing volatiles in selected foods, compared with the total number of volatiles, are shown in Table I. These data have been obtained from the TNO lists of volatile compounds in foods *(5)*. It is interesting to note that foods from cereals and other plant sources appear to have many more nitrogen-containing than sulfur-containing volatiles, whilst in meat the opposite trend applies. Presumably, this reflects the higher protein-content of meat and, therefore, the greater availability of sources of sulfur in the form of the sulfur amino acids.

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Table I. Number of Sulfur- and Nitrogen-Containing Compounds Reported in Selected Foods

Type of Food	*Nitrogen Compounds*	*Sulfur Compounds*	*Total Number Volatiles*
Cooked rice	19	8	153
Wheat bread	73	17	296
Roast peanut	67	45	319
Cocoa	188	23	558
Black tea	67	19	590
Coffee	229	100	845
Chicken, cooked	7	19	189
Chicken, roast	24	10	186
Chicken, fried	38	31	152
Beef, boiled	92	124	487
Beef, roast	50	48	340
Mutton, boiled	1	7	133
Pork, boiled	6	16	108
Pork, roast	25	27	66

The classes of compounds comprising the sulfur volatiles in these foods are shown in Table II. In the foods derived from plant sources, thiazoles are the most abundant class, and coffee is the only one of these foods, which contains a significant number of other sulfur classes. This may reflect the more severe thermal treatments which coffee undergoes during processing. However, coffee is one of the most researched foods in respect of flavor chemistry, and this probably has some bearing on the number of compounds identified. In meat, there have been many more publications on the flavor of beef than of other species, and this is reflected in the larger number of aroma volatiles. Nevertheless, all the meats show a higher proportion of thiols, sulfides, thiophenes and thiophenones than other foods.

Table II. Classes of Sulfur Compounds Found in Selected Foods

Type of Food	*Thiols*	*Sulfides and Polysulfides*	*Alkylthiophenes*	*Other Thiophenes*	*Thiophenones*	*N/S heterocycles (Thiazoles, etc)*	*Thiolanes and Thianes*	*Others*
Cooked rice	1	3	–	–	–	4	–	–
Roast peanut	1	16	1	3	2	21	–	1
Wheat bread	1	4	3	5	–	2	–	2
Cocoa	–	11	–	–	–	9	–	3
Black tea	–	2	4	4	–	7	–	2
Coffee	5	26	8	16	3	27	2	13
Chicken, cooked	4	11	1	–	–	–	2	1
Chicken, roasted	1	2	3	–	–	3	–	1
Chicken, fried	1	1	4	1	–	19	5	–
Beef, boiled	25	39	11	11	2	19	10	7
Beef, roast	–	10	9	4	–	20	1	4
Mutton, boiled	–	2	–	1	–	1	3	–
Pork, boiled	3	7	2	–	–	2	1	1
Pork, roast	1	10	3	1	1	11	–	–

The concentrations of most sulfur-compounds in the volatiles of cooked foods are generally very low. However, many of these compounds are very powerful odorants with exceptionally low odor threshold values, which are often below 1 ppb (μg/kg) (Table III). Some of the lowest known odor threshold values are associated with sulfur compounds; for example bis (2-methyl-3-furyl) disulfide, which has an aroma characteristic of cooked meat, has been shown to have an odor threshold value of 20 pg/L in water *(6)*. No compound has been reported to have a lower odor threshold. The very low concentrations of such compounds in foods have made their isolation and identification particularly difficult.

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Table III. Odor Threshold Values for Some Sulfur Compounds in Water

Compound	*Odor Threshold (μg/L)*
3,5-Dimethyltrithiolane	10
Methional	1.8
2-Acetylthiazole	1
Methanethiol	0.2
Dimethyl disulfide	0.16
2-Acetylthiophene	0.08
2-Furfurylthiol	0.005
2-Methyl-3-furanthiol	0.005
Bis(2-methyl-3-furyl)-disulfide	0.00002

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Sources of Sulfur for Volatile Compounds in Heated Foods

For the majority of foods the main source of sulfur, from which sulfur-containing aroma compounds may derive, are the sulfur amino acids, cysteine, cystine and methionine. Certain foods, such as vegetables in the Brassica and Allium families, contain biosynthesized sulfur compounds including other sulfur amino acids, but in meat, fish, milk, coffee, tea, cocoa, bread and other baked cereals, cysteine and methionine are the most abundant sources of sulfur. Thiamin, found in meats and cereals, is another source of sulfur, which has received some attention from flavor chemists.

Thiamin-Derived Aroma Compounds

The thermal degradation of thiamin has been studied by a number of researchers. Yeasts contain relatively high concentrations of thiamin and yeast extracts are used as flavorings in a range of food products. Van der Linde et al. found 5-(2-hydroxyethyl)-4-methylthiazole, among the primary products formed when thiamin was heated at 130 °C *(7)*. This then could break down to give other thiazoles. Another important degradation product was shown to be 5-hydroxy-3-mercapto-2-pentanone, which is the key intermediate in the formation of a number of furanthiols and thiophenes, some of which have savory aromas *(7,8)*. The main breakdown pathways for the thermal degradation of thiamin are shown in Figure 2. More recently Güntert et al. have undertaken a detailed study of the degradation products of thiamin and identified many novel sulfur-containing heterocycles *(9,10)*. Some of these are summarized in Figure 3. A number of bicyclic compounds have been found among the volatiles, including 1-methylbicyclo[3.3.0]-2,4-dithia-8-oxaoctane, which was also found to be a product of the photolysis of thiamin *(11)*. When first isolated this compound was believed to be responsible for the odor of the photodegraded thiamin and a very low threshold was reported. However, it was shown subsequently that a trace of bis (2-methyl-3-furyl)-disulfide, with an even lower odor threshold (see Table III), was present in the isolate and this was responsible for the characteristic aroma *(6)*. Subsequently this disulfide has been found in meat and it is believed to be very important in meaty aromas *(12)*.

In autolyzed yeast and yeast extracts, which have been subjected to heat, sulfur-containing volatiles derived from thiamin make a major contribution to the aroma. Although thiamin is present in a number of foods, especially meat and cereals, the quantities are small (Table IV). The extent to which these low levels of thiamin contribute to aroma in the cooked food is not known. However, other sources of sulfur, in particular the sulfur-containing amino acids, are present in much higher quantities in meat than thiamin. In such circumstances, the contribution from thiamin to the sulfur-containing volatiles may be small in comparison to amino acids such as cysteine.

Table IV. Thiamin Content of Some Foods

Food	*Thiamin mg/100g*	*Food*	*Thiamin mg/100g*
Beef	0.05	Rice	0.08
Pork	0.59	Peanuts	0.23
Lamb	0.09	Coffee	0
White bread	0.18	Yeast extract	3.10
Brown bread	0.24		

Flavor Formation in the Maillard Reaction

The Maillard reaction, which occurs between amino compounds and reducing sugars, is one of the most important routes to flavor compounds in cooked foods. This reaction is complex and provides a large number of oxygen-, nitrogen- and sulfur-compounds that contribute to flavor *(13-16)*. Cysteine, and to a lesser extent methionine, are very important amino acids in the reaction and provide precursors for most of the sulfur-containing aroma compounds in heated foods. The initial stages of the Maillard reaction have been studied in detail and involve the condensation of the carbonyl group of a reducing sugar with an

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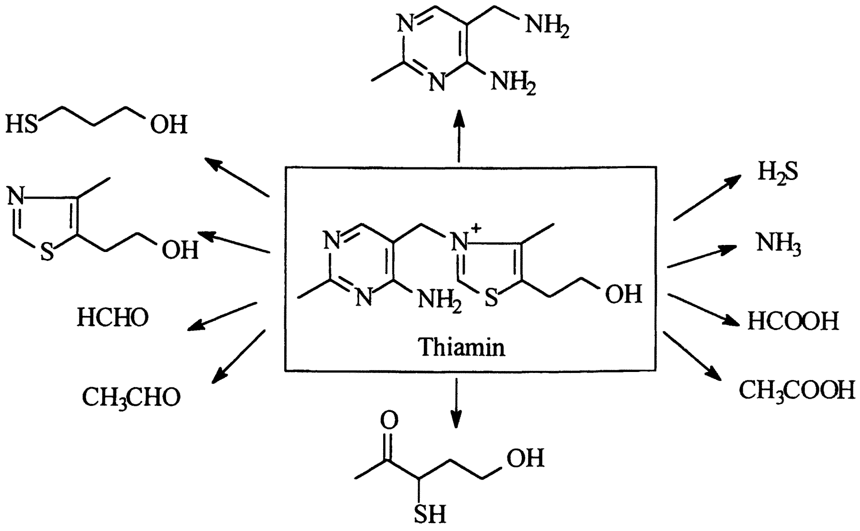
HS
OH
OH
HCHO
CH₃CHO
NH₂
NH₂
Thiamin
H₂S
NH₃
HCOOH
CH₃COOH
OH
SH

Figure 2. Main pathways for the thermal degradation of thiamin. (Adapted from reference (10).

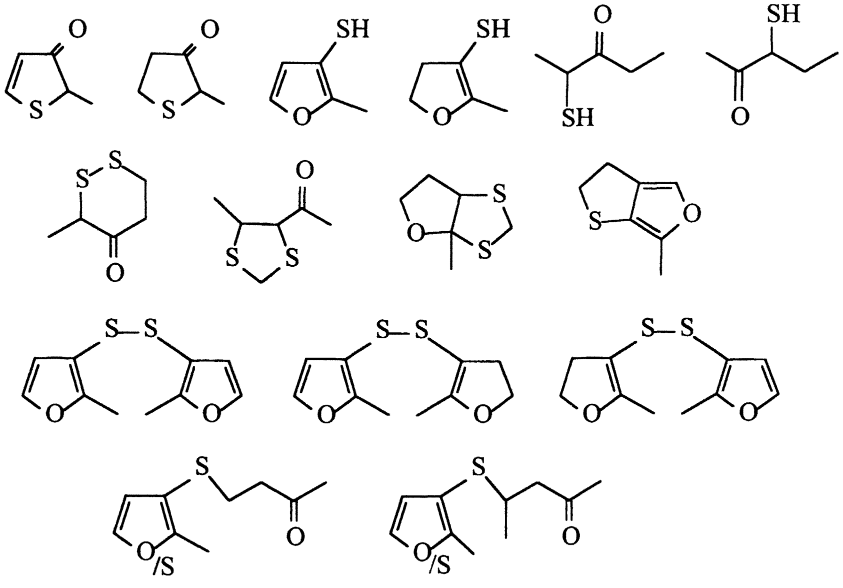

Figure 3. Some sulfur-containing aroma compounds formed from thiamin.

amino compound to give a glycosylamine. Subsequently, this rearranges and dehydrates, *via* deoxyosones, to various sugar dehydration and degradation products such as furfural and furanone derivatives, hydroxyketones and dicarbonyl compounds *(17,18)*. The subsequent stages of the Maillard reaction involve the interaction of these compounds with other reactive components such as amines, amino acids, aldehydes, hydrogen sulfide and ammonia, and it is these interactions which provide many of the aroma compounds which characterize cooked foods.

An important associated reaction is the Strecker degradation of amino acids by dicarbonyl compounds formed in the Maillard reaction. The amino acid is decarboxylated and deaminated forming an aldehyde, whilst the dicarbonyl is converted to an α–aminoketone or aminoalcohol. If the amino acid is cysteine, Strecker degradation can lead to the production of hydrogen sulfide, ammonia and acetaldehyde, while methionine will yield methanethiol (Figure 4). These compounds, together with carbonyl compounds produced in the Maillard reaction, provide a rich source of intermediates for reactions resulting in the formation of aroma compounds, including sulfur- compounds such as thiophenes, thiazoles, trithiolanes, thianes, thienothiophenes and furanthiols and disulfides.

Hydrogen sulfide is an essential intermediate in the formation of many heterocyclic sulfur compounds. Figure 5 summarizes the reactions between hydrogen sulfide and other simple intermediates formed in other parts of the Maillard reaction. Other reactions, which occur during heating of food, may also provide intermediates for these reactions. Lipid degradation products have been shown to participate in the Maillard reaction. In particular long-chain unsaturated aldehydes, which result from lipid oxidation, will react with hydrogen sulfide, ammonia and Maillard derived carbonyl compounds to form thiophenes, thiapyrans, pyridines, thiazolines and thiazoles *(19-21)*.

The Occurrence and Formation of Some Important Sulfur-Containing Aroma Compounds

The sulfur-containing volatiles found in foods comprise both heterocyclic and sulfur-substituted aliphatic and alicyclic compounds, as discussed above. Most have odors at low concentrations and, therefore, are potential contributors to food aromas. There have been a number of reviews dealing with the occurrence of these compounds in foods, including a series of reviews in a book edited by Maarse *(22)*, which was written to complement the TNO lists of volatile compounds in foods *(5)*. To discuss the contribution of all sulfur-compounds to food aroma is too large an undertaking for this present review

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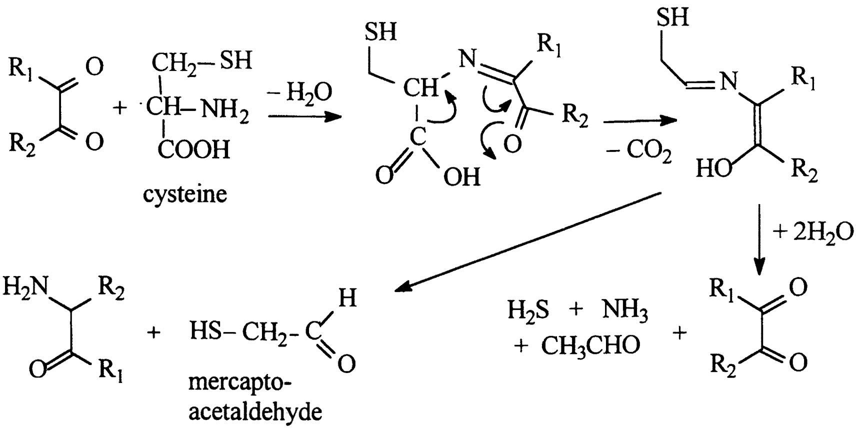
cysteine
$-H_2O$
$-CO_2$
$+2H_2O$
mercapto-acetaldehyde
$H_2S + NH_3$
$+ CH_3CHO$

Figure 4. Strecker degradation of cysteine.

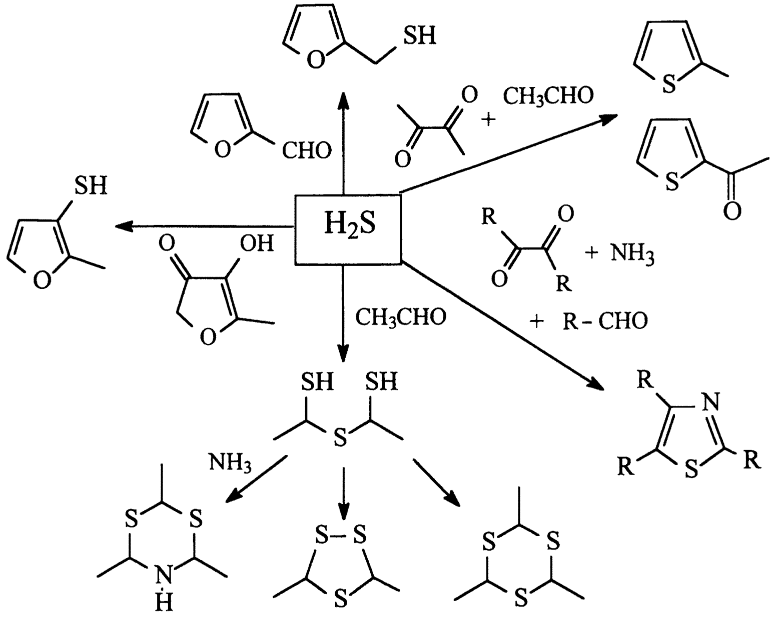
H_2S
CH_3CHO
$+ NH_3$
$+ R-CHO$
NH_3

Figure 5. Scheme for formation of heterocyclic aroma compounds from the reaction of hydrogen sulfide with Maillard derived dicarbonyls and ammonia.

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and, therefore, we have selected for discussion three important classes of sulfur-compounds that contribute to characteristic aromas in heated foods.

Furanthiols and Sulfides

A number of furans with thiol, sulfide or disulfide substitution have been reported as aroma volatiles, and these are particularly important in meat and coffee. In the early 1970s, it was shown that furans and thiophenes with a thiol group in the 3-position posses strong meat-like aromas and exceptionally low odor threshold values *(23-25)*. However, it was over 15 years before such compounds were reported in meat itself. In 1986, 2-methyl-3-(methylthio)-furan was identified in cooked beef and it was reported to have a low odor threshold value (0.05 μg/kg) and a meaty aroma at levels below 1 μg/kg *(26)*. Gasser and Grosch identified 2-methyl-3-furanthiol and the corresponding disulfide, bis-(2-methyl-3-furanyl) disulfide, as major contributors to the meaty aroma of cooked beef *(12)*. As discussed above, the odor threshold value of this disulfide has been reported as 0.02 ng/kg, one of the lowest known threshold values *(6)*. Other thiols, which may contribute to meaty aromas, include mercaptoketones, such as 2-mercaptopentan-3-one. 2-Furylmethanethiol (2-furfurylmercaptan) has also been found in meat, but is more likely to contribute to roasted rather than meaty aromas. Disulfides have also been found, either as symmetrical disulfides derived from two molecules of the same thiol or as mixed disulfides from two different thiols *(27)*.

Disulfides and thiols containing a furan ring have also been found among the volatiles of coffee, however, those containing the 2-furylmethyl moiety are more abundant than compounds with the 2-methyl-3-furyl moiety. 2-Furylmethanethiol was first described as an important constituent of coffee in a patent published in 1926 *(28)*. Since then the 5-methyl homologue and various other thiols and disulfides have been found *(29)*. Both these thiols have coffee-like characteristics at low concentrations, but are sulfurous and unpleasant at higher concentrations. Odor-port evaluation of a series of thiols and disulfides containing 2-methyl-3-furyl or 2-furylmethyl moieties confirmed that compounds of the former type had meaty characteristics, while those with 2-methylfuryl groups had roast, nutty, burnt characteristics *(30)*. The odor thresholds and odor characters of a number of thiols and disulfides are shown in Table V.

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Table V. Odor Characteristics of Selected Thiols, Sulfides and Disulfides

Compound	*Threshold μg/L*	*Odor Description*
Dimethyl sulfide	0.3-1	Sulfurous, sickly, cooked cabbage
Dimethyl disulfide	0.1-12	Sulfurous, sickly, cooked cabbage
Methional	0.2	Boiled potato
2-Furfurylthiol	0.005 - 0.12	Roasted, coffee (low conc.), sulfurous (higher conc.)
2-Furfuryl methyl disulfide	0.04	Bread crust, roasted
Bis(2-furfuryl) disulfide		Onions, roasted
2-methyl-3-furanthiol	0.005	Meat-like
2-Methyl-3-furyl methyl sulfide	0.05	Meat-like
Bis(2-methyl-3-furyl) disulfide	0.00002	Meat-like

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The routes involved in the formation of the various furan sulfides and disulfides are likely to be the interaction of hydrogen sulfide with dicarbonyls, furanones and furfurals. Possible pathways are shown in Figure 6. Furanthiols have been found in heated model systems containing hydrogen sulfide or cysteine with pentoses *(19,33,34)*. 2-Methyl-3-furanthiol has also been found as a major product in the reaction of 4-hydroxy-5-methyl-3(2*H*)-furanone with hydrogen sulfide or cysteine *(35,36)*. This furanone is formed in the Maillard reaction of pentoses. Alternatively, it has been suggested that it may be produced by the dephosphorylation and dehydration of ribose phosphate, and that this may be a route to its formation in cooked meat *(35,37)*.

Thiazoles and Thiazolines

Most cooked foods contain thiazoles. Simple alkyl substituted thiazoles generally have odor threshold values in the range 1-1000 μg/L. Odor descriptions include green, vegetable-like, cocoa, nutty, and some are claimed to have meaty characteristics (Table VI). Although most alkylthiazoles result from thermal reactions, some, such as 2-isobutylthizole, are biosynthesized. This compound makes a very important contribution to the aroma of fresh tomatoes *(38)*.

Table VI. Odor Characteristics of Selected Thiazoles and Thiazolines

Compound	*Threshold μg/L*	*Odor description*
2-acetyl-4-methylthiazole	300	anthranilic, burnt
2-acetylthiazole	10	cereal, bready, popcorn-like, strong, nutty, roasted
4-acetylthiazole	170	hazelnut, earthy, cracker-like
2,4-diethylthiazole	6.5	ethereal, musty, earthy
2,5-diethylthiazole	0.09	slightly skunky and green peppery
2,4-dimethylthiazole	0.1-18	skunky-oily, meat, cocoa
4,5-dimethylthiazole	470-500	meaty, boiled poultry
5-ethyl-4-methylthiazole	20	green vegetables, unroasted hazelnuts
2-isobutylthiazole	2-3.5	strong green, tomato leaf
2,4,5-trimethylthiazole	50	cocoa, hazelnut, dark chocolate, green vegetables
2-acetyl-2-thiazoline	1.3	cracker-like, fresh baked bread crust
2,4-dimethyl-3-thiazoline	20	nutty, roasted, vegetable
2,4,5-trimethyl-3-thiazoline	500	meaty, nutty, onion-like

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2-Acetylthiazole has been reported in a number of cooked foods, including meats, shell-fish, coffee, nuts, cereals, and some heated vegetables, and it probably makes important contributions to roast, nutty aromas in cooked foods. Mulders proposed a pathway for its formation in which the mercaptoiminenol intermediate in the Strecker degradation of cysteine and 2-oxopropanal (Figure 4) undergoes ring closure by nucleophilic attack of the thiol group on the double bond of the enol *(39)*.

The route to alkylthiazoles probably involves the reaction of α-dicarbonyls, such as 2,3-butanedione or 2-oxopropanal, with ammonia and hydrogen sulfide (Figure 7). This mechanism requires the participation of an aliphatic aldehyde, whose alkyl chain becomes substituted in the 2-position of the thiazole. This aldehyde may be acetaldehyde or a simple Strecker aldehyde, resulting from Strecker degradation of an amino acid. Alternatively, it may be a lipid oxidation product, such as hexanal or nonanal. Several thiazoles with C_4-C_8 n-alkyl substituents have been found in the volatiles of cooked meat *(21,40,41)* and, recently, forty-eight 2-alkyl-3-thiazolines were reported in the headspace

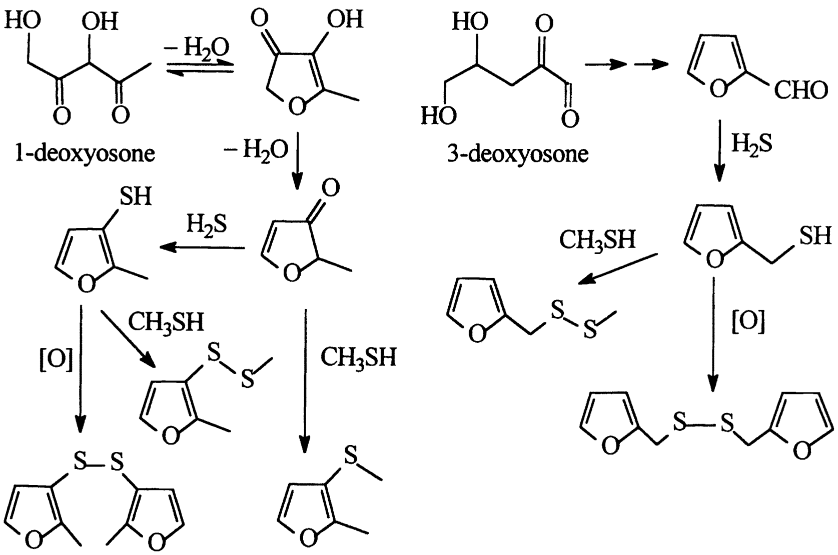
1-deoxyosone
3-deoxyosone
- H_2O
H_2S
CH_3SH
[O]
CHO
SH

Figure 6. Pathways for the formation of furanthiols, sulfides, and disulfides from intermediates of the Maillard reaction.

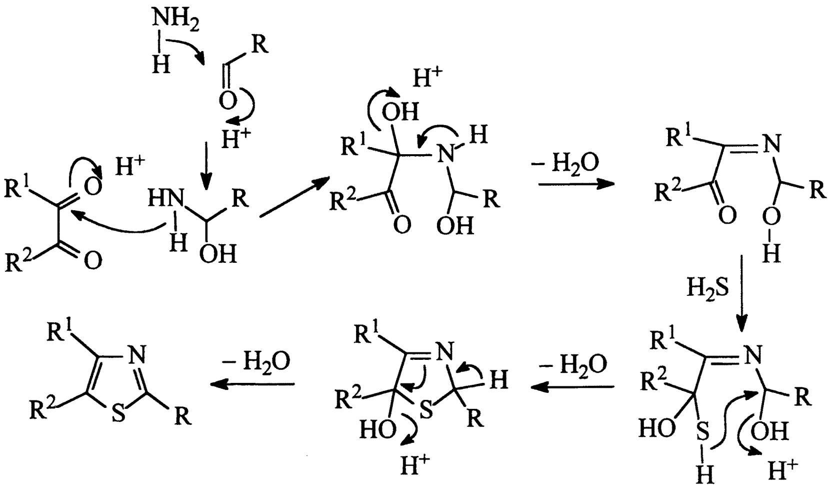
NH_2
H^+
- H_2O
H_2S
R^1
R^2
R

Figure 7. Pathway for the formation of thiazoles from intermediates of the Maillard reaction.

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volatiles of boiled beef from animals in which the meat contained raised levels of polyunsaturated fatty acids *(21)*. However, these compounds with long alkyl chains were not found to be potent odorants.

Dithiazines

Thialdine (2,4,6-trimethyldihydro-1,3,5-dithiazine) is a six membered heterocyclic compound containing sulfur and nitrogen in the ring. It was first reported in a food product in 1972 by Brinkman et al., who identified it in heated pork *(42)*. Subsequently it has been found in the other meat species, as well as peanuts, dry red beans, soybeans, boiled shrimp and several other seafoods *(43)*. Thialdine was reported as the major volatile product obtained from a sample of boiled mutton *(44)*. Thialdine was first reported over 150 years ago by Wöhler and Liebig *(45)* who showed that it was formed by the reaction of acetaldehyde, hydrogen sulfide, and ammonia (Figure 8). The reaction occurs very readily without heating and, therefore, it is possible that it is formed during the extraction procedure. In our laboratory we found thialdine in a series of cold traps attached to the outlet of a simultaneous steam distillation solvent extraction system containing boiled meat, with the largest quantities in the last and coldest of three traps. This indicated that thialdine was formed in the trap where acetaldehyde condensed and reacted with hydrogen sulfide and ammonia. Despite such findings, there is evidence that dihydrodithiazines do occur in food products and contribute to aroma *(46)*.

In the 1980s, several other dithiazines were identified in Antarctic krill *(47)* and later in shrimp *(48)* and dried squid *(49)*. They were considered to make important contributions to the aroma of these seafoods. Over 40 different dithiazine derivatives have now been identified in other foods, including beef, pork, chicken, grilled liver, roast peanuts, peanut butter, and cocoa *(43,50)*. Some of these are given in Table VII. In addition, at least 47 dithiazines have been found in yeast extracts *(43)*.

The occurrences and sensory properties of these compounds have been discussed in an excellent review by Werkhoff et al. *(43)*. They also discuss the formation of these compounds in model systems comprising aldehydes, ammonia and hydrogen sulfide. The odor thresholds are reported to be in the range 5-500 μg/L and the odor properties of 42 synthesized dithiazines are given in the review. Typical odor descriptors are roasted, onion, garlic-like, meaty, roast peanut, egg-like, sulfury.

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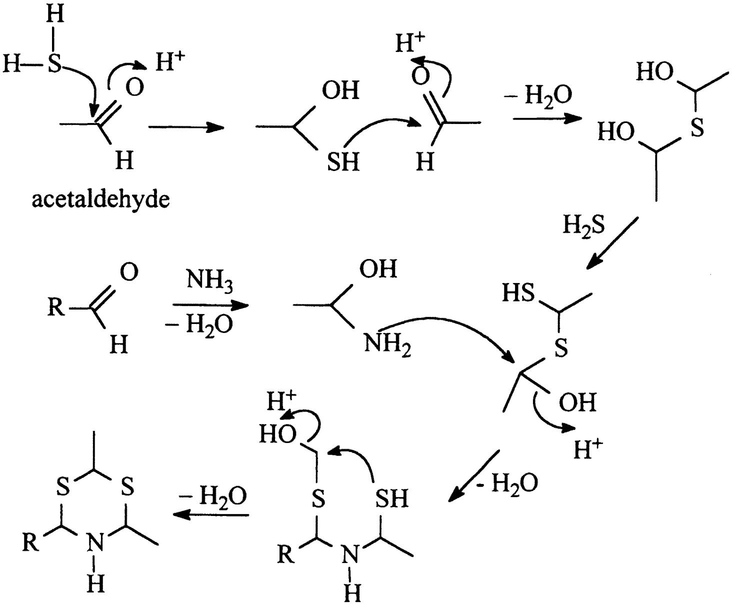
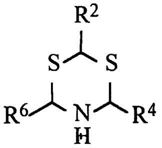
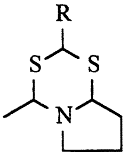

H
H—S
O
H+
H
acetaldehyde
OH
SH
H+
O
H
- H_2O
HO
HO
S
H_2S
R
O
H
NH_3
- H_2O
OH
NH_2
HS
S
OH
H+
- H_2O
H+
HO
S
SH
R
N
H
- H_2O
S
S
R
N
H

Figure 8. Pathway for the formation of thialdine (R=CH_3) and other dihydrodithaizines from acetaldehyde, ammonia, and hydrogen sulfide.

Table VII. Selected Dithiazines Found in Various Food Products

Compound	*Found in*
	Dihydro-1,3,5-dithiazines
4,6-dimethyl	boiled shrimp
2,4,6-trimethyl	beef, pork, mutton, chicken, dry red beans, boiled Antarctic krill, boiled shrimp, dried squid, roast peanuts, peanut butter, cocoa
2-ethyl-4,6-dimethyl	boiled Antarctic krill, boiled shrimp, dried squid, pork, beef, chicken, peanut butter
2,6-dimethyl-4-ethyl	boiled Antarctic krill, boiled shrimp, dried squid, pork, beef, chicken, peanut butter
2,4,6-triethyl	boiled shrimp
2-*n*-propyl-4,6-dimethyl	pork, chicken, roast peanuts, peanut butter, cocoa
2-isopropyl-4,6-dimethyl	dried squid, pork, roast peanuts
2,6-dimethyl-4-isopropyl	dried squid, pork, roast peanuts
2-*n*-butyl-4,6-dimethyl	dried squid, pork, chicken, roast peanuts, peanut butter
2-isobutyl-4,6-dimethyl	dried squid, pork, roast peanuts
2,6-dimethyl-4-isobutyl	pork, beef, chicken, roast peanuts, cocoa
2-*n*-pentyl-4,6-dimethyl	pork, chicken, roast peanuts, peanut butter
2-*n*-hexyl-4,6-dimethyl	pork, roast peanuts
2-*n*-heptyl-4,6-dimethyl	pork, roast peanuts
	Tetrahydropyrrolo[2,1-d]-1,3,5-dithiazines
2,4-dimethyl	dried squid, pork, beef, chicken, roast peanuts, peanut butter, cocoa
2-ethyl-4-methyl	pork, peanut butter
2-isopropyl-4-methyl	Pork
2-isobutyl-4-methyl	dried squid, pork

SOURCE: Data are from references 43, 47-51.

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Conclusions

Volatile sulfur compounds are among the most potent odorants in foods. Nearly all aliphatic and heterocyclic sulfur-containing volatiles are odoriferous. They occur in both heated and unheated foods, but most sulfur-containing heterocyclic aroma compounds are formed during heating of foods. The main sources of sulfur for their formation are the amino acids cysteine and methionine, and to a lesser extent thiamin. Sulfur-containing heterocyclics are particularly important in meat. Although many sulfur-heterocycles are found in coffee and cereals, they do appear to be as important to the overall the aroma as they are in meat.

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Chapter 5

Novel Sulfur Compounds from Lipid–Maillard Interactions in Cooked Meat

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A number of 3-thiazolines, thiazoles, thiapyrans, and thiophenes with 2-alkyl substituents have been found in cooked beef and lamb. These compounds derive from the interaction of lipid autoxidation products, such as saturated and unsaturated aldehydes, with simple intermediates of the Maillard reaction, such as hydrogen sulfide, ammonia, and dicarbonyls. Although the aromas of these compounds are weak, they may influence flavor by modifying the formation of other compounds in the Maillard reaction or autoxidation of lipid.

The main reactions occurring during the cooking of meat, which are responsible for the characteristic flavor, are the Maillard reaction between amino acids and sugars, and the thermal oxidation of lipid. Sulfur-containing aroma compounds make particularly important contributions to meat flavor and the main source of sulfur for these compounds is the amino acid cysteine. In previous work using heated model systems containing ribose and cysteine, we demonstrated that the addition of phospholipid to these systems resulted in compounds formed by the interaction of lipid degradation products with intermediates of the Maillard reaction *(1-3)*.

In Heteroatomic Aroma Compounds; Reineccius, G., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2002.

A number of compounds that could be formed from the interaction of lipid with the Maillard reaction have also been found in the volatiles of cooked foods *(4)*. These compounds include O-, N- or S-heterocycles containing long *n*-alkyl substituents (C_5-C_{15}). The alkyl groups usually derive from aliphatic aldehydes, obtained from lipid oxidation, while amino acids are the source of the nitrogen and sulfur.

Meat volatiles have been found to contain a number of such compounds. Several thiazoles with C_4-C_8 *n*-alkyl substituents in the 2-position have been reported in roast beef *(5)* and in fried chicken *(6)*. Other alkylthiazoles with much longer 2-alkyl substituents (C_{13}-C_{15}) have been reported in the volatiles of heated beef and chicken, with the highest concentrations in beef heart muscle *(7,8)*. Other heterocyclic compounds with long *n*-alkyl substituents found in meat include 2-alkylthiophenes with C_4-C_8 alkyl substituents reported in pressure cooked beef *(9,10)*, and 5-butyl-3-methyl-1,2,4-trithiolane and its 5-pentyl homologue reported in fried chicken *(11)* and pork *(12)*.

This paper reviews some other sulfur heterocycles, which have recently been found in beef and lamb, and discusses the relationship to the fatty acid composition of the meat.

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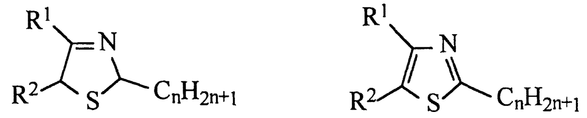
Effect Fatty Acid Composition on Meat Volatiles

In recent years there has been increased interest in modifying the fatty acid composition in meat and milk to provide products that possess a fatty acid profile more appropriate to current human dietary guidelines *(13,14)*. These recommend an increase in the polyunsaturated to saturated fatty acid ratio (P:S ratio), while decreasing the *n*-6 :*n*-3 ratio. The fatty acids deposited in beef tissues are relatively saturated, giving a low P:S ratio in the meat, but the ratio of *n*-6:*n*-3 fatty acids is beneficially low. Hence, strategies to improve the nutritional quality of beef need to increase the P:S ratio, while keeping the *n*-6:*n*-3 ratio low. This may be achieved by altering the diet of cattle to increase the levels of components containing sources of long chain polyunsaturated fatty acids (PUFA) or by modifying the extent of the hydrogenation in the rumen. However, an increase in PUFA concentrations in meat may compromise oxidative stability resulting in flavor changes in the processed meat.

Recently we examined the volatiles from cooked beef and lamb that had been fed diets containing linseed or fish oil. These diets gave enhanced levels of *n*-3 PUFA in the triglycerides (C18:3 *n*-3) and in the phospholipids (C18:3 *n*-3, C20:5 *n*-3, C22:6 *n*-3). The volatiles from both cooked lamb and cooked beef with the increased *n*-3 PUFA showed increased levels of aliphatic aldehydes compared with control samples *(15,16)*. This was due the enhanced autoxidation of the unsaturated fatty acids. However, sensory panels only found small differences in flavor in the cooked meat *(17)*.

Thiazoles and Thiazolines in Beef and Lamb

A series of alkylthiazoles and alkyl-3-thiazolines were found in the volatiles of cooked beef and lamb from these studies. The number and concentration of these heterocycles were higher in the meat from the linseed and fish oil diets than in controls. A total of 46 alkyl-3-thiazolines were found in beef and 12 in lamb *(15,16,18)*. Most contained methyl or ethyl groups in the 4 and 5 positions with long alkyl chains (C_4-C_9) in the 2-position (Figure 1). It was the first time any of these compounds had been reported in a food product, except for 2-isobutyl-4,5-dimethyl-3-thiazoline, which had been found in a yeast extract *(19)*. It was shown that these thiazolines were the main products in the reaction between α-hydroxycarbonyls, such as 1-hydroxy-2-butanone or 3-hydroxy-2-butanone, ammonium sulfide and an n-alkanal or a Strecker aldehyde, such as 2-methylbutanal *(20)*. In these thiazolines the alkyl chain of the alkanal occupied the 2-position on the ring. In the cooking of meat, the Maillard reaction could give the hydroxycarbonyls, ammonia and hydrogen sulfide would be produced from cysteine, while the alkanals derive from lipid oxidation products or the Strecker degradation of amino acids. A smaller number of alkylthiazoles were also found in the beef and lamb, but generally at lower concentrations than the corresponding 3-thiazolines.



$R^1 = H, CH_3$ or C_2H_5
$R^2 = CH_3$ or C_2H_5
$n = 3 - 9, 15$

R^1 and $R^2 = CH_3$ or C_2H_5
$n = 4 - 8, 15$

Figure 1. Alkyl-3-thiazolines and alkylthiazoles found in cooked beef and lamb.

The odors of thiazolines and thiazoles with 2-*n*-alkyl substitution were described as slightly fatty, but they did appear to have low odor thresholds and could not be detected by GC-olfactometry in any of the volatile extractions of the cooked meat. Thiazolines and thiazoles with methyl or acetyl substituents

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possess thresholds in the low µg/kg range, but it appears that the larger molecules with long alkyl substituents are not such potent odorants.

Thiophenes and Thiapyrans

Until recently only one alkylthiapyran had been reported in food: 2-pentylthiapyran in cooked beef heart *(7)*. However, 2-alkyl-(2*H*)-thiapyrans with C_2-C_7 alkyl chains, along with their 2-alkylthiophenes isomers with C_3-C_8 alkyl chains, were isolated from cysteine/ribose/lecithin reaction mixtures *(21)*. 2-Pentylthiapyran and 2-hexylthiophene were identified as major products when 2,4-decadienal and hydrogen sulfide were reacted in aqueous solution at pH 8 *(22)*.

In our recent work examining meat with modified PUFA content, we found six 2-alkylthiophenes and six 2-alkyl-(2*H*)-thiapyrans in volatiles of both cooked beef and lamb (Figure 2) *(23)*.

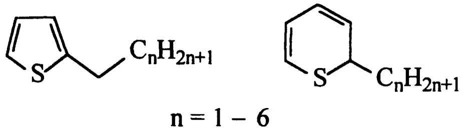

Figure 2. n-Alkylthiophenes and n-alkyl-(2H)-thiapyrans found in the volatiles of cooked beef and lamb.

Although these compounds were found in all samples of meat examined, both diet and species affected the quantities obtained. Quantities of both thiophenes and thiapyrans were higher in the meat of animals fed PUFA supplements, corresponding to higher quantities of *n*–3 PUFA found in the muscles of these animals. These compounds may be formed by the reaction of 2,4-alkadienals with hydrogen sulfide (Figure 3). The higher levels of lipid breakdown products, such as 2,4-dienals, in the cooked meat with increased levels of PUFA were considered to be responsible for the larger quantities of thiophenes and thiapyrans.

In order to confirm the identities of these thiapyrans, 2,4-alkadienals were reacted with hydrogen sulfide in aqueous solution. 2-Alkylthiophenes and 2-alkyl-(2*H*)-thiapyrans were produced in all of these reactions. However, the quantities of alkylthiapyrans recovered were up to 100 times greater than those of the equivalent isomeric thiophenes. The relative quantities of thiophenes and thiapyrans in meat were similar, suggesting that a mechanism other than that given in Figure 3 may be required to explain the formation of alkylthiophenes.

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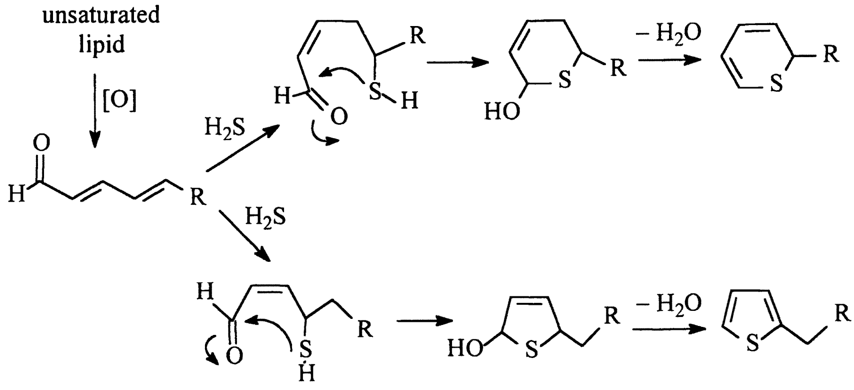

Figure 3. Pathways for the formation of 2-alkylthiophenes and 2-alkyl-(2H)-thiapyrans from 2,4-alkadienals and hydrogen sulfide.

The aromas of the alkylthiapyrans were weak and thiophene-like, and the lower molecular weight thiapyrans possessed unpleasant garlic-like notes. The weak odor intensities suggest that it is unlikely that any of these compounds contribute directly to cooked meat aroma. However, the presence in the meat confirms that lipid-Maillard interactions do take place during the cooking of meat. Such interactions will modify the profile of aroma compounds produced by the Maillard reaction and by lipid degradation and, therefore, have an indirect affect on the aroma profile. For example, the reaction of 2,4-alkadienals with hydrogen sulfide, to form 2-alkyl-(2*H*)-thiapyrans, leads to decreases in the concentrations of the dienals, which are potent aroma compounds, whilst forming compounds with low aroma significance.

Alkylthiophenes and Alkyl-(2*H*)-thiapyrans Formed in Model Systems Containing Linoleic and Linolenic Acids

The two main series of naturally occurring polyunsaturated fatty acids, *n*-3 and *n*-6, will give different profiles of volatile aldehydes and other products during autoxidation. Aldehydes from the former will generally be unsaturated because of the double bonds at the ω3 and ω6 positions on the alkyl chain. However, the *n*-6 series, will give both saturated and unsaturated aldehydes, and the latter may have up to seven saturated carbon atoms in the alkyl chain. Figure 4 shows some of the saturated and unsaturated aldehydes which may form during the autoxidation of 18:2 *n*-6 and 18:3 *n*-3. If 2-alkylthiophenes and 2-

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alkyl-(2*H*)-thiapyrans are formed in meat from the reaction of dienals with hydrogen sulfide (Figure 3), then the relative amounts of different alkylthiophenes and alkylthiopyrans formed will depend on the proportions of 18:2 *n*-6 and 18:3 *n*-3 fatty acids in the meat.

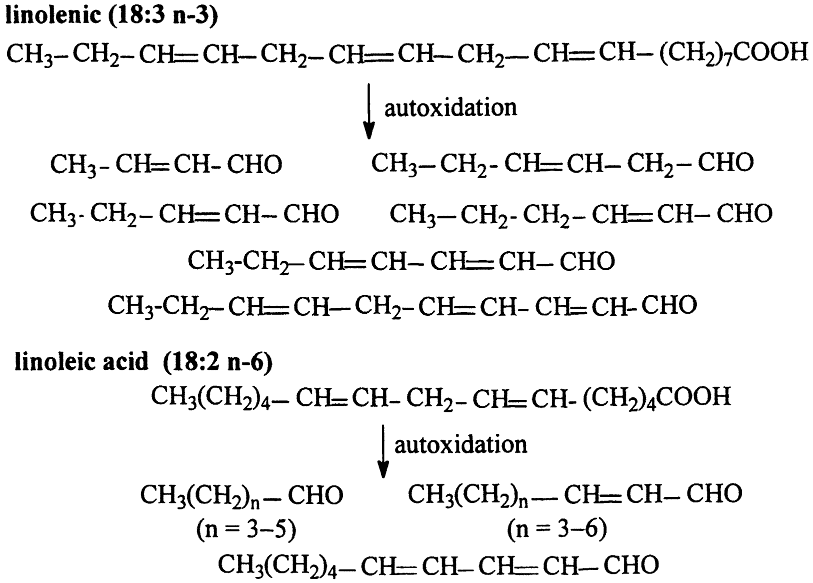
linolenic (18:3 n-3)
$CH_3-CH_2-CH{=}CH-CH_2-CH{=}CH-CH_2-CH{=}CH-(CH_2)_7COOH$
autoxidation
$CH_3-CH{=}CH-CHO$
$CH_3-CH_2-CH{=}CH-CH_2-CHO$
$CH_3-CH_2-CH{=}CH-CHO$
$CH_3-CH_2-CH_2-CH{=}CH-CHO$
$CH_3-CH_2-CH{=}CH-CH{=}CH-CHO$
$CH_3-CH_2-CH{=}CH-CH_2-CH{=}CH-CH{=}CH-CHO$
linoleic acid (18:2 n-6)
$CH_3(CH_2)_4-CH{=}CH-CH_2-CH{=}CH-(CH_2)_4COOH$
autoxidation
$CH_3(CH_2)_n-CHO$
(n = 3–5)
$CH_3(CH_2)_n-CH{=}CH-CHO$
(n = 3–6)
$CH_3(CH_2)_4-CH{=}CH-CH{=}CH-CHO$

Figure 4. Some aldehydes formed in the autoxidation of linolenic and linoleic acids.

We have examined the formation of 2-alkylthiophenes and 2-alkyl-(2*H*)-thiapyrans in two aqueous model systems, both containing cysteine and ribose as a source of hydrogen sulfide. One contained linoleic acid (18:2 *n*-6) methyl ester and the other contained α-linolenic acid (18:3 *n*-3) methyl ester. The systems were heated at 140°C in a sealed container for 30 min, then the headspace volatiles were extracted using solid phase microextraction and were analyzed by GC-MS. The profiles of thiapyrans and thiophenes produced were compared with those in the headspace of cooked beef and lamb from animals fed on diets containing either linseed or fish oil supplements (Table I).

In the model systems containing 18:3 *n*-3 methyl ester, the main thiapyran was 2-ethyl-(2*H*)-thiapyran, which could be formed from 2,4-heptadienal, a major autoxidation product of 18:3 *n*-3. Small amounts of the methyl and propyl homologues were also found, but no thiapyrans with longer alkyl chains. In the 18:2 *n*-6 reaction systems these thiapyrans containing shorter alkyl chains were absent and the reaction favored alkylthiophenes, with 2-pentylthiophene

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the most abundant. In the meat, both beef and lamb fed with linseed or fish oil had raised levels of *n*-3 fatty acids. The cooked meat from these diets had higher levels of 2-ethyl-, 2-methyl- and 2-propylthiapyrans compared with the control meats.

Table I. Quantities of Alkylthiophenes and Alkylthiapyrans Found in Beef and Lamb Fed on PUFA Rich Diets, Compared with Quantities Produced in Model Reaction Systems

	Lamb			Beef			Model	
Compound	Control	Linseed	Fish	Control	Linseed	Fish	18:2	18:3
2-alkylthiophenes								
ethyl	13	14	32	5	6	15	3	99
propyl	2	3	4	tr	tr	tr	7	9
butyl	2	4	4	–	tr	tr	8	tr
pentyl	4	3	4	tr	tr	tr	21	tr
hexyl	4	4	5	tr	tr	tr	9	tr
2-alkyl-(2H)-thiapyrans								
methyl	3	3	6	–	–	–	–	19
ethyl	22	59	131	3	6	14	–	399
propyl	2	4	15	–	tr	2	–	3
butyl	tr	tr	2	–	–	–	tr	–
pentyl	5	4	8	–	tr	tr	tr	–

Quantities in headspace above meat are ng/100g meat, quantities in headspace above model reaction mixture are ng per 0.5 mmol fatty acid; – not detected, tr = trace.

Model systems: cysteine + ribose + fatty acid methyl ester (18:2 *n*–6 or 18: *n*–3); 0.5 mmol each.

Conclusions

The interaction of products of the Maillard reaction with saturated and unsaturated aldehydes from the autoxidation of lipids results in the formation of heterocyclic sulfur compounds, such as thiazolines, thiazoles, thiapyrans, and thiophenes, all with alkyl substituents derived from the aldehyde. All these classes of compounds have recently been found in cooked meat. Although these compounds may not have significant aromas, the reactions by which they are formed may influence the relative quantities of other odor compounds in the aroma profiles of foods.

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Chapter 6

Gas Chromatography–Olfactometry and Chemiluminescence Characterization of Grapefruit Juice Volatile Sulfur Compounds

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GC-Olfactometry (GC-O) identified five sulfur-containing odorants among 32 odor-active compounds detected in commercial not-from-concentrate (NFC) grapefruit juice. These sulfur compounds imparted characteristic and supporting aroma attributes to the grapefruit juice. Two compounds, 3-mercaptohexyl acetate and 3-mercaptohexan-1-ol, are reported for the first time in grapefruit juice. Over 20 sulfur-containing components were detected in grapefruit juice extracts by sulfur chemiluminescence (SCD). GC-SCD analysis demonstrated that pasteurization of grapefruit juice qualitatively and quantitatively altered the profile of the volatile sulfur compounds. Our study is one of the first couplings of GC-O with SCD. This analytical combination revealed which odor-active compounds in NFC grapefruit juice contain sulfur, and conversely, that not all of the sulfur compounds detected are odor-active.

In Heteroatomic Aroma Compounds; Reineccius, G., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2002.

Introduction

Volatile sulfur compounds are important flavor constituents of many foods and beverages. The contribution of sulfur compounds to the aroma characteristics of grapefruit has been a subject of interest from the early 1980s. Demole et al. (*1*) isolated 1-*p*-menthene-8-thiol and the cyclization product 2,8-epithio-*cis*-*p*-menthane from grapefruit juice. The former was shown to be a potent character-impact compound of grapefruit, which occurred at the ppb-level or lower in juice. The authors reported that the terpene thiol had an odor threshold of 0.1 ppt, which made it one of the most potent aroma compounds found in a food product. The epithio was also found to have a grapefruit-like note but with a higher odor threshold (9 ppb). Other previously identified grapefruit juice sulfur compounds include: hydrogen sulfide, carbonyl sulfide, methyl sulfide, methanediol and sulfur dioxide (*2*). Cadwallader and Wu (*3*) analyzed volatile components in fresh grapefruit juice using purge and trap GC but reported no sulfur compounds. Recently, Buettner and Schieberle (*4*) identified 1-*p*-menthene-8-thiol, 4-mercapto-4-methyl-2-pentanone and methional in fresh grapefruit juice using aroma extract dilution analysis (AEDA) and GC-O. 4-Mercapto-4-methyl-2-pentanone produced one of the highest dilution factors (FD = 128) relative to other volatiles detected in their grapefruit juice extract. The same authors (*5*) reported that the amounts of this thiol and the terpene thiol were 0.8 and 0.01 μg/L in hand squeezed white grapefruit juice using isotope dilution assay.

The objective of this work was to couple GC-O with a sulfur specific detector, determine in a grapefruit juice extract which intense GC-O peaks contain sulfur, and study the effect of thermal processing (pasteurization) on the sulfur compounds in grapefruit juice.

Experimental

Grapefruit Juice

NFC grapefruit juice samples were purchased from a local supermarket. In the study of the effect of pasteurization on sulfur compounds, juices were prepared from the whole fruit using FMC extractors. Pasteurization was performed at 90.6°C, with a flow rate of 1 gallon per minute.

Chemicals

Standard compounds 4-heptadecanone, S-methylthiobutanoate, acetic acid, methional, Furaneol, vanillin, isoeugenol, 2-methylbutanoic acid, 1-octen-3-one,

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(*E*)-2-nonenal, (*E*)-2-undecenal, (*E,E*)-2,4-nonadienal, and (*E,E*)-2,4-decadienal were purchased from Aldrich (Milwaukee, WI). 3-Mercaptohexyl acetate and 3-mercaptohexan-1-ol were bought from Interchim (Montlucon, France). Myrcene, limonene, octanal, decanal, nootkatone, linalool, and dihydronootkatone were gifts from SunPure (Lakeland, FL). *p*-1-Menthen-8-thiol and β-damascenone were obtained from Givaudan (Lakeland, FL). 4-Mercato-4-methyl-2-pentanone was synthesized in our laboratory. (*Z*)-2-Nonenal was found in (*E*)-2-nonenal at a 5-10 % level and *trans*-4,5-epoxy-(*E*)-2-decenal was found in an oxidized sample of (*E,E*)-2,4-decadienal. The identities of these components were confirmed by retention indices and odor qualities.

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Sample Preparation

For GC-O-FID analysis, volatiles were extracted from the juices with pentane-ethyl acetate (1/1, v/v) and for GC-SCD and GC-O-SCD analysis, extractions were made with ethyl acetate. Known amounts of an internal standard (4-heptadecanone or S-methylthiobutanoate) were added to 8, 10 or 25 mL of juice and the juices extracted with solvent (2x10 mL) using a Mixxor-like apparatus consisting of two joined 50 mL syringes. The extracts were concentrated to 25-100μL before chromatographic analysis.

Gas Chromatography-Olfactometry

GC-O was performed using an HP-5890 GC (Palo Alto, CA), equipped with a sniff port (DATU, Geneva, New York) and FID detector. A splitter was connected at the end of the capillary column to split the effluent between the FID and sniff port in a 1:2 ratio. Assessors rated the odor intensity of eluting compounds continuously throughout the chromatographic separation process using a linear potentiometer. Aroma descriptors along with approximate retention times were recorded and combined later with the time-intensity data using Chrom Perfect (Justin Innovations, Palo Alto, CA) software. A DB-Wax column (30 m × 0.32 mm i.d. × 0.5 μm), DB-1 column (30 m × 0.32 mm i.d. × 0.25 μm), or a DB-5 column (30 m × 0.32 mm i.d. × 0.5 μm), all from J&W Scientific (Folsom, CA), were used to separate the juice volatiles. GC conditions were as follows: DB-Wax column initial oven temperature 40°C, ramped to 240 °C at 7 °C/min, and held at 240 °C for 5 min.; DB-1 column 40 to 290 °C at 7 °C/min; and DB-5 column 35 to 275 °C at 6 °C/min. Helium was used as the carrier gas at a flow rate of 1.55 mL/min. A Gerstel (Baltimore, MD) cooled injection system model CIS-3 was used to introduce volatile samples into the columns. Initial injector temperature was set at 40°C, and then balistically heated to 225 °C. Detector temperature was 240 °C. A 0.5 μL aliquot of juice extract was injected into the column in the splitless injection mode.

Linear retention index (Kovat's) values were established using a series of alkane standards from C5-C25.

Sulfur Chemiluminescence Detection (SCD)

A Sievers sulfur chemiluminescence detector, Model 350 B with a SCD™ Flameless Interface Model 355 (Sievers Instruments Inc., Boulder, CO), was installed in an HP-5890 GC and operated under the conditions described above. The internal temperature of the SCD burner was 780 °C. Oxygen and hydrogen was maintained at 8 and 100 mL/min respectively. Cell pressure was 4-6 torr and the ozone pressure was set at 8 psi. The effluent of GC was either totally directed to a SCD or spilt between a SCD and the sniff port (DATU, Geneva, New York) using a Gerstel splitter.

Results and Discussion

Aroma Impact Compounds of a Pasteurized NFC Grapefruit Juice

The sensory impression of the juice extract was perceived as grapefruit-like in aroma character. In order to understand which compounds were responsible for a characteristic grapefruit aroma, GC-O was utilized to evaluate the individual odor-active components in the extract. A time-intensity procedure similar to the OSME system described by da Silva (*6*) was used to obtain the aromagram shown in Figure 1. (In Figure 1, the aromagram is inverted to make visual comparisons easier with the corresponding FID chromatogram). Since the olfactory assessors provided a continuous aroma intensity response during the course of the chromatographic run, the resulting time-intensity data set is considered an aromagram just as the corresponding instrumental time-intensity data set is a chromatogram. Thirty-two odor-active components were detected in the concentrated pentane-ethyl acetate juice extract. The relative intensity of each odor-active compound is represented by the peak height in the aromagram. Each of the aroma peaks in Figure 1 is numbered and the corresponding sensory descriptors are given in Table I.

The aroma qualities of the odorants 4, 7, 9, 12, 14, 15, 20, 21, 22 and 32 are summarized as sulfury-grapefruit, fresh-citrusy and sweet and posses many aroma characteristics of the original juice. These defining aroma attributes were reinforced by the odorants 5, 6, 8, 10, 11, 16, 17, 18, 23, 24, 26, 28 and 31. Least intense or characteristic grapefruit-like odorants 1, 2, 3, 19, 27 and 29, may not contribute greatly to the characteristic grapefruit juice aroma; rather, these compounds may serve to impart a subtle fullness or naturalness to the juice aroma.

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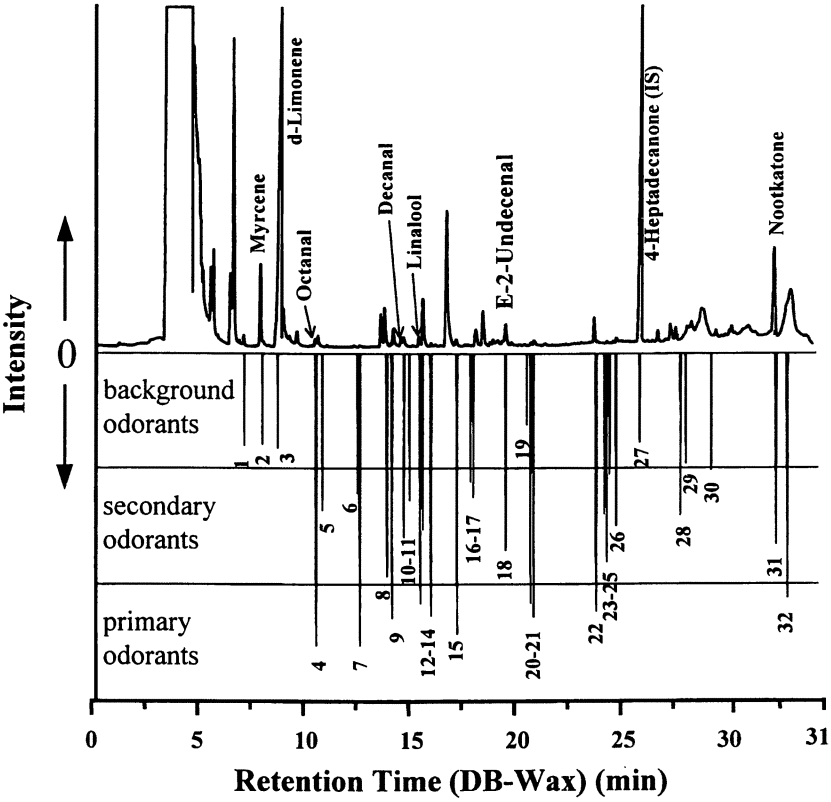

Figure 1. A FID-chromatogram (top) and a time-intensity aromagram (bottom) of a pentane-ethyl acetate (1/1, v/v) aroma extract of a commercial NFC grapefruit juice.

The chromatogram and corresponding aromagram in Figure 1 show that only odorants 1, 2, 3, 4, 8, 10, 12, 18, 27 and 31 have measurable FID signals. These compounds were identified based on MS, retention indices, and matching sensory descriptors. However, the remaining 22 odorants had negligible FID signals. Extensive additional concentration and fractionization were subsequently required to obtain MS data. However, GC-O and calculated retention indices proved more powerful tools to identify most of the remaining unknown aroma compounds.

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Table I. Aroma Impact Compounds Identified in a Pentane-Ethyl Acetate (1/1, v/v) Aroma Extract of a Commercial NFC Grapefruit Juice

#	*Compound*	*Descriptor*	*KI (DB-Wax)*	*KI (DB-1)*
1	Unknown	Fishy	1112	-
2	Myrcene	Geranium	1170	991
3	Limonene	Terpene-like	1210	1030
4	Octanal	Fresh, minty	1298	985
5	1-Octen-3-one	Mushroom	1311	945
6	1,(*Z*)-5-Octadien-3-one	Fresh	1380	-
7	4-Mercapto-4-methyl-2-pentanone	Sulfury, catty	1386	910
8	Acetic acid	Sour	1456	-
9	Methional	Potato	1465	867
10	Decanal	Soapy, beefy	1508	1185
11	(*Z*)-2-Nonenal	Soapy	1520	1125
12	Linalool	Citrusy	1554	1098
13	(*E*)-2-Nonenal	Soapy, fatty	1560	1139
14	*p*-1-Menthen-8-thiol	Grapefruit	1612	1264
15	2-Methylbutanoic acid	Sweaty	1673	932
16	(*E,E*)-2,4-Nonadienal	Fatty	1718	1191
17	3-Mercaptohexyl acetate	Grapefruit	1724	1225
18	(*E*)-2-Undecenal	Geranium	1770	1344
19	3-Mercaptohexan-1-ol	Grapefruit	1820	1107
20	(*E,E*)-2,4-Decadienal	Fatty, fried	1828	1293
21	β-Damascenone	Honey, sweet	1839	1363
22	*trans*-4,5-Epoxy-(*E*)-2-decenal	Green, metallic	2020	1337
23	Unknown	Dish water	2057	-
24	Furaneol	Burnt sugar	2060	1075
25	Unknown	Spicy	2065	-
26	Homofuraneol	Seasoning	2076	-
27	4-Heptadecanone (IS)[a]	Dried fruit	2251	-
28	Unknown	Vanilla, sweet	2355	-
29	Isoeugenol	Pepper	2368	1425
30	Dihydronootkatone	Grapefruit	2396	1681
31	Nootkatone	Grapefruit	2595	1782
32	Vanillin	Vanilla	2617	1360

[a] IS=Internal Standard

The remaining odorants were identified based on aroma descriptors and retention indices on DB-Wax and DB-1 columns. The identities of the odorants are summarized in Table I along with the aroma descriptors and retention indices. Of the 32 odorants, 28 odorants were identified and 4 remain unknown. However, of the 32 identified, 1-(*Z*)-5-octadien-3-one and homofuraneol were only tentatively identified because these two compounds were not detected on the DB-1 column. Odorants with the highest FD values were identified as octanal, 4-mercapto-4-methyl-2-pentanone, methional, linalool, *p*-1-menthen-8-thiol, 2-methylbutanoic acid, (*E,E*)-2,4-decadienal, β-damascenone, *trans*-4,5-epoxy-(*E*)-2-decenal and vanillin. Odorants with relatively intermediate FD values were identified as 1-octen-3-one, 1,(*Z*)-5-octadien-3-one, acetic acid, decanal, (*E,E*)-2,4-nonadienal, 3-mercaptohexyl acetate, Furaneol, homofuraneol and nootkatone.

Recently, the most odor-active volatiles in hand-squeezed grapefruit juice were characterized by GC-O (*4*). Odorants 2, 3, 4, 5, 6, 7, 8, 9,10, 11, 12, 13, 14, 15, 22, 24, 31 and 32 from this study were also reported in the fresh juice study. However, odorants 16, 17, 18, 19, 20, 21, 26, 29 and 30 identified in pasteurized NFC juice had not been reported in the hand-squeezed (unpasteurized) juice study. It is worth noting that seven esters, four aldehydes, two vinyl ketones, and a few miscellaneous odorants, identified in the unpasteurized juice study, were not detected in this work. In addition to the qualitative differences in odor-active compounds mentioned above, the relative odor intensities, expressed in FD factors or aroma intensities, were also considerably different for the majority of the aroma active compounds. However, odor activities of the most potent aroma components such as *p*-1-menthen-8-thiol, 4-mercapto-4-methyl-2-pentanone and *trans*-4,5-epoxy-(*E*)-2-decenal were similar in both studies. Obviously, heat treatments, fruit quality, time of storage, the manner in which the juice was produced from the fruit, plus the procedures used to extract the aroma volatiles from the juice are probable sources for the qualitative and quantitative differences between the two studies.

Of the 24 odorants identified, five odorants were sulfur-containing compounds. The chemical structures of these compounds are illustrated in Figure 2. In addition to *p*-1-menthen-8-thiol, 4-mercapto-4-methyl-2-pentanone and methional, identified in fresh, hand-squeezed grapefruit juice (*4*), 3-mercaptohexyl acetate and 3-mercaptohexan-1-ol were detected for the first time in grapefruit juice. The aroma of these two compounds has been described as grapefruit, passion fruit, and box tree-like and the perception thresholds in alcohol solution were reported to be 4 and 60 ng/L, respectively (*7*). These compounds have previously been identified in both Sauvignon Blanc wines (*7*) and passion fruit (*8*). The relatively low aroma intensities (Figure 1) and the aroma attributes of these two compounds suggest that the contributions to

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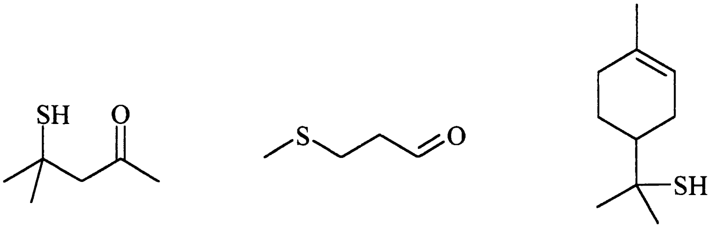
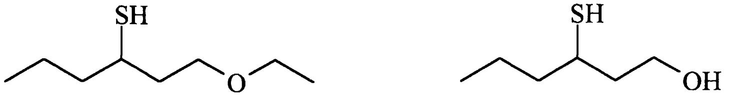
grapefruit juice flavor may be more of a background role to round out, add complexity, and naturalness to the grapefruit juice aroma.



4-mercapto-4-methyl-2 -pentanone (7)

Methional (9)

p-1-menthen-8-thiol (14)



3-mercapto hexyl acetate (17)

3-mercapto hexan-1-ol (19)

Figure 2. Chemical structures of the sulfur-containing odorants found in the pasteurized NFC grapefruit juice.

Effect of Thermal Processing on Sulfur Compounds

Since many sulfur-containing compounds are thermally unstable, changes in sulfur components due to pasteurization were examined. Ethyl acetate was found to extract more sulfur compounds than solvent mixtures of pentane-ethyl acetate or pentane-ethyl ether and as a result was used exclusively for sulfur analysis. SCD-chromatograms of unpasteurized and a pasteurized early season grapefruit juice extracts are compared in Figure 3 with the larger sulfur peaks numbered 1-15. Peaks 1, 6, 11, 12, 14, 15 increased as a result of pasteurization. It is worth noting that the increase in peak 15 was profound. A few sulfur peaks (5, 7 and 10) cannot be detected with any certainty in the unpasteurized extract and appear to be formed due to heat treatment. They are most likely formed from reactions involving sulfur containing amino acids such as cysteine, glutathione (*9*) and methionine (*10*). All three amino acids have been reported in fresh grapefruit juice. Thermal reactions of these non-volatile sulfur-containing amino acids must be responsible for the general increase in sulfur compounds after thermal treatment. On the other hand, a few sulfur compounds decreased in concentrations most likely due to thermal

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decomposition. Peak 6 was tentatively identified as *p*-1-menthen-8-thiol by co-injection of the standard compound.

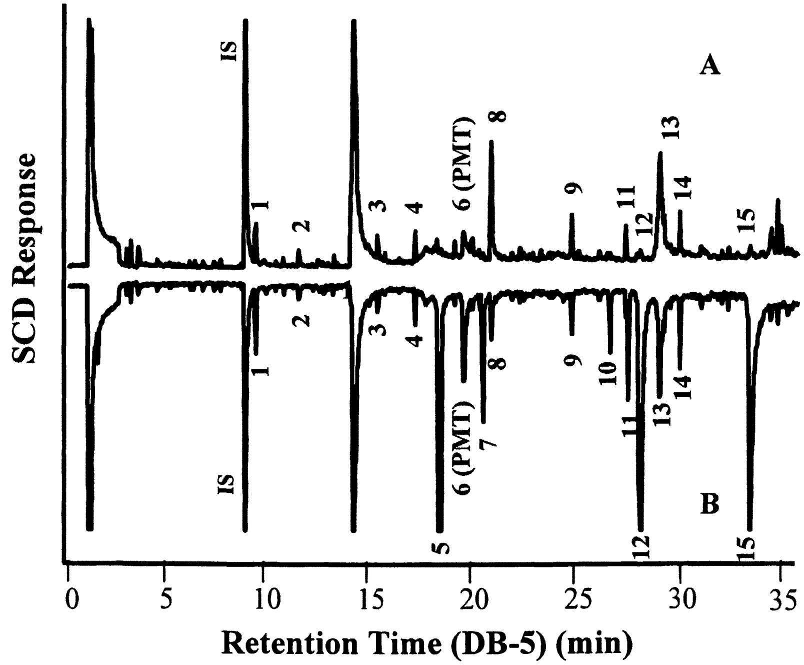

Figure 3. Effect of pasteurization on sulfur compounds in early season grapefruit juice: A) unpasteurized, B) pasteurized. Larger sulfur peaks are numbered, peak 6 = PMT = p-1-menthen-8-thiol. IS = internal standard.

Odor-Active Sulfur Compounds by GC-O and SCD Detection

GC-O was coupled with SCD to determine which odor-active compounds in grapefruit juice contained sulfur. Commercial pasteurized NFC (25 mL) was extracted with ethyl acetate and then concentrated to 25 μL. GC effluent was split into the sniff port and SCD. A time-intensity aromagram and a sulfur chromatogram were obtained simultaneously and the results are compared in Figure 4.

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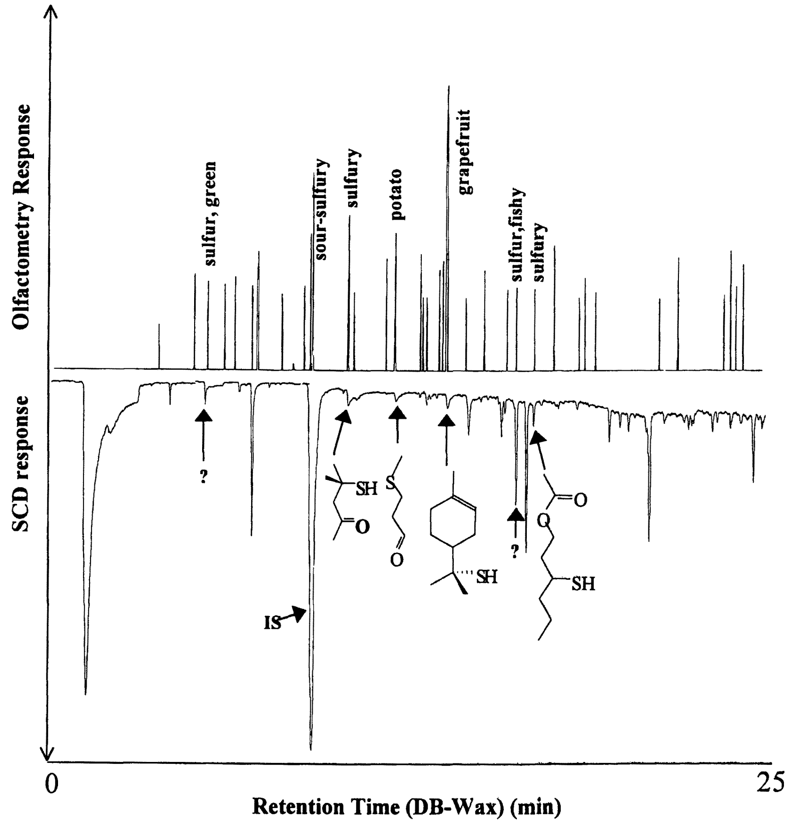

Figure 4. A time-intensity aromagram (top) and a GC-SCD chromatogram of a pasteurized NFC juice obtained simultaneously by coupling of GC-O with SCD.

The SCD chromatogram compared to the GC-O aromagram show at least seven sulfur peaks coincide with odor-active peaks. One of these peaks was the internal standard. There were also two other peaks that could possibly be considered as sulfur compounds, but they will not be discussed further in this study. Four of the remaining six were identified as 4-mercapto-4-methyl-2-pentanone, methional, *p*-1-menthen-8-thiol and 3-mercaptohexyl acetate. Small SCD peaks could be assigned to these compounds. However, this should be confirmed by analysis on a column with a different stationary phase. Whereas the combination of SCD and GCO can be used as demonstrated to determine which peaks have aroma activity, it is also worth noting that several large sulfur peaks were devoid of a corresponding GC-O peak. Therefore, it can be seen that not all sulfur compounds in grapefruit juice are odor-active

Conclusions

GC-O characterization of odor-active compounds in grapefruit juice resulted in the identification of five sulfur-containing odorants, two of which are newly identified in grapefruit juice. The sensory attributes of these compounds (aroma quality, threshold and intensity) suggest these compounds add to the perceived characteristics of grapefruit juice aroma. Thermal pasteurization of grapefruit juice has a significant effect on the sulfur-containing volatiles. GC-O, GC-SCD and, especially, coupling of GC-O with SCD demonstrate good utility for both qualitative and quantitative characterization of sulfur-containing odorants in grapefruit juice. This approach can be easily applied to study the importance of sulfur to aroma in other foods and beverages.

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Chapter 7

Review of Analytical Methodologies for Volatile Nitrogen Heterocycles in Food

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Numerous techniques are employed to isolate nitrogen-containing heterocyclic aroma compounds for chemical characterization and/or quantitation. These include: solvent extraction, adsorption and ion exchange chromatography, molecular and steam distillation, static and dynamic headspace sampling and solid phase micro-extraction. Gas chromatography-mass spectrometry is used most commonly to identify the isolated compounds. However, other analytical techniques used to characterize and/or quantify nitrogen heterocycles include multidimensional chromatography and gas chromatography-olfactometry, as well as gas chromatography with various types of nitrogen-specific detection or atomic emission detection. The most commonly employed of these techniques are described, and examples are cited of their use in analyzing food and beverage flavors for nitrogen heterocycles.

In Heteroatomic Aroma Compounds; Reineccius, G., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2002.

Introduction

Volatile heterocyclic compounds are very important contributors to the flavors of foods and beverages of which the nitrogen-containing constituents make up an important sub-class. They are produced by heat via the Maillard reaction involving sugars and amino acids, by pyrolysis of food components such as proteins, or by enzymatic pathways as in bell peppers, tomatoes and ripened cheeses (*1*). Pyrazines, pyridines, pyrroles, thiazoles, oxazoles and the partially or fully hydrogenated derivatives are the most significant nitrogen-containing heterocyclic compounds that contribute to the flavors of foods. The increased resolution brought about by advances in capillary gas chromatography (GC) have led to its almost exclusive use in the analysis of volatile aroma compounds. Advances in analytical instrumentation led to the identification of pyrazines in roasted and baked foods in the 1960s. Alkylpyrazines were detected in cocoa beans (*2*), coffee (*3*), peanuts (*4*) and potato chips (*5*). Pyrazines, pyridines and thiazoles are also found in essential oils and flowers (*6*). However, until the early 1970's there were very few reports on the flavor properties of the pyrazines. The structure of a compound determines its aroma character. For example, the alkylpyrazines are described as having a roasted nut-like flavor (*7*), while the acetylpyrazines are considered to have a popcorn-like character. A total of approximately 195 pyrazines, 75 pyridines, 140 pyrroles, 100 thiazoles and 85 oxazoles have been identified in natural and processed products (*8*).

The methods used for the isolation and detection of nitrogen heterocycles have much in common with those generally used in flavor analysis. Therefore, this paper is an overview of the more popularly used techniques, both classical and contemporary, with an emphasis on modifications made particularly for nitrogen heterocycles.

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Physicochemical Properties of Nitrogen Heterocycles: Influence on choice of Analytical Methodology

Examination of the flavor research literature reveals a seemingly limitless number of analytical methods designed for the determination of flavor components in foods and beverages (e.g. *9*). However, the vast majority of these techniques are based on different combinations and permutations of what might be regarded as isolation, concentration, separation, identification and quantitation unit operations. The optimum analytical procedure for a given situation depends ultimately on the product matrix being analyzed (e.g., whether continuous or particulate in nature, wet or dry, its fat content, the presence of

Maillard reaction precursors, etc.) and the type of flavor constituents likely to be present (e.g., volatility, water- and fat-solubility and chemical stability).

Table I. Physicochemical Data for Nitrogen Heterocycles and Several other Flavor Components Commonly Found in Foods and Beverages

Compound	*Boiling Point (°C)*	*Water solubility (@ pH 7)*	*Pka of Parent Molecule (and/or Conjugate Acid)*
oxazole	70	soluble	(0.8 estimated value)
pyrazine	115	soluble	(0.65)
2,5-dimethylpyrazine	155	soluble	(1.85)
pyridine	115	miscible	(5.25)
2-ethylpyridine	149	soluble	(5.90)
pyrrole	130	sl. soluble	17.0 estimated value (-0.3 estimated value)
pyrrolidine	87	miscible	(11.27)
thiazole	118	sl. soluble	(2.44)
thiazolidine	165	miscible	(6.2 estimated value)
benzaldehyde	179	sl. soluble	N/A
caproic acid	205	sl. soluble	4.85
d-limonene	176	insoluble	N/A
ethyl acetate	77	soluble	N/A

SOURCE: Most data are from Reference (*10*)

Methods for the analysis of nitrogen heterocycles in foods and beverages should therefore be designed specifically to take advantage of the particular physical and chemical properties associated with these molecules. Table I lists various measured and calculated physicochemical parameters for a number of nitrogen heterocycles frequently found in foods and beverages, together with those for a few other common flavor constituents listed for comparison. In general the majority of nitrogen heterocycles are of only medium volatility; isolation and concentration methods should therefore be chosen with this in mind. As mentioned above, many nitrogen heterocycles are Maillard reaction products derived from the effects of heating sugars and amino-nitrogen compounds. Thus, use of atmospheric distillation methods involving heat, where such precursors could be present, are likely to yield analytical artifacts and should be avoided in favor of vacuum distillation.

Nitrogen heterocycles also tend to be of moderate water-solubility but, more importantly, most have basic characteristics resulting from the presence of

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one or more nitrogen atoms, though, exceptionally, pyrroles behave as weak acids. Thus, as discussed below, some of the most selective and sensitive methods for analyzing nitrogen heterocycles include pH adjustment steps for removing potentially interfering neutral and/or acidic constituents. This can be particularly beneficial in the case of a food or beverage containing, for example, ppb-levels of nitrogen heterocycles in the presence of ppm-levels of neutral and acidic constituents. Improved selectivity notwithstanding, pH adjustment is frequently *not* used, either because of practical difficulties in the case of solid samples, such as meat, or because at the outset the work was not especially aimed at targeting nitrogen heterocycles. An additional caveat is that analytical artifacts can result from changes in the pH of a food or beverage matrix, for example, the formation of Maillard reaction products. Also, pyrroles, oxazolines, thiazolines and thiazolidines are all unstable in acid. Aside from effects on the heterocycles themselves, however, changing the pH of a food or beverage can also have a significant effect on other, major components (e.g., proteins) releasing additional volatiles and altering the binding/release patterns of others.

Isolation and Concentration of Nitrogen Heterocycles

Numerous techniques for flavor isolation are available to the flavor analyst. However, it must be borne in mind that the selected procedure should yield an isolate that accurately reflects the organoleptic quality of the original product. Some of the more commonly used techniques, namely, extraction, distillation, simultaneous steam distillation/extraction, headspace methods and solid phase micro-extraction, in some cases with modifications made specifically for nitrogen heterocycles, are described below.

Distillation

The distillation process separates volatile components from the non-volatile materials of a food matrix. A variety of distillation procedures are available to the chemist and include steam distillation for high boiling compounds with low solubility in water, to molecular distillation carried out under vacuum for constituents that have high boiling points or could decompose at elevated temperatures (*11*). Maga and Sizer (*12*) reviewed the formation theories of pyrazines and the isolation methods used. The authors listed a number of ambient pressure and vacuum distillation techniques used by various researchers. Distillation was normally followed by concentration and fractionation with HCl and then NaOH. The basic fraction was extracted with solvents such as hexane, diethyl ether and dichloromethane. While simple distillation only separates compounds with a wide difference in boiling points, say, 50°C or more, fractional distillation using a spinning band column can separate compounds with a boiling point difference of as little as 0.5°C. Methoxypyrazines in red wines were isolated by atmospheric pressure steam distillation followed by ion exchange resin extraction (*13*). Thiazoles were identified in pressure-cooked beef by atmospheric pressure distillation followed by liquid/liquid extraction with diethyl ether (*14*).

A recent study by Hérent and Collin (*15*) described optimization of vacuum distillation and solvent extraction methods to maximize the recoveries of 18 pyrazines and 9 thiazoles from an aqueous solution. The researchers found that vacuum distillation for five hours and pH adjustment to 0.1 with HCl, followed by four dichloromethane extractions (yielding the acid/neutrals fraction) and then adjustment of the pH of the distillate to 12 with NaOH followed by three extractions with dichloromethane (yielding the base fraction) gave optimum recoveries for most compounds. Recovery factors were correlated to lipophilicity, steric hindrance and electronic density parameters. During distillation, for example, lipophilicity was better correlated to the volatility of the pyrazines and thiazoles than was boiling point.

Solvent Extraction

Solvent extraction may be carried out employing a liquid/liquid procedure, e.g., using a separatory funnel or continuous extractor in the case of beverages and other aqueous systems, or it may be carried out by means of a liquid/solid procedure, e.g., employing a Soxhlet or supercritical fluid extractor in the case of solid foods or suitably immobilized liquids. A number of extractors are available to suit various sample types, sample amounts and solvents.

Solvent extraction may be carried out as a batch or as a continuous process, depending on the water solubility of analytes and the availability of equipment. Continuous extraction methods generally yield higher recoveries but are more time consuming to carry out. In the case of batch extractions, for a given amount of solvent, multiple extractions increase the efficiency compared to a single extraction. Solvents that have very low boiling points are used to facilitate the concentration step. The choice of solvent should be based on the sample type as well as class(es) of compounds of specific interest. For example, with alcoholic beverages a relatively non-polar solvent is preferred. Dichloromethane is considered to be a good solvent, especially for extraction of compounds having an enolone structure, such as maltol and Furaneol®. Addition of sodium chloride increases extraction efficiency by salting out

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organic compounds. Precautions should be taken to minimize emulsion formation through the addition of salts or by pH adjustment.

The isolation of volatile compounds by direct solvent extraction is generally applied in the case of foods that are lipid-free, such as most fruit juices, fruits and vegetables and alcoholic beverages, because lipids are soluble in the solvents normally used such as pentane, diethyl ether and dichloromethane. Nitrogen heterocycles are normally formed in processed food products that also contain lipids. The co-extraction of lipids necessitates the use of a technique such as SDE (see below) or molecular distillation as an additional step to isolate the volatile compounds. Reineccius and Anandraman (*16*) reviewed in detail the various solvent extraction and distillation procedures available for volatile flavor compounds. For example, cocoa beans were placed in a glass chromatographic column and extracted with diethyl ether (*17*). Acid/base fractionation was carried out and the pyrazines quantitated in the basic fraction. Akochi-K *et al.* (*18*) extracted pyrazines from the basic fraction of maple syrup with dichloromethane. As illustrated in Figure 1, acid/base fractionation simplifies the chromatogram by removing most acid and neutral compounds (*19*); additionally, a method for removal of aldehydes and ketones is through formation of sodium bisulfite adducts.

In the work of Hérent and Collin (*15*) referred to earlier, during liquid/liquid extractions the less basic compounds such as 2-acetylpyrazine and 2-isopropyl-3-methoxypyrazine were recovered to a greater extent in the acid/neutral fraction. Polar compounds such as pyrazinylethanethiol were not extracted well by dichloromethane. Basicity, as well lipophilicity, determined partitioning into the organic and aqueous phases. The researchers theorized that the low basicity of compounds such as the methoxy and the acetyl derivatives was due to nitrogen lone pair availability being decreased by steric hindrance.

An optimized supercritical carbon dioxide extraction of brewed coffee (immobilized on a solid support) yielded an extract with reduced proportions of pyrazines as compared to headspace SPME or either dichloromethane or pentane extraction (*20*). Use of an appropriate density and temperature of extraction in the case of peanuts yielded a pyrrole and several pyrazines (*21*). The addition of organic modifiers (e.g., methanol) can increase the efficiency of extraction of polar compounds with supercritical carbon dioxide, which is otherwise a low polarity solvent.

Simultaneous Steam Distillation/Extraction (SDE)

Simultaneous steam distillation and solvent extraction, a method originally developed by Likens and Nickerson (*22*), is one of the most frequently used methods for isolating flavor volatiles. Precautions have to be taken to minimize

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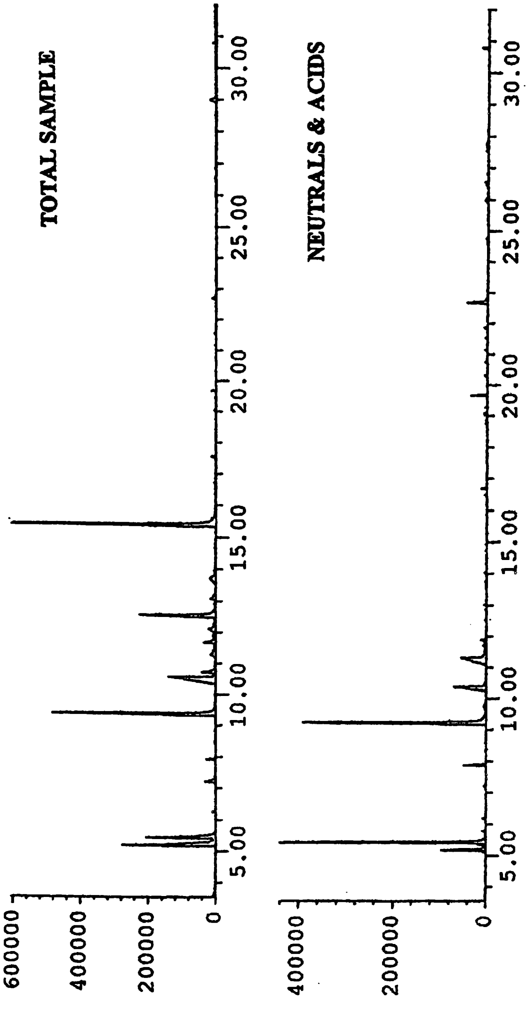

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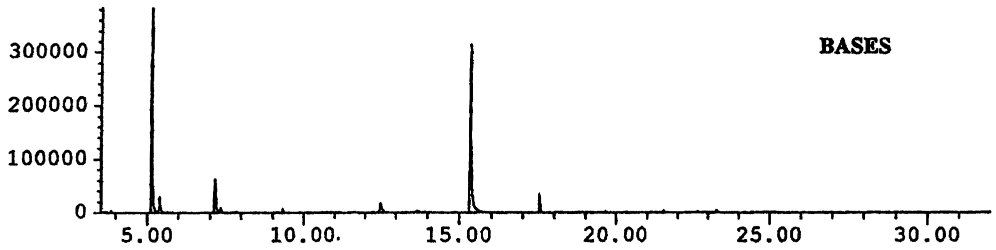

Figure 1. Simplification of the analysis of a Maillard reaction system by pH adjustment and solvent extraction. (Adapted from Reference 19. Copyright 1998 American Chemical Society.)

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contamination and artifact formation due to chemical changes. Accordingly, several modifications to the apparatus have been made to minimize artifacts as well as speed up the process and increase the efficiency of extraction (*23, 24*). An additional approach to minimize artifact formation is to perform solvent extraction prior to distillation, while another is to use vacuum distillation. Molecular distillation is applied to lipid containing food matrices (*25*). Thirty-three pyrazines were identified in pressure-cooked beef after SDE was carried out over 2 days followed by preparative chromatography (*26*). In a variation of the Likens-Nickerson SDE apparatus, Hashim and Chaveron (*27*) used steam-distillation with a micro-distillation apparatus to extract and quantify methylpyrazines in cocoa beans; a pentane-diethyl ether mixture was used as the extracting solvent. Highest recoveries were obtained in forty-five minutes of extraction.

Although SDE has certain drawbacks, such as the possibility of artifact formation and contamination from various sources, it is a powerful technique that has found wide application in the analysis of heterocyclic compounds.

Ion Exchange

The fact that most nitrogen heterocycles identified in foods are basic, with the notable exception of pyrroles, means that ion exchange can be used to isolate them from acidic and neutral flavor constituents. This provides an alternative method to straightforward solvent extraction employing pH adjustment, as described above. However, judicious choice of both resin (e.g., strong or weak cation exchanger) and elution reagent allows a further separation of nitrogen heterocycles of differing basicity. Thus, Peppard and Halsey (*28*) used a column of weakly acidic cation-exchange resin followed by elution with aqueous sodium chloride to isolate pyridines from vacuum steam distillates of beer; pyrazines, being relatively weaker bases than pyridines, remained with the acidic and neutral beer constituents. Work with a model system showed that certain thiazoles could also be isolated by this means, and that use of a strong cation-exchanger could be used to isolate pyrazines, though with rather poor recoveries. Allen *et al.* also used ion exchange to isolate methoxypyrazines from steam distillates of red wine (*13*).

Dynamic Headspace (Purge and Trap) Sampling

Due to inadequate volatility, static (equilibrium) headspace sampling provides insufficient sensitivity for detecting the majority of nitrogen heterocycles. A more sensitive method is based on dynamic headspace sampling (*29*). This technique typically involves passing an inert gas such as helium or nitrogen over or through a sample, and then trapping the extracted volatiles by subsequent passage of the gas stream through a column of adsorptive material, such as charcoal, Tenax® or C_{18}-bonded silica. Volatiles may then be eluted from the trap using solvent, or desorbed by means of heating, with subsequent analysis by GC.

Dynamic headspace sampling represents an excellent qualitative or semi-quantitative method of extracting nitrogen heterocycles having a wide range of volatilities from difficult-to-extract matrices. While reliable quantitation can be problematic, especially in the case of solid samples and where no suitable "blank" is available, standard addition techniques can sometimes be used with good results. Disadvantages include the relatively high cost of equipment needed, the difficulty of optimizing method parameters (choice of adsorbent and trap size, purge gas flow rate and time, desorption temperature and time, etc.) and possible carryover effects between samples. Advantages of the method, on the other hand, are ease of sample preparation and the fact that automation is possible.

There are many examples of the use of this technique for determining nitrogen heterocycles in foods and beverages. For example, Bredie *et al.* used Tenax® with thermal desorption to identify and quantify many pyrroles, pyridines, pyrazines and thiazoles generated during the extrusion cooking of maize (corn) flour (*30*) while Ramarathnam *et al.* used both Florisil and cold traps with solvent extraction to determine various nitrogen heterocycles in pork, beef and chicken (*31, 32*). As mentioned above, the technique can be used in combination with pH adjustment to achieve enhanced selectivity over interfering neutral or acidic constituents, as demonstrated by Kuo *et al.* in the case of a series of alkylpyrazines identified in potato chips (*33*). However, Baudron *et al.*, using Porapak Q with thermal desorption and a fractional factorial designed experiment to optimize several purge and trap parameters, found optimum recoveries of several pyrazines from culture media occurred at pH 5.3 rather than at alkaline pH (*34*). We have also observed, when working with coffee extracts, that raising the pH of the medium does not necessarily improve recoveries of alkylpyrazines achieved by dynamic headspace sampling.

Solid Phase Micro-extraction (SPME)

This is a relatively new technique (*35*) that is based on adsorption of flavor volatiles onto a short length of fused silica fiber coated with a thin layer of adsorptive material. The fiber is mounted on a syringe-like device and several types of coated fiber are available, each differing in selectivity. Commercially available SPME fibers include those coated with: poly(dimethylsiloxane),

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polyacrylate, a poly(dimethylsiloxane)/divinylbenzene blend, a poly(dimethylsiloxane)/Carboxen® blend, a Carbowax®/divinylbenzene blend and a divinylbenzene/Carboxen®/poly(dimethylsiloxane) blend. Adsorption of compounds can take place in the headspace above a solid, semi-solid or liquid sample, or alternatively can occur following immersion of the coated fiber into an aqueous sample. Analytes are then desorbed by heat in the injection port of a gas chromatograph.

SPME-GC can detect trace levels of flavor constituents, including those of low to medium volatility such as the majority of nitrogen heterocycles. It is a very rapid and easy method to perform, and it does not require the use of solvents. The technique is not equally selective for all flavor materials, as selectivity depends on choice of fiber coating. Competition from high concentrations of ethanol, for example in alcoholic beverages, can also be a problem. Results obtained using SPME can be hard to quantify reliably unless great care is taken to ensure appropriate and consistent procedures are followed. Carried out manually, SPME is a very inexpensive technique. However, because the method is equilibrium-based, better results will normally be obtained using a more expensive automated system, properly optimized for parameters such as fiber type, sample contact time and temperature, pH adjustment, addition of salts, desorption time and temperature, etc.).

There are several publications where SPME has been used to determine nitrogen heterocycles in foods and beverages, though in most cases researchers have not made deliberate pH adjustments. Sala *et al.* used headspace SPME to measure levels of 3-alkyl-2-methoxypyrazines in wine musts (*36*). More recently Bicchi *et al.* used SPME with both headspace and liquid sampling to characterize pyridines, alkylpyrazines and other volatile constituents in roasted coffee and coffee beverages (*37*) while Mindrup reported on the use of headspace SPME for the detection and quantitation of numerous pyrazines and one pyrrole in peanut butter (*38*). Coleman studied analytical performance parameters of SPME for a number of Maillard reaction products, including pyrazines, pyridines and thiazoles, in model systems (*39*).

Separation and Detection of Nitrogen Heterocycles

The qualitative and quantitative analysis of volatile nitrogen heterocycles, isolated and concentrated using methods such as those described above, is generally performed using gas chromatography. Crude pre-fractionation may

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also be carried out using column chromatography on adsorbents such as alumina or silica (e.g. *40*), or by employing high pressure liquid chromatography (HPLC). However, the basic property of nitrogen heterocycles that can be utilized to good effect in separating them from neutral and acidic components can also lead to potential problems in chromatography. Thus, attempting separation using materials having an acidic surface can lead to irreversible adsorption or peak tailing, for example, when using alumina for column chromatography. The same phenomenon also tends to occur in GC using wall-coated open tubular glass capillary columns, although this is much less of a problem of late. Solutions in such cases are to use, respectively, basic alumina and bonded phase fused silica capillary columns that have additionally been base deactivated. Since nitrogen heterocycles are sufficiently volatile to be amenable to GC, and because nitrogen selective detectors are available, derivatization has not generally been required for analysis.

GC is generally carried out using a non-selective detector, such as a flame ionization detector (FID) or, less commonly, a thermal conductivity detector (TCD). However, the use of a nitrogen-selective detector can greatly simplify the task of identifying nitrogen-containing heterocycles in complex flavor samples. Available nitrogen-selective detectors include:

1. Nitrogen chemiluminescence detector (CLND)
2. Nitrogen-phosphorus detector (NPD)
3. Pulsed flame photometric detector (PFPD)
4. Atomic emission detector (AED)
5. Electrolytic conductivity detector (ELCD)

The important operating characteristics of these detectors, together with indications of cost and ease of use, are summarized in Table II. The NPD has been used extensively in flavor studies (*36, 42*) for over 20 years. The ELCD and the AED have not found widespread acceptance due to lack of convenience and, in the case of the latter, cost. The CLND and the PFPD are relatively new detectors and their strengths and drawbacks are also listed in the table.

A practical application of nitrogen-selective detection is illustrated in Figure 2. Benn *et al.* (*43*) demonstrated the usefulness of a CLND in identifying a trace adulterant in a peach flavor. The adulterant, 2-isopropyl-4-methylthiazole (peak A), while buried within a mass of peaks, was easily pinpointed by the CLND detector.

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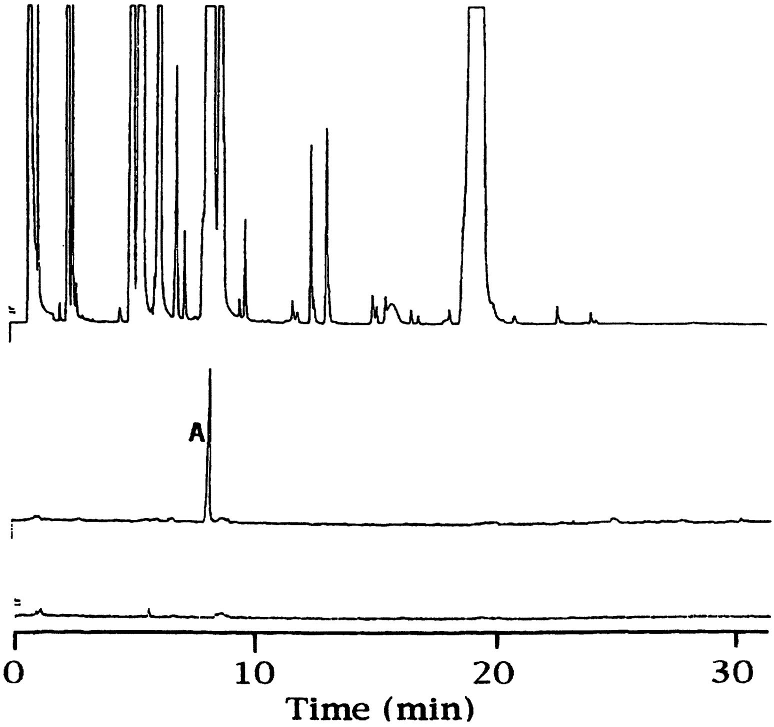

Figure 2. GC-FID and GC-CLND chromatograms of peach flavor with and without the addition of 2-isopropyl-4-methylthiazole (peak A). Upper trace: Peach flavor plus 2-isopropyl-4-methylthiazole by FID. Middle trace: Peach flavor plus 2-isopropyl-4-methylthiazole by CLND. Lower trace: Peach flavor alone by CLND. (Reproduced from Reference 43. Copyright 1993 Elsevier Science.)

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Table II. Characteristics of Nitrogen-Specific Detectors

Detector	*CLND*	*NPD*	*PFPD*	*AED*	*ELCD*
Sensitivity (g.N/s)	10^{-12}	10^{-13}	10^{-11}	10^{-12}	10^{-11}
Selectivity (N/C)	10^{7}	10^{4}-10^{5}	10^{5}-10^{6}	10^{4}-10^{5}	~10^{4}
Linear Response	Yes	Yes	No	Yes	Yes
Linear Range	10^{4}-10^{5}	>10^{4}	10^{3}	10^{4}-10^{5}	10^{4}-10^{5}
Equimolarity	Yes	Yes	No	Yes	Yes
Quenching	Limited	No	Yes	No	No
Ease of use	Simple	Moderate	Moderate	Difficult	Difficult
Cost	Medium	Low	Medium-low	High	Low

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It is also possible to use a mass spectrometer as a specific detector for certain groups of nitrogen heterocycles, as illustrated in Figure 3. This shows an *m/z* 107 selected ion monitoring trace obtained during analysis of the basic extract of a commercial dark beer spiked with 0.1 – 1 ppb levels of nine ethyl- and dimethylpyridines (*28*). Notably, the section of chromatogram shown contains no significant peaks, other than those originating from these pyridines. When nitrogen heterocycles are well resolved from other compounds the mass spectrometer can act as a compound specific detector, yielding a characteristic spectrum. For compounds such as pyrroles, thiazoles, pyridines and derivatives thereof, the odd molecular ion provides a helpful diagnostic tool. For substances which contain an additional sulfur atom, e.g., thiazoles, the relative abundance of the M+2 ion indicates the presence sulfur. When a nitrogen heterocycle is present at trace levels, and especially when it co-elutes with other compounds, identification using a mass spectrometer becomes more difficult. To overcome this difficulty multi-dimensional gas chromatography (MDGC) together with GC-olfactometry may be applied to good effect; the co-eluting or trace compound of interest may be heart-cut from a pre-column onto an analytical column to obtain better resolution, as demonstrated for 2-

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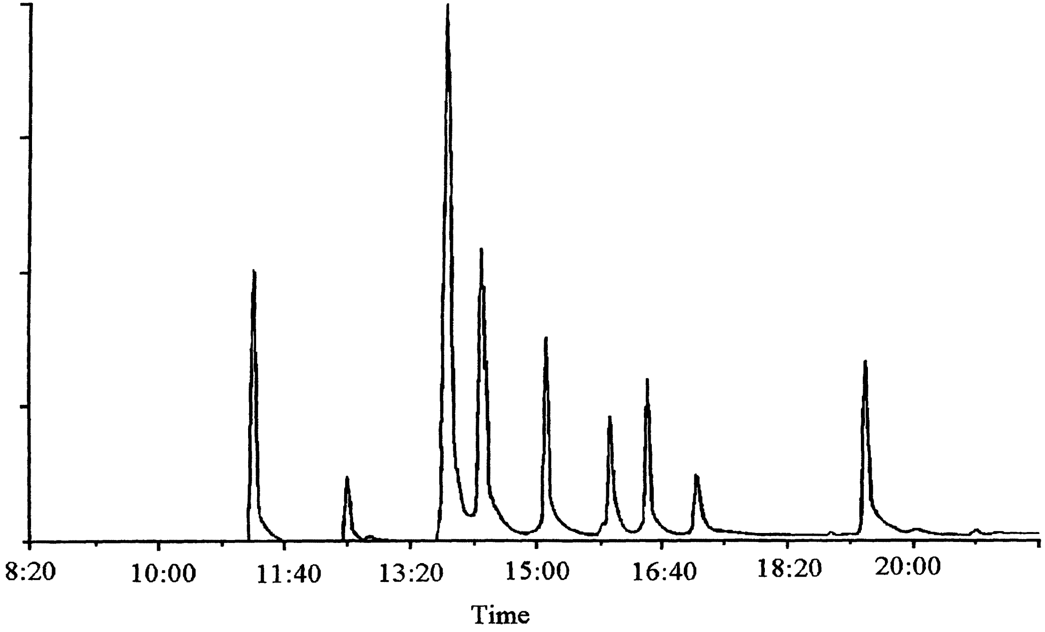

Figure 3. Selected ion monitoring trace (m/z 107) obtained during analysis of commercial beer spiked with nine ethyl- and dimethylpyridines. (Reproduced from Reference 28. Copyright 1980 Elsevier Science.)

acetylpyrroline in basmati rice (*44*). Additionally, mass-spectrometry software that can deconvolute co-eluting trace peaks is now available, thus simplifying the task of analyte detection and identification.

Recently, Schieberle *et al.* (*45*) used chemosensors to selectively detect different classes of compounds at varying temperatures. The researchers indicated that a ZnO/Pt sensor at 300°C was able to detect 2-acetylpyrroline selectively.

As described previously, class separation may be used to good effect with a non-specific detector such as an FID. However, the combination of class separation with use of a nitrogen-specific detector greatly simplifies the task of identifying trace level nitrogen heterocycles in foods and beverages.

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Chapter 8

Biosynthesis of Aroma Compounds Containing Nitrogen

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The characteristic aromas of many natural foodstuffs of plant and animal origin are strongly influenced by trace quantities of volatile nitrogen compounds like amines, imines, pyrazines and pyrroles. The biosynthesis of these and related compounds begins with the conversion of nitrogen gas or nitrate into ammonia. Many nitrogenous volatiles are formed as minor byproducts of amino acid biosynthesis. Some heteroaromatic compounds like pyrazines also appear to be formed under biogenic conditions by a combination of enzyme-catalyzed and non-enzyme-catalyzed reactions. The overall driving force for the latter reactions is probably an exothermic, irreversible final step leading ultimately to stable aromatic nuclei.

In Heteroatomic Aroma Compounds; Reineccius, G., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2002.

Introduction

The importance of nitrogen-containing volatiles in food flavor has been well recognized by food technologists and hundreds, if not thousands of these substances have been catalogued to date. In general the nitrogen-containing volatiles may be formed either thermally, enzymatically or by a combination of thermal and enzyme-catalyzed reactions. Thermally produced nitrogen-containing volatiles are prevalent in heat-processed foods like coffee, cocoa and roasted meat and pathways leading to their formation such as the Maillard reaction have been well investigated (*1*). Nitrogen-containing volatiles also occur in uncooked fruits, vegetables and meat and their formation has, in some cases, been shown to be due to enzyme-catalyzed reactions. Nitrogen-containing volatiles accumulate in trace quantities in many uncooked foods apparently as secondary products of normal metabolism or catabolism. In spite of their low concentrations in foods, many nitrogen-containing volatiles are still flavor-significant because of their low olfactory thresholds. The purpose of this article is to discuss the chemistry and origin of volatile nitrogen compounds that are formed in foods under biogenetic conditions.

In a sense all volatile nitrogen compounds in foods are biologically derived since their precursors, ammonia and amino acids, must first be produced enzymatically from atmospheric nitrogen or nitrate in the soil. Further transformation of ammonia and amino acids under biogenetic conditions leads to diverse nitrogen-containing volatiles including amines, imines, oximes, isocyanates, isothiocyanates, nitriles and nitroalkanes. In addition, some heterocyclic compounds like pyridines, pyrazines, oxazoles, thiazoles and pyrroles also occur in uncooked foods. Perhaps with the exception of methoxypyrazines, the mechanisms for the biogenesis of nitrogen heterocycles are still largely undefined and much work remains to be done in the area.

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Biochemical Origin of Nitrogen-Containing Volatiles Found in Foods

Formation of Ammonia and Amino Acids

In the so-called nitrogen cycle of nature, atmospheric nitrogen is reduced to ammonia and incorporated into nitrogen compounds in plants and animals. After death, these compounds undergo conversion to ammonia during decay and ammonia is eventually oxidized to nitrite/nitrate by soil organisms. Finally, other soil organisms reduce the nitrate back to ammonia (or nitrogen) to complete the cycle. Apropos to the biogenesis of nitrogen-containing flavor volatiles is the formation of ammonia from molecular nitrogen, i.e., nitrogen fixation (*2*). Utilizing hydrogen from water and energy from ATP, an enzyme complex in Rhizobium bacteria found in root nodules of leguminous plants catalyzes the efficient reduction of nitrogen to ammonia under biological conditions. The enzyme, a nitrogenase complex, consists of a reductase and a nitrogenase. Electrons originating in ferridoxin gain enhanced reducing potential in the reductase aided by ATP. With enhanced potential the electrons flow into the nitrogenase reducing the bound nitrogen into ammonia. In the overall process two moles of ammonia are formed from nitrogen at the expense of twelve moles each of water and ATP. Naturally under biological conditions, ammonia is generated in the form of ammonium ions.

In the next step for incorporation of nitrogen into living species, ammonia is utilized in amino acid biosynthesis. The synthesis of all natural amino acids begins with the synthesis of glutamate. Basically, α-ketoglutarate, a citric acid cycle intermediate, undergoes reductive aminination with ammonia to form glutamate in a coupled redox process catalyzed by *glutamate dehydrogenase.* The enzyme co-factor in these reactions is nicotine adenine dinucleotide (NAD^+) whose reduced form, NADH, provides hydrogen ions and electrons (the biochemical equivalent of hydride ions) to affect the reduction. Beginning with glutamate, the nitrogen atom is further transferred to diverse metabolic intermediates to form all twenty amino acids found in nature. Pathways to individual amino acids are complex and beyond the scope of this review (*3*); however, the basic process of transamination is discussed below since related reaction intermediates lead to the formation of volatile amines.

Biosynthesis of Amines and Imines

An excellent review by Maga (*4*) cites the occurrence of a prodigious number of simple, mostly aliphatic amines in a wide variety of foodstuffs. Within twelve food categories mentioned to contain amines, the six with the largest numbers of different amines were poultry [24], meat [23], cereal products [21], vegetables [19], fruits [16] and fish/fish products [13]. Structurally, most of the amines found in foods are primary amines, however, a small number of secondary amines and a still smaller number of tertiary amines exhibit widespread occurrence. For the primary amines a homologous series is observed extending from methylamine to octyl- and benzylamine and many structures are indicative of amino acid decarboxylation as their mode of formation, e.g. valine => isopropylamine, etc. Secondary and tertiary amines

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mostly contain a multiplicity of the same kinds of alkyl groups found in primary amines. Besides the simple amines, polyamines like putrescine (1,4-diaminobutane) also occur in foods and whose odors one usually associate with putrification. The well-known fishy, ammoniacal odor of amines is usually suppressed at normal food pH by salt formation perhaps with the exception of compounds with extremely low aroma thresholds, i.e. like trimethylamine in fish products. Also, the fact that amines bind tightly to carbohydrates might further limit their odor display in foods (*5*).

The biosynthesis of many primary amines found in foods is closely related to amino acid biosynthesis (*3*). Several biogenetic pathways are governed by a group of remarkably versatile enzymes called pyridoxyl phosphate-enzymes that contain the same prosthetic group, pyridoxal phosphate (POP). Normally, the pyridoxal moiety is bound to the enzymes through an imine linkage at the ε-amino group of lysine. During biotransformation, amino acid substrates, $RCH(NH2)CO_2H$ exchange with protein-bound lysine to form imine (Schiff base) linkages with POP (Figure 1).

In normal amino acid metabolism an *amino acid transaminase* facilitates C-H bond breakage at (A) leading to transamination and formation of an α-keto acid derived from the original α-amino acid. The by-product of path A (not shown) is pyridoxamine phosphate (PAP) which can react to form Schiff bases with different α-ketoacids to create new amino acids, for example PAP + pyruvate => POP + alanine. So, in general, *amino acid transaminases* catalyze the interconversion of specific α-amino acids and α-keto acids. In foods, PAP may react chemically with simple carbonyl compounds to generate amines, but the involvement of enzyme catalysis is questionable. In the catabolic reaction, path B, an *amino acid decarboxylase* facilitates C-CO2 bond breakage at (B) leading to loss of carbon dioxide and the formation of a primary amine.

Structures of resultant amines are those of the parent amino acids minus the carboxyl carbon atom. Likewise the diamines, like putrescine and cadaverine, result from decarboxylation of the diamino acids ornithine and lysine, respectively. Higher polyamines, like spermidine (a triamine) and spermine (a tetraamine), are biosynthesized from diamines and occur in many plants and animals. However, the relatively high basic strength of polyamines usually precludes any participation in normal food aroma. Most simple amines are minor products in the normal biochemistry of plants and animals and the enzymes required for their formation are usually found in bacteria associated with decay. Pressor amines (compounds which raise blood pressure), like benzylamine, tyramine, 2-phenylethylamine, histamine and serotonin, are also formed by amino acid decarboxylation, but their presence in foods has not been associated with food aroma.

Not all amines found in foods are formed from amino acid precursors. However, amines of all classes and structure types can be formed by reductive

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amination of carbonyl compounds via a putative biochemical route such as shown in Figure 2. Starting with carbonyl compounds from other biosynthetic pathways and ammonia (or a primary amine), water is eliminated to form an imine (a Schiff's base); or, in the case of secondary amines, an iminium salt. Coupled reduction with suitable reductases and co-factors (like NADH) can then explain the formation of certain secondary and tertiary amines in foods and the occurrence of amines whose carbon skeletons do not resemble those of amino acids. The fact that twenty-one diverse amines of all three classes were detected in a uniform bacterial culture, i.e., *Streptococcus lactis,* lends support to such a mechanism (*6*).

Extensive studies have shown that trimethylamine and dimethylamine observed frequently in fishery products are derived from trimethylamine oxide (TMO) contained in the muscle tissue and visceral organs of sea fish and invertebrates (*7*). The origin of TMO is uncertain, but conceivably it is formed by hydrolysis of betaine (carboxymethyltrimethylammonium hydroxide, inner salt) which occurs along with TMO in the body fluids of saltwater fish. Post-mortem cleavage of TMO proceeds in two directions; reduction and demethylation. Enzymes responsible for both reduction and demethylation of TMO have been isolated from the liver of the lizard fish, *Saurida tumbil* (*8*), however, more recent studies have shown that a demethylation reaction leading to dimethylamine and formaldehyde may also be catalyzed by catabolic products of cysteine, i.e., cysteinesulfinic acid and ferrous ion (*9*). The subject of amines in fishery products has been thoroughly reviewed (*10*).

Arylamines, like methyl anthranilate, methyl N-methylanthranilate and 2-aminoacetophenone, are significant aroma compounds in several natural products including labrusca grapes (*11*). The volatile anthranilates appear to be secondary metabolites of anthranilic acid, a normal intermediate in tryptophane biosynthesis (*3*). Conceivably, methylation of anthranilic acid takes place via a non-specific *methyltransferase* and S-adenosylmethionine. Other esters of anthranilic acid probably are products of subsequent transesterification by ambient alcohols. 2-Aminoacetophenone is probably a degradation product of kynurenine, a well-known tryptophane catabolite (Figure 3). Transamination of kynurenine would afford an α,γ-diketoacid whose acid-catalyzed fragmentation can produce 2-aminoacetophenone. The acid catalysis may be facilitated by ammonium salt formation.

Imines, a.k.a. Schiff's bases, have received little attention as aroma compounds with the exception of acylated cyclic analogs like 2-acetyl-1-pyrroline, which occur in many heat-treated foods (*12*). Recently, 2-acetyl-1-pyrroline was isolated in fermenting cocoa and a biochemical origin was proposed for its formation (*13*). Labeling experiments with ^{13}C and ^{15}N showed that *Bacillus cereus* growing in cocoa masses is able to synthesize 2-acetyl-1-pyrroline from glucose, glutamic acid and proline. Since *B. cereus* occurs

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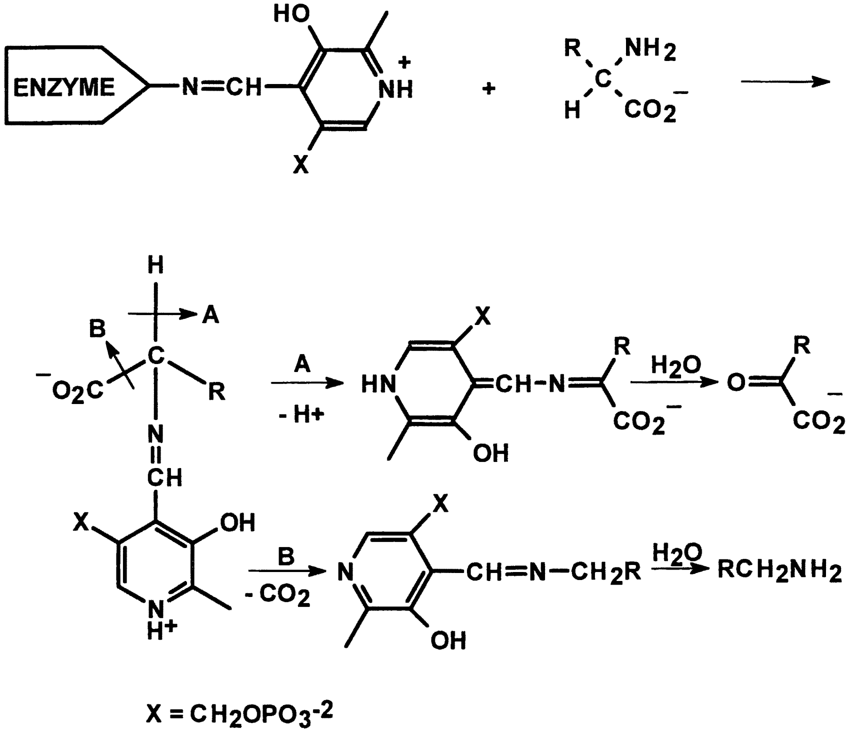
ENZYME
N=CH
HO
+
NH
X
+
R
NH2
C
H
CO2
-
H
A
B
C
-O2C
R
N
CH
X
OH
N
H+
A
- H+
HN
X
=CH-N=
R
CO2-
OH
H2O
O=
R
CO2-
B
- CO2
N
X
CH=N-CH2R
OH
H2O
RCH2NH2
X = CH2OPO3-2

Figure 1. Transamination and Decarboxylation of α-Amino Acids

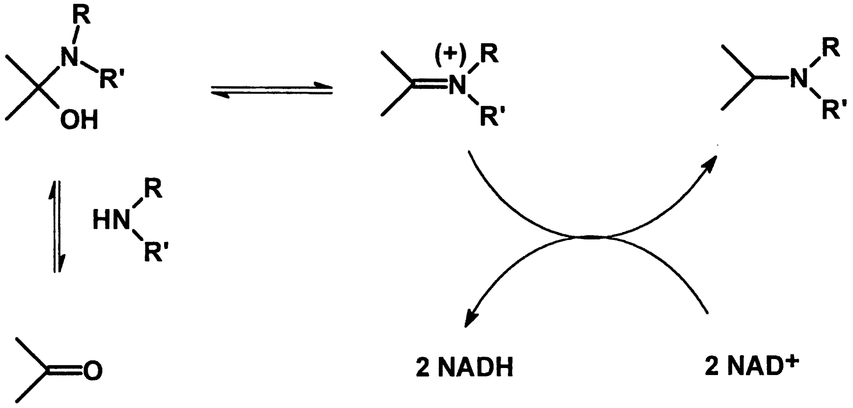
R
N
R'
OH
(+) R
N
R'
R
N
R'
HN
R
R'
O
2 NADH
2 NAD+

Figure 2. Reductive Amination Scheme

naturally in many unprocessed cereal grains, it was claimed that specific strains might have been an unrecognized factor with 2-acetyl-1-pyrroline formation. Aliphatic imines can also have potent food-like odors, but to date few examples have been observed in food and these appear to be products of Maillard reactions (*14*). Aliphatic imines of pure biochemical origin have not yet been reported in foods, although a cyclic analog, 1-pyrroline is a known biological oxidation product of spermidine, which itself is a minor constituent of many foods (*4*).

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Glucosinolates and Related Decomposition Products

Glucosinolates are non-volatile thioglycosides which are biosynthesized in plants of the *Cruciferae* family and are hydrolyzed to form nitrogen-containing volatiles when plant tissues are disrupted or damaged (*15, 16*). Among the *Cruciferae,* the food plant subgroup *Brassica* includes common varieties with well-known pungent flavors like mustard, horseradish and brussel sprouts. The pungent flavor of these foods is due in part to volatile isothiocyanates, thiocyanates and nitriles that are formed as a result of glycoside hydrolysis.

Biosynthesis of Glucosinolates, Oximes and Nitroalkanes

The biosynthesis of glucosinolates has been suggested by radioactive tracer studies to proceed according to the sequence shown in Figure 4 (solid arrows), with key intermediates identified as aldoximes, thiohydroximates and desulfoglucosinolates (*17*). α-Amino acids are N-hydroxylated and undergo oxidative decarboxylation to form aldoximes in a pathway shared by the biosynthesis of cyanogenic glycosides (*18*). In a way yet to be defined, sulfur is transferred from cysteine to aldoximes to form thiohydroximic acid (salts) which are bioglucosylated with UDP-glucose to form desulfoglucosinolates. Ultimately, sulfation occurs, catalyzed by a sulfotransferase, and glucosinolates are formed (*17*). Some glucosinolates are clearly derived from protein amino acids, i.e., with R = methyl, or isopropyl coming from alanine and valine; however, the majority have side chains that require some degree of prior modification such as homologation, dehydrogenation or hydroxylation. Ample evidence exists (3) that such processes can occur along the biosynthetic pathway. Aldoximes are intriguing intermediates since they appear to participate in several biosynthetic pathways. Besides their involvement with glucosinolates and cyanogenic glucosides, they may also serve as precursors of nitroalkanes. Many years ago it was suggested that aldoximes might be oxidized to nitroalkanes and that the tautomeric (aci-forms) might be an

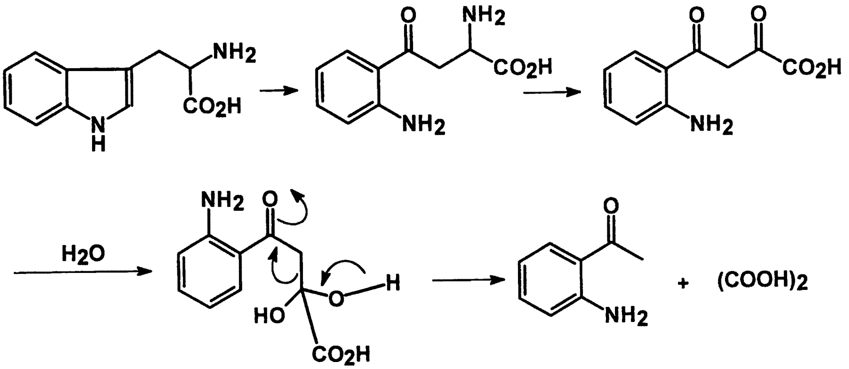
NH2
CO2H
N
H
O
NH2
CO2H
NH2
O
O
CO2H
NH2
H2O
NH2
O
HO
O
H
CO2H
O
NH2
+ (COOH)2

Figure 3. Formation of 2-Aminoacetophenone

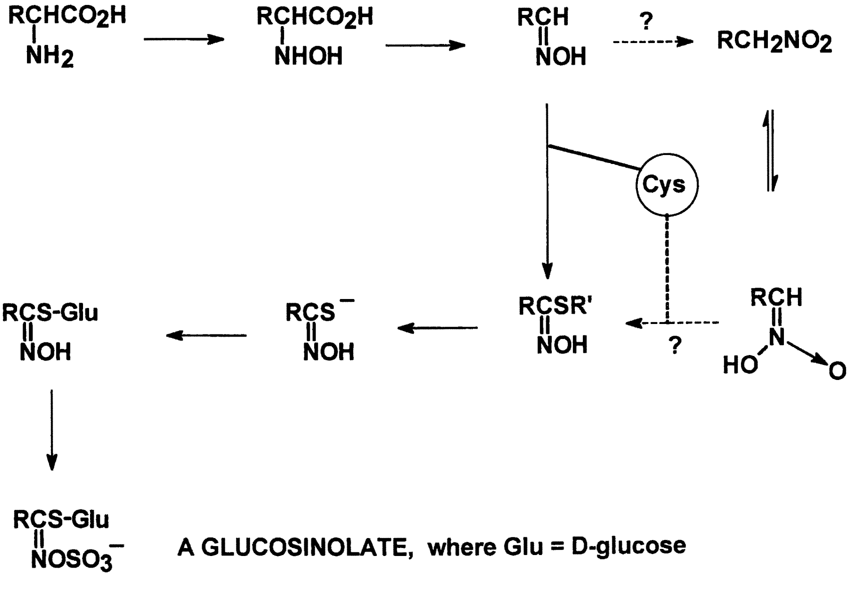
RCHCO2H
NH2
RCHCO2H
NHOH
RCH
NOH
?
RCH2NO2
Cys
RCSR'
NOH
?
RCH
N
HO
O
RCS⁻
NOH
RCS-Glu
NOH
RCS-Glu
NOSO3⁻
A GLUCOSINOLATE, where Glu = D-glucose

Figure 4. Biosynthesis of Glucosinolates and Related Compounds

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alternative entry point for cysteine sulfur in glucosinolate biosynthesis (Figure 4) (*19*). Nitroalkanes have not been detected in glucosinolate biosynthesis, however, they do co-occur naturally with structurally related aldoximes and nitriles in flower volatiles (*20, 21*) and in fresh tomato fruit (*22*). Buttery has proposed that nitroalkanes found in tomato are formed by oxidation of oximes in a modification of the known pathway to cyanogenic glycosides. Although he did not detect oximes in tomato, he did observe nitroalkanes, aldehydes and nitriles whose structures are obviously derived from valine, leucine and phenylalanine and whose co-existence support the proposed mechanism.

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Formation of Isothiocyanates, Thiocyanates and Nitriles from Glucosinolates

When Cruciferous plants are crushed, glucosinolates rapidly undergo a series of enzymatic and non-enzymatic reactions to form flavor-significant volatile products including isothiocyanates, thiocyanates and nitriles (Figure 5). Initially a thioglucosidase (myrosinase) catalyzes hydrolysis and formation of chemically unstable aglycones which are thiohydroximate-O-sulfonates. Myrosinases are known to be groups of isoenzymes within individual plants and some data suggest that enzymes from one plant can accept a wide range of glucosinolate substrates (*23*). The most commonly formed products of non-enzyme catalyzed aglycone decomposition are isothiocyanates and nitriles. At neutral pH a rearrangement similar to the Lossen rearrangement of acylhydroximates occurs by migration of R-groups from carbon to nitrogen with concomitant loss of sulfate ion to form isothiocyanates (path A in Figure 5). Nitriles are formed more readily at acidic pH and in the presence of ferrous ions by a redox process in which both sulfate ion and elemental sulfur are eliminated. Thiocyanate production is relatively rare and occurs only with allyl-, benzyl- and 4-(methylthio)butylglucosinolates. A recently referenced mechanism for thiocyanate formation (Figure 5, path B) suggests that an isomerase catalyzes Z => E isomerization of the natural Z-aglycone isomers, and that only E-aglycones with stable cationic R-groups are able to undergo rearrangement to thiocyanates (*23*).

Approximately eighty glucosinolates have been reported in plants with diverse structures where R = alkyl, hydroxyalkyl, arylalkyl, alkenyl, hydroxyalkenyl, ω-(methylthio)alkyl, ω-(methylsulfinyl)alkyl and ω-(methylsulfonyl)alkyl and most of whose decomposition has been shown to produce structurally related isothiocyanates and nitriles. Some alkenylglucosinolates decompose to form epithioalkyl cyanides instead of the predicted alkenyl cyanides plus elemental sulfur. Also, 2-hydroxy-ethylglucosinolates do not afford the predicted 2-hydroxylethylisothiocyanates, but yield instead cyclized products, oxazolidine-2-thiones. A compilation of food relevant glucosinolate decomposition products has recently appeared (*16*) and will not be reproduced here.

Biosynthesis of Volatile N-Heteroaromatic Compounds

The occurrence and flavor significance of N-heteroaromatic compounds in foods has a long history (*24*). Usually heteroaromatics are associated with heat-processed foods, but occasionally they appear in raw or unprocessed foods and appear to be the products of biosynthesis either in the food itself or in microorganisms allied with the food. Biosynthetic routes have been proposed for numerous pyridines, pyrazines and pyrroles, but in many cases detailed formation mechanisms still remain obscure. Most often it has been suggested that biologically derived N-heteroaromatics arise from a combination of enzyme-catalyzed and spontaneous, non-enzyme-catalyzed reactions.

Pyridines

Pyridine and many of its volatile derivatives occur widely in both processed and unprocessed foods (*25*). A recent review emphasized the likelihood of pyridine biogenesis in foodstuffs by listing the occurrence of sixty pyridine derivatives in the essential oils of fruits, vegetables and herbs (*20*). Most of the pyridines were mono- or disubstituted alkyl or alkenyl derivatives with straight or branched sidechains ranging in size from methyl to octyl. The final step in the formation of pyridines in foods has been explained by invoking reactions of aldehydes and amines, i.e., the so-called Chichibabin reaction (Figure 6). In this reaction, unsaturated aldehydes react with ammonia or primary amines to form dihydropyridines or dihydropyridinium salts, respectively. Since the starting aldehydes can themselves be products of aldol condensation/dehydration reactions between two or more carbonyl compounds, the variety of pyridines possible is virtually endless. Exactly which, if any, elements of this sequence is enzyme catalyzed is unknown. Mechanistically it's possible that biologically derived amines and aldehydes exist in equilibrium with dihydropyridines and that these in turn are oxidized to pyridines. The driving force for pyridine formation is very likely the thermodynamic preference for an aromatic (benzenoid) ring in the final step.

Evidence for the sequence in Figure 6 is circumstantial, yet convincing. For example 2-pentylpyridine present in orange oil (*20*) can be formed in the reaction of 2,4-decadienal known to be present in the oil and ammonia. Previous model studies suggested that 2-pentylpyridine is formed thermally from 2,4-decadienal and ammonia (*26*). In orange oil the dienal precursor may

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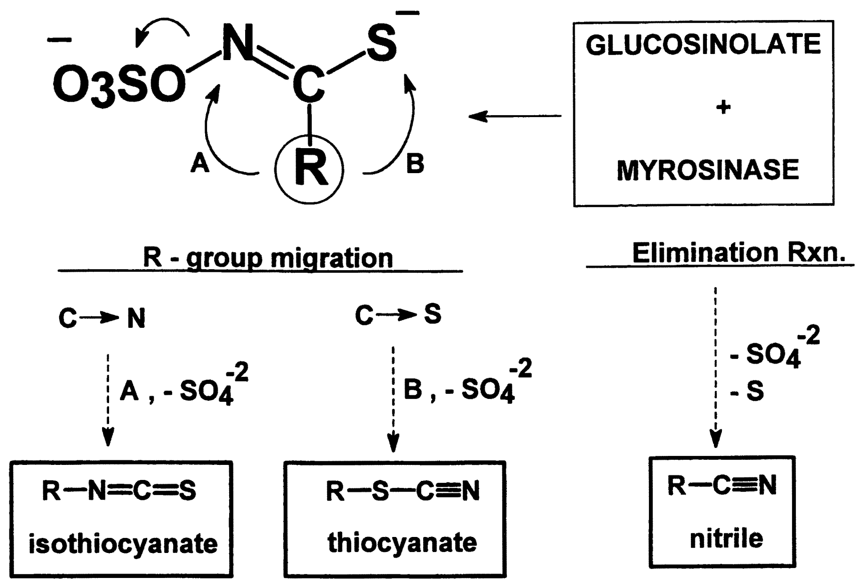
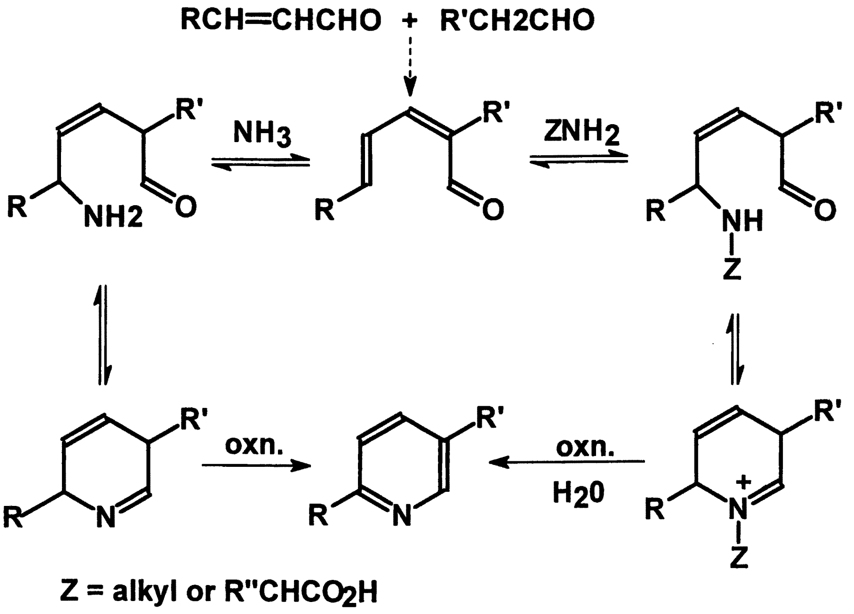


$^{-}O_3SO$
N
C
S
R
A
B
GLUCOSINOLATE + MYROSINASE
R - group migration
Elimination Rxn.
C → N
C → S
A , - SO_4^{-2}
B , - SO_4^{-2}
- SO_4^{-2}
- S
R—N=C=S
isothiocyanate
R—S—C≡N
thiocyanate
R—C≡N
nitrile

Figure 5. Non-enzymic Reactions of Thiohydroximate - O - sulfonates



RCH=CHCHO + R'CH2CHO
NH3
ZNH2
R
R'
NH2
O
NH
Z
oxn.
oxn.
H2O
N
+
Z = alkyl or R"CHCO2H

Figure 6. Pyridine Synthesis

have arisen from lipoxygenase catalyzed linoleate oxidation. In a similar way 2-isopropyl-5-methylpyridine reported in parsley and carrot seeds can result from a reaction of 2-isopropyl-2,4-hexadienal and ammonia. In this case the dienal can result from aldol condensation/dehydration of 3-methylbutanal (from leucine) and 2-butenal (from lipid oxidation or aldol condensation of acetaldehyde).

Pyrazines in General

Pyrazines have been recognized as important food flavor components for at least seventy-five years and many reviews have documented their occurrence and modes of formation. Pyrazines are formed in heat-treated foods by completely non-enzymic processes and in uncooked foods or living organisms by what appears to be a combination of non-enzymatic and enzyme-catalyzed processes. The biogenesis of pyrazines has also been the subject of detailed reviews (*27, 28, 29*) and will be briefly considered here. From a mechanistic standpoint, pyrazine biogenesis falls into two distinct categories, namely alkylpyrazines and methoxypyrazines. Historically, alkylpyrazines have long been associated with heat-treated foods and are only occasionally mentioned in connection with living organisms or fermented foods. Conversely, methoxypyrazines rarely occur as heat-induced artifacts and appear more like natural secondary metabolites.

Alkylpyrazines

Naturally occurring alkylpyrazines are probably formed in spontaneous chemical reactions from biologically produced α-hydroxycarbonyls, ammonia and/or aminoacetone (Figure 7). The construction of the pyrazine ring can begin with an α-hydroxycarbonyl precursor like hydroxyacetone as shown, or one of its homologs collectively known as acyloins. Acyloins accumulate in foods under certain circumstances like those induced by bacteria in cheese ripening (*30*). Starter cultures containing Leuconostoc strains of Streptococci develop excess pyruvate and acetaldehyde due to overload of the TCA cycle in the lactose-rich medium. To offset the buildup, thiamine pyrophosphate enzymes (TPP-enzymes) convert excess carbonyl compounds into the acyloin, acetoin (2-hydroxy-3-butanone), which accumulates. Acetoin is formed either from pyruvate via α-acetolactate followed by decarboxylation or from acetaldehyde via acyloin condensation with acetyl-TPP. Acetoin is the most likely precursor of naturally ubiquitous tetramethylpyrazine.

Acyloins like hydroxyacetone and acetoin are aminated to form α-

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aminocarbonyls which cyclodimerize with loss of water to yield dihydropyrazines and pyrazines. The amination of acyloins to form pyrazines is not known to be enzyme catalyzed, but it does take place spontaneously in model systems under biological conditions (*31*). The structural diversity of natural alkylpyrazines can be partially explained by acyloin chemistry, since a few acyloins can generate many pyrazines. Thus, due to ene-diol tautomerism, a single unsymmetrical acyloin like hydroxyacetone yields two isomeric aminocarbonyls, i.e., aminoacetone and 2-aminoacetaldehyde, and, therefore, (after cyclodimerization/oxidation) two isomeric pyrazines: 2,5- and 2,6-dimethylpyrazine. In a similar way two unsymmetrical acyloins can form six alkylpyrazines and so on to generate a wide variety of di, tri and tetrasubstituted alkylpyrazines.

In lieu of acyloins, aminoacetone formed by alternative pathways can also act as a pyrazine precursor. Aminoacetone is unstable and reacts spontaneously to form dihydropyrazines and pyrazines at room temperature (*31*). Aminoacetone is a natural catabolic product of L-threonine and it has been detected in *S. Marcescens*, an organism known to produce a rich variety of pyrazines (*28*). Whereas self-condensation of aminoacetone should lead only to 2,5-dimethylpyrazine, in fact, the process is more complex and higher alkylated pyrazines have been observed (*31*). In addition, it has been shown that intermediate dihydropyrazines will react with added aldehydes to form trisubstituted derivatives (Figure 7) (*28*).

The driving force for the formation of alkylpyrazines seems to be the irreversible formation of the thermodynamically stable pyrazine ring. The oxidative conversion of dihydropyrazines to pyrazines is not well understood except for it being a very facile process and one not always requiring molecular oxygen (*32*). More work is needed to identify the reduction products of this reaction and to clarify the reaction mechanism.

Methoxypyrazines

Methoxypyrazines occur widely in nature, but in contrast to alkylpyrazines, their structural diversity is rather limited (*28*). In fact, with few exceptions, the field of methoxypyrazines is dominated by three compounds, 2-methoxy-3-isopropyl-, 2-methoxy-3-isobutyl- and 2-methoxy-3-sec.-butylpyrazine. The similarity in methoxypyrazine side chain structures with side chains of protein amino acids, valine, leucine and isoleucine, immediately suggested amino acid involvement in their biosynthesis.

In vivo biosynthesis of 2-methoxy-3-alkylpyrazines was studied in several *Pseudomonas spp.* and conclusive evidence was found for an amino acid precursor. When L-[1-^{13}C]valine was added to a growing culture of *P.*

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taetrolens at the time when the production of 2-methoxy-3-isopropylpyrazine (MIP) was optimum, the valine carbon skeleton was incorporated intact into the pyrazine (*33*). Analysis of ^{13}C-NMR data on the isolated methoxypyrazine proved that C-2 in the pyrazine ring was derived from the carboxyl carbon of valine.

In a related study, cultures of *P. perolens* were grown using ^{13}C-labelled pyruvate as the sole source of carbon and isolated MIP was analyzed by NMR (*27, 34*). The labeling pattern observed in MIP indirectly confirmed the incorporation of valine. Thus, the carbon atoms in MIP attributed to valine were labeled in the same way that valine would be labeled biosynthesized from pyruvate according to accepted pathways. Carbons at C-5 and C-6 in MIP were also labeled with ^{13}C but the ratio of their NMR signal intensities was reversed from what was predicted had C-5 and C-6 been derived from glycine (via pyruvate). Based on these results the authors concluded that glycine was not involved and that a diketopiperazine derived from valine and glycine was not an intermediate in MIP biosynthesis. The authors also proposed an alternate pathway for MIP suggesting glyoxylic acid as a source for carbons C-5 and C-6. The glyoxylic acid mechanism explains the NMR data but is inconsistent with the fact from previous work (*33*) that glyoxylic acid neither supported the growth of *P. perolens* nor led to the production of MIP *in vivo*. Significant labeling of the methoxy group in MIP was observed with both C-2 and C-3 labeled pyruvate. Since methyl-labeled methionine is known to be biosynthesized from 3-^{13}C-pyruvate the authors proposed that the methoxy group in MIP was introduced enzymatically via S-adenosyl-L-methionine.

A mechanism for MIP formation that is consistent with results of labeling studies is shown in Figure 8. Pyruvate is assimilated to generate amino acids including valine and serine. Serine is transaminated to β-hydroxypyruvate, which exists in equilibrium with 2-hydroxy-3-oxopropionic acid. Ambient valine reacts with the aldehyde to form a Schiff's base and cyclization takes place to form a γ-lactone. The lactone undergoes decarboxylation and amination to yield a dihydro [1-H]pyrazinone. Finally O-methylation and oxidation (-2H) [or the reverse sequence] occurs to produce MIP.

According to this mechanism C-2 labeled pyruvate will introduce the label at C-6 in MIP and C-3 labeled pyruvate will introduce the label at C-5. In practice both C-2 and C-3 labeled pyruvates led to labeling at both C-5 and C-6 in MIP. However, in accord with Figure 8, C-2 labeled pyruvate introduced about twice as much label at C-6 vs. C-5 in MIP and the reverse was observed with C-3 labeled pyruvate. The lack of complete labeling specificity can be explained in terms of relative reaction rates and the chemical nature of the intermediates. If 2-hydroxy-3-oxopropionic acid undergoes decarboxylation faster than it reacts with valine, hydroxyacetaldehyde will be formed. Through enolization, hydroxyacetaldehyde can form a symmetrical ene-diol structure

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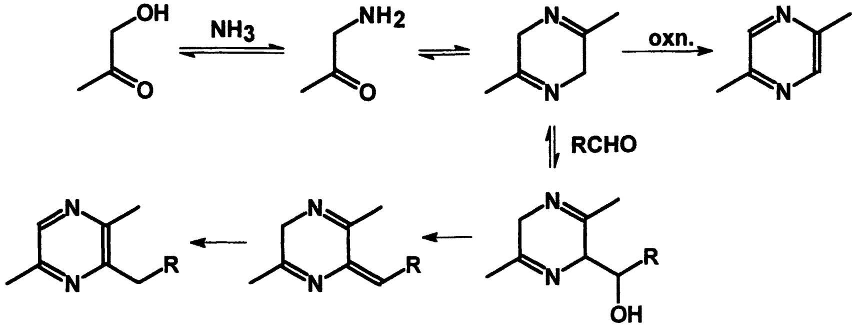
OH
NH3
NH2
O
N
oxn.
RCHO
R
OH

Figure 7. Formation of Alkylpyrazines

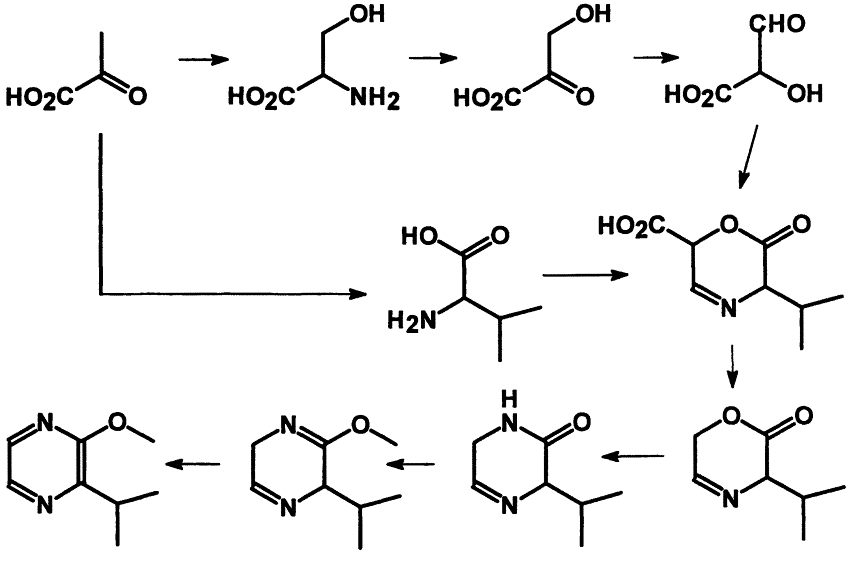
HO2C
O
OH
NH2
CHO
HO
H2N
N
H

Figure 8. Putative Biosynthesis of 2-Methoxy-3-isopropylpyrazine

(1,2-dihydroxyethene) and thereby lead to partial scrambling of the ^{13}C label before it finally reacts with valine. The result of label scrambling is a tendency to produce equal labeling at C-5 and C-6 in MIP and this effect is superimposed on the ideal specificity of Figure 8 leading to the observed results.

The new mechanism is consistent with the observation that glycine (in addition to valine) enhanced MIP formation in *P. tetrolens*, and the fact that glycine incorporation could not be detected in the pyrazine (*33*). Since glycine is a known precursor of serine in biosynthesis it too should serve as a precursor of MIP. However, since 1-^{13}C-glycine was used in the earlier labeling study, the label would have been predictably lost during decarboxylation.

The mechanism in Figure 8 does not explain the rare occurrence of 2-methoxy-3,5-dialkylpyrazines; however, it can with minor modification as shown in Figure 9. Combinations of α-amino acids and aminocarbonyls should generate pyrazinone intermediates similar to one in Figure 8. For example, a reaction between aminoacetone and alanine can lead regiospecifically to 2-methoxy-3,5-dimethylpyrazine, a compound produced by Gram negative bacteria (*35*) and recently found in raw coffee (*36*).

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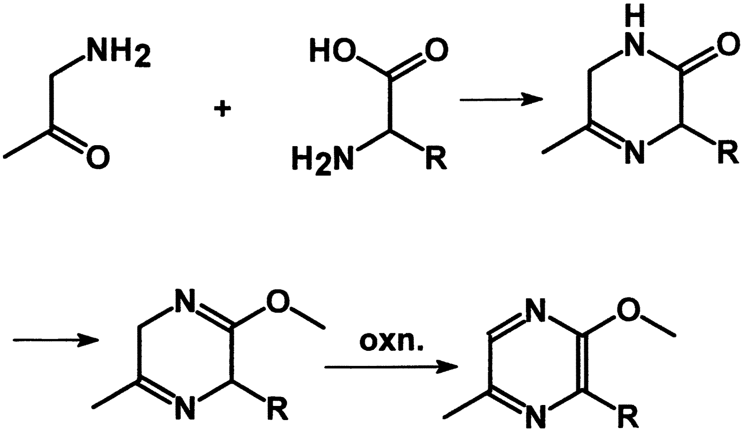

Figure 9. Formation of 2-Methoxy-3-isopropyl-5-methylpyrazines

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Chapter 9

The Chemistry of the Most Important Maillard Flavor Compounds of Bread and Cooked Rice

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The Maillard reaction *i.e.* the non-enzymatic browning reaction of proteins or amino acids and reducing sugars, produces a variety of azaheterocyclic compounds, which have a major impact on the flavor profile of foodstuffs. This overview covers the chemistry and flavor characteristics of cracker-like flavors, such as 6-acetyl-1,2,3,4-tetrahydropyridine and 2-acetyl-1-pyrroline. Focus will be given to the mechanism of formation, their instability, and the synthesis of these most important Maillard flavor compounds. The so-called Hodge mechanism on the generation of the major bread flavor component will be critically evaluated. Finally, the formation of these flavors by fermentation processes will be discussed.

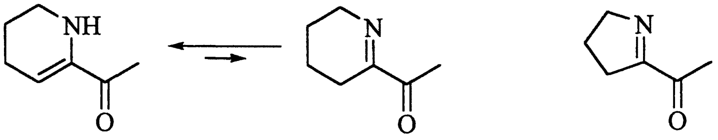
In this review several aspects of the most important flavor compounds of bread and cooked rice will be covered. Attention will be paid to the occurrence of 6-acetyl-1,2,3,4-tetrahydropyridine **1** and 2-acetyl-1-pyrroline **3** in foodstuffs (Figure 1). The chemistry, synthesis and biotransformation processes towards

In Heteroatomic Aroma Compounds; Reineccius, G., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2002.

these compounds will be discussed, while focus will be given to the mechanism of formation of these flavor compounds.

The Maillard reaction is the well-known reaction of proteins or amino acids with reducing sugars. It concerns a very complex set of reactions leading to a whole variety of degradation compounds. This non-enzymatic browning reaction contributes to a great extent to the flavor profile of foodstuffs (*1*). That it is a complex reaction becomes clear from the fact that single amino acids react with a great range of sugars under heating (model reactions) to give rise to hundreds of amino acid specific compounds. This reaction has been studied over the years in great detail (*2-7*) because of the great impact on foodstuffs. The Maillard reaction occurs during food processing at higher temperatures, but operates also at much lower temperatures, such as during storage, although proceeding then at a much slower rate. The importance of the Maillard reaction to food and to the quality of food is related to several aspects, including safety, the nutritional value, the texture and the flavor impact (*8*). The debate on the safety aspect is still going on, based on mutagenic and anti-cancerogenic activities reported for Maillard compounds. In a recent paper (*9*) the formation of mutagens after heating of a sugar-casein model system was examined using the Ames test. Mutagenicity could be fully ascribed to Maillard reaction products and strongly varied with the kind of sugar.

The nutritional value and the texture of a foodstuff undergoes a major influence during cooking, reflecting the process of the Maillard reaction. However, this review will cover only in detail the flavor aspects of the Maillard reaction, with special focus on the most important flavor compounds of bread and cooked rice, *i.e.* 6-acetyl-1,2,3,4-tetrahydropyridine **1** (and its tautomer **2**) and 2-acetyl-1-pyrroline **3**.



1 **2** **3**

Figure 1. The most important flavor compounds of bread and cooked rice.

Sugars undergo a series of degradation reactions, mainly based on retro-aldol reactions, to produce small reactive fragments, *e.g.* hydroxylated carbonyl

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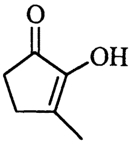
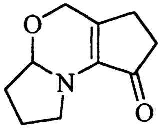
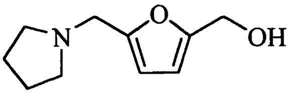
compounds and *α*-diketo compounds, which react with *α*-amino acids to give labile intermediates (*4*). These labile intermediates quite often give rise to aspecific condensations or degradation reactions affording heterocyclic substances, some of which display profound flavor characteristics. Various types of heterocyclic Maillard flavor compounds have been identified so far, including pyridines, thiazoles, pyrazines, furans, oxazoles, pyrroles and all their respective derivatives (partially or fully reduced, fused, etc. ...). As an example, some proline-specific Maillard compounds **4** (*10*, *11*), **5** (*12*), **6** (*13*), **7** (*14*), **8** (*9*, *15*) and **9** (*11*) from a glucose/proline (1:1) model system are given below (Figure 2).



4 Corylone or
Cyclotene



5 Maltoxazine



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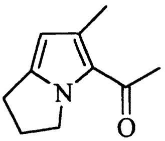

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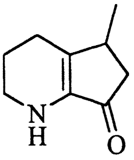

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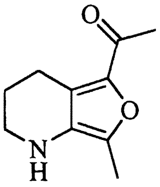

9

Figure 2. Proline-specific Maillard compounds.

If such heterocyclic compounds contain an imino function or enamino function to which an acetyl group is substituted at the imino carbon or the *α*-carbon of the enamine (see subunits **10** and **11**, Figure 3), it was postulated and observed that roasty, biscuit- or cracker-like odor characteristics were associated with these compounds (*16*). This structural requirement applies also for acyclic *α*-iminoketones but not for *N*-substituted *α*-acetyl cyclic enamines **12** (R = alkyl), which do not show the roasty flavor characteristics at all (*17*).

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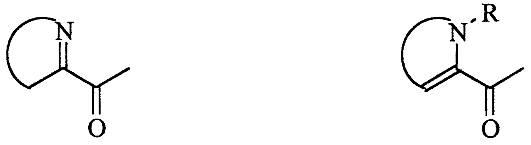

10

11 R = H
12 R = alkyl

Figure 3. Structural requirements for roasty, bisquit or cracker-like odor characteristics.

In more detail, several aspects of the occurrence in foodstuffs and the chemistry of 2-acetyl-1-pyrroline **3** and 6-acetyl-1,2,3,4-tetrahydropyridine **1** (and tautomer **2**) will be given.

2-Acetyl-1-pyrroline

2-Acetyl-1-pyrroline **3** has been found in a whole series of varieties of cooked rice in the range of 6-90 ppb (*18*). It has been identified in wheat bread crust (*19*), rye bread (*19*), cooked beef (*20*), sweet corn (*21*), corn tortillas (*22*), pearl millet (*23*), chempedak fruit (*24*), air-dried sausages (*25*), pandanus leaves (*26*) and roasted wild mango seeds (*27*). It can be produced by certain *Bacillus cereus* strains (*vide infra*) while it appears to play a role in the sexual and territorial connotations of the Indian tiger (*28*). It is an important and powerful flavor compound with an odor threshold value of 0.1 ppb (in water). It can also be considered as the popcorn flavor compound. An analogous thio-analogue, *i.e.* 2-acetylthiazoline **13** has a similar low threshold value of 1.3 ppb (in water) while the corresponding aromatic analogues have a much higher threshold value of 170 ppm for 2-acetylpyrrole **15** and 10 ppb for 2-acetylthiazole **14** (both in water) (Figure 4). Bread prepared without yeast does not contain 2-acetyl-1-pyrroline. In popcorn, proline plays a major role in the generation of 2-acetyl-1-pyrroline while in bread crust it seems to be ornithine that takes over this role. It has been proven that phosphorylated sugar degradation products like 1,3-dihydroxyacetone monophosphate and fructose-1,6-diphosphate (and subsequent aldolase treatment) give rise to a pronounced formation of 2-acetyl-1-pyrroline upon reaction with proline in dilute aqueous medium (*29*). This influence of phosphate stimulates the generation of propane-1,2-dione **22**, which combines with 1-pyrroline **21**.

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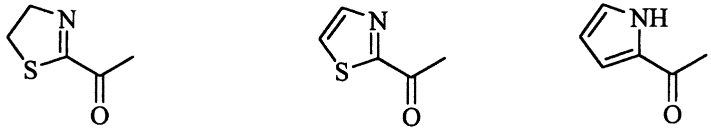

13 **14** **15**

Figure 4. Analogues of 2-acetyl-1-pyrroline.

The latter unstable cyclic imine originates from a Strecker degradation of proline. That both propane-1,2-dione **22** and 1-pyrroline **21** play a major role in the generation of 2-acetyl-1-pyrroline becomes clear from the fact that in model reactions between both reactants, 72 % of the volatile fraction consisted of 2-acetyl-1-pyrroline **3**. Various syntheses for 2-acetyl-1-pyrroline have been described in the literature. The first synthesis (Figure 5) of the flavor compound **3** consisted of an oxidation of 2-(1-hydroxyethyl)pyrrolidine **16** with a large excess of silver carbonate on Celite in benzene. However, the reaction mixture consisted of a large number of products, of which only 10 % (by GC) appeared to be 2-acetyl-1-pyrroline **3** (*18*).

A flexible method for the synthesis of not only 2-acetyl-1-pyrroline **3**, but also of other 2-alkanoyl-1-pyrrolines **18** (R ≠ Me), entailed the addition of a Grignard reagent to imidoyl cyanide **17** and subsequent hydrolysis (*30*) (Figure 6). Three methods entailing the cyclization of 6-amino- or 6-azidohexane-2,3-diones **19** to form 2-acetyl-1-pyrroline **3** have been published (*31-33*).

Of fundamental importance is the finding that 2-acetylpyrrolidine **20** is oxidized spontaneously in aqueous medium at neutral pH under the influence of oxygen (air) to afford 2-acetyl-1-pyrroline **3** (*34*) (Figure 7). This finding led to the proposal that the oxidation step is essential in the generation of 2-acetyl-1-pyrroline **3** in foodstuffs.

Accordingly, it was proposed that 1-pyrroline **21** condensed with 1,2-propanedione **22** to generate 2-(1,2-dioxopropyl)pyrrolidine **23**, which oxidizes with oxygen to the corresponding 1-pyrroline, the latter undergoing an addition of water and subsequent semibenzilic rearrangement. The β-ketoacid **25** thus formed decarboxylates to 2-acetylpyrrolidine **20**, affording 2-acetyl-1-pyrroline **3** upon air oxidation (*35*). The air oxidation of 2-acetyl-1-pyrrolidine **20** to 2-acetyl-1-pyrroline **3** is similar to the known oxidation of α-aminoketones and α-aminoimines to α-iminoketones and α-diimines, respectively (*36-39*) (Figure 8).

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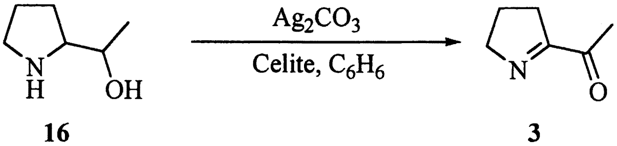
Ag$_2$CO$_3$
Celite, C$_6$H$_6$
N
H
OH
N
O
16
3

Figure 5. First reported synthesis of 2-acetyl-1-pyrroline (18).

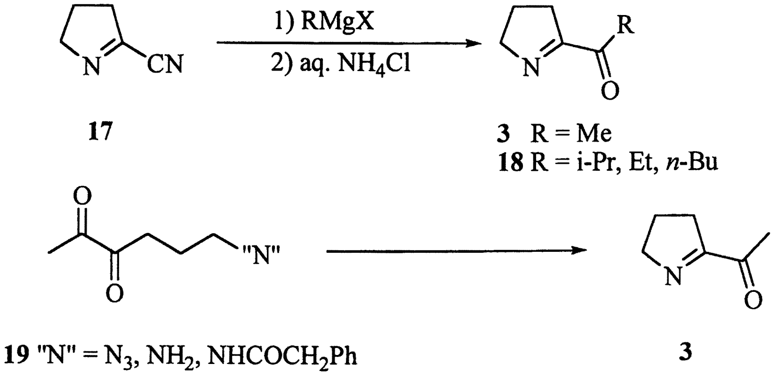
N
CN
1) RMgX
2) aq. NH$_4$Cl
N
R
O
17
3 R = Me
18 R = i-Pr, Et, *n*-Bu
O
O
"N"
N
O
19 "N" = N$_3$, NH$_2$, NHCOCH$_2$Ph
3

Figure 6. Methods for the synthesis of 2-acetyl-1-pyrroline and other 2-alkanoyl-1-pyrrolines (30-33).

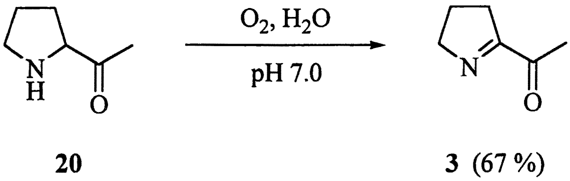
O$_2$, H$_2$O
pH 7.0
N
H
O
N
O
20
3 (67 %)

Figure 7. Oxidation of 2-acetylpyrrolidine to 2-acetyl-1-pyrroline (34).

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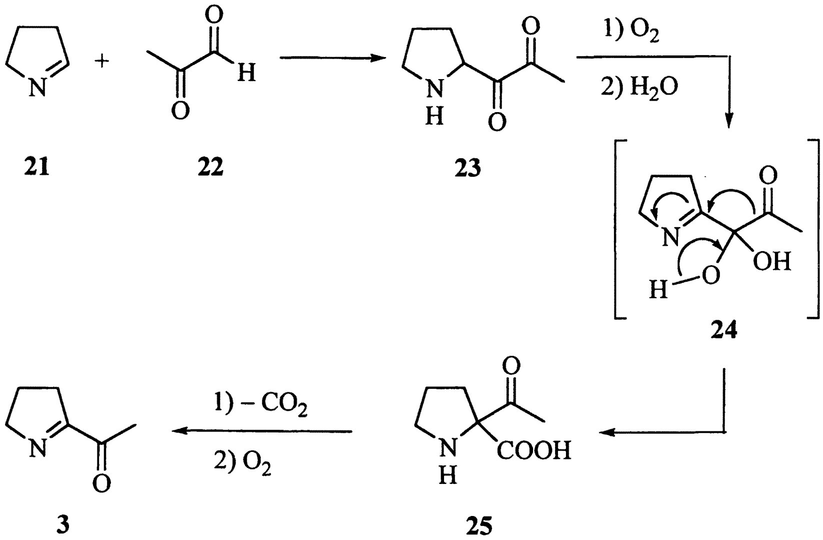


Figure 8. Proposed reaction mechanism for the formation of 2-acetyl-1-pyrroline from 1-pyrroline and 1,2-propanedione (36-39).

5-Acetyl-2,3-dihydro-1,4-thiazine

5-Acetyl-2,3-dihydro-1,4-thiazine **27** (Figure 9) was found recently in a ribose/cysteine model system and has a very intense, popcorn-like Maillard odorant (*40*, *41*). This heterocycle was synthesized from diacetyl **26** *via* 1-bromo-3,3-dimethoxy-2-butanone utilizing cysteamine or *N*-(*t*-butoxycarbonyl)cysteamine (*42*).

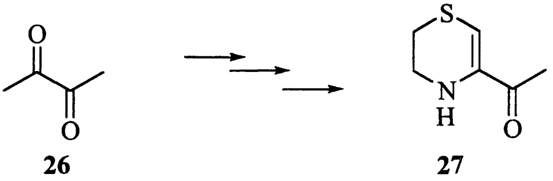
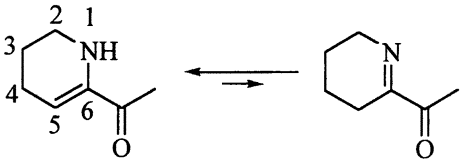


Figure 9. Synthesis of 5-acetyl-2,3-dihydro-1,4-thiazine (42).

6-Acetyl-1,2,3,4-tetrahydropyridine

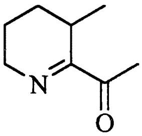
The carba-analogue of 5-acetyl-2,3-dihydro-1,4-thiazine **27** is 6-acetyl-1,2,3,4-tetrahydropyridine **1** which occurs in tautomeric equilibrium with 6-acetyl-2,3,4,5-tetrahydropyridine **2** (Figure 10) (*43*). This important Maillard flavor compound was suggested as a flavorant for bakery goods (*44-46*). It was identified in potato chips (*47*), rye bread crust (*48*), popcorn (*49*) and corn tortillas (*50*).



1 **2**

Figure 10. Tautomeric equilibrium of 1,2,3,4-tetrahydropyridine 1 and 6-acetyl-2,3,4,5-tetrahydropyridine.

Alkylation of this substance **1** at the 5-position results in a dramatic shift of the tautomeric equilibrium to the imino tautomer **2**, leaving the enamine for about 3 % (see **28**) (*35*) (Figure 11). The presence of a sulfur atom in 5-acetyl-2,3-dihydro-1,4-thiazine **27** causes the equilibrium to shift completely to the enamine tautomer (*42*). The influence of an electron-withdrawing substituent at the α-carbon of an imine on the tautomeric equilibrium has been studied in detail for α-cyanoenamines which are in equilibrium with imidoyl cyanides (*51-53*). The electron distribution in α-cyanoenamines is such that C-alkylation at the β-carbon cannot be obtained in a preparative useful way. Consequently, *N*-alkylation is the favored process (*54*).



28

Figure 11. 6-Acetyl-2,3,4,5-tetrahydro-5-methylpyridine.

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The socalled Hodge mechanism on the generation of 6-acetyl-1,2,3,4-tetrahydropyridine **1** from proline **29** and propane-1,2-dione **22** (Figure 12) was an established explanation of this flavor compound in text books on food chemistry (*55-57*). However it has been proven that the Hodge mechanism is most probably not operative, based on the fact that the intermediate 4-(*N*-acetonyl)aminobutanal **30** did not give rise to this compound under various reaction conditions (*58*).

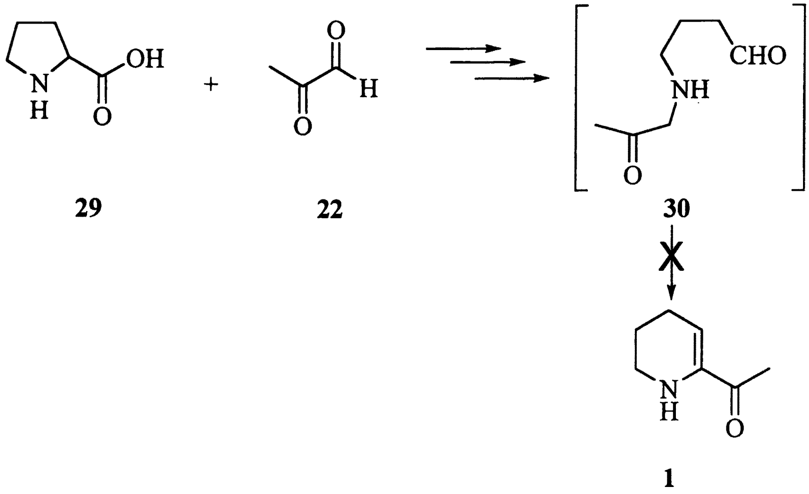

Figure 12. Failure of Hodge mechanism to generate 6-acetyl-1,2,3,4-tetrahydropyridine from 4-(N-acetonyl)aminobutanal (58).

The most recent proposal on the generation of 6-acetyl-1,2,3,4-tetrahydropyridine **1** stems from the condensation of 1-pyrroline **21** and hydroxyacetone **31**, the nucleophilic addition product being cleaved spontaneously to form 7-aminoheptane-2,3-dione **33** (Figure 13). The latter δ-aminoketone undergoes cyclization to the bread flavor component **1**. Some of the reaction steps are supported by experimental facts (*35*).

Several syntheses of 6-acetyl-1,2,3,4-tetrahydropyridine **1** (in equilibrium with its imino tautomer **2**) have been reported in the literature. The classical Hunter experiment, *i.e.* the thermal condensation of proline with 1,3-dihydroxyacetone in the presence of $NaHSO_3$, gave the bread flavor component only in an extremely low yield, together with other condensation products (*44*). The oxidation of 2-(1-hydroxyethyl)piperidine with a large excess of silver carbonate afforded 6-acetyl-1,2,3,4-tetrahydropyridine **1** in a useful way (*59*). The addition of Grignard reagents to 6-cyano-2,3,4,5-tetrahydropyridine **34**

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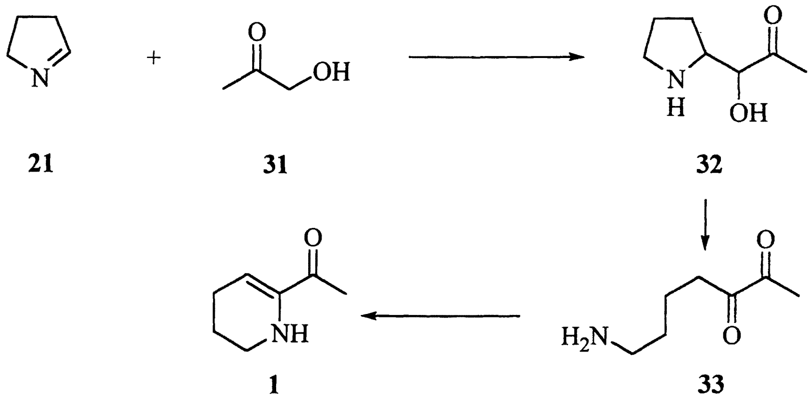

Figure 13. Proposed condensation of 1-pyrroline and hydroxyacetone.

opened the door to the bread flavor component **1** (Figure 14), but also to a higher analogue, *e.g.* **35**, which is also a major flavor impact compound in bread (*60*).

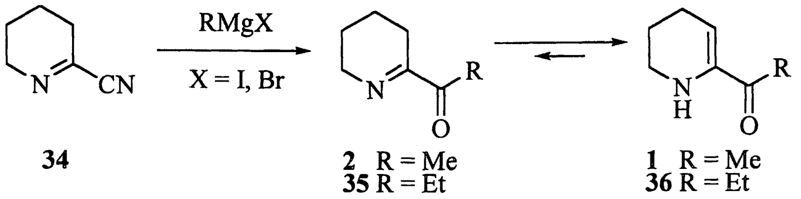

Figure 14. The addition of Grignard reagents to 6-cyano-2,3,4,5-tetrahydropyridine to form important bread-like flavor compounds (60).

The *ω*-aminoketone approach for the synthesis of 6-acetyl-1,2,3,4-tetrahydropyridine **1** was worked out several times, including *via* aza-Wittig cyclization of 7-azidoheptane-2,3-dione **37** (*32*) or 7-azido-2,2-dimethoxy-3-heptanone **38** (*61*). The spontaneous oxidation of 2-acetylpiperidine **39** with oxygen at pH 7 to provide 6-acetyl-1,2,3,4-tetrahydropyridine **1** is the most recent synthetic pathway for the latter Maillard flavor compound (*34*).

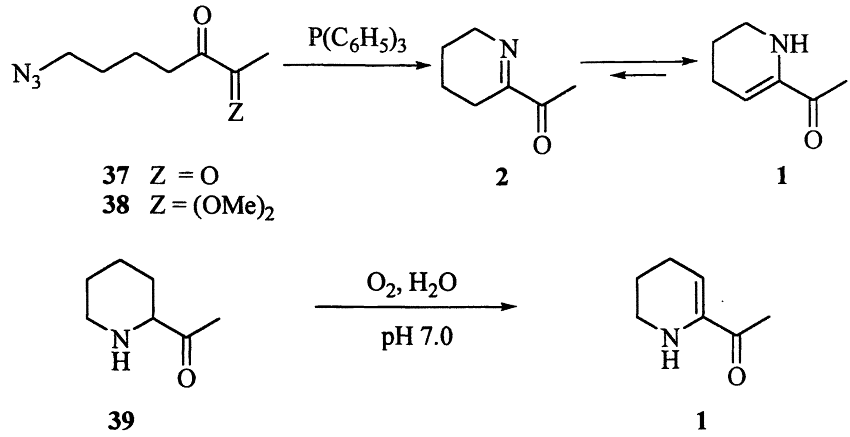

Figure 15. Recent synthetic pathways for 6-acetyl-1,2,3,4-tetrahydropyridine (34).

Production of Pyrazines and 2-Acetyl-1-pyrroline by Bacteria

The first evidence that microorganisms are able to produce pyrazines was provided in the early sixties (*62*). It was shown that tetramethylpyrazine is present in natto, a Japanese fermented soybean food. This compound is responsible for the characteristic odour of this food. *Bacillus subtilis* grown on a sucrose:asparagine medium produced tetramethylpyrazine (*63*). *Penicillium caseicolum* isolated from Camembert and Brie cheese surfaces, when grown in a defined medium (*64*), produced 2-methoxy-3-isopropylpyrazine, which imparted an earthy, nutty, potato-like flavor. *Bacillus subtilis* grown on coconut produced tetramethylpyrazine and 2,3,5-trimethylpyrazine: compounds assumed to be responsible for the pungent odour (*64*). Volatile compounds produced by *Pseudomonas perolens* ATCC 10757 in sterile fish muscle included 2-methoxy-3-isopropylpyrazine, which was primarily responsible for the musty, potato-like odour in the fish tissue (*65*). The biosynthesis of 2-methoxy-3-isopropylpyrazine in *Pseudomonas perolens* has been studied (*66*). On the basis of the results of labeling experiments, endogenous valine, glycine, and methionine were considered to be the precursors of 2-methoxy-3-

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isopropylpyrazine produced by *P. perolens*. The formation of 3-alkyl-2-methoxypyrazine **45** (Figure 16) is explained by condensation of an α-amino amide **41**, derived from an α-amino acid **40**, and glyoxal **42** (*67*).

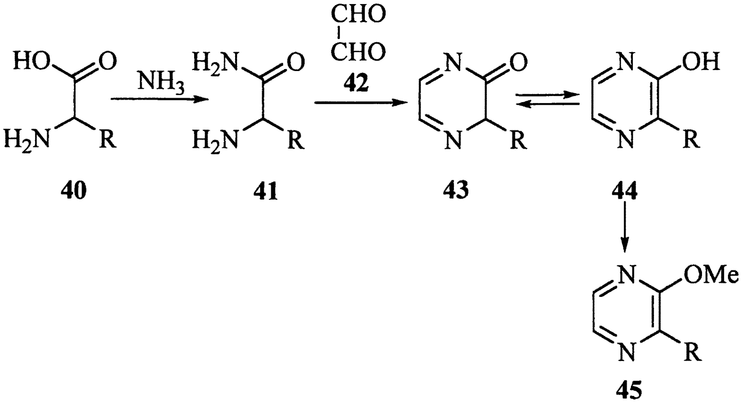

Figure 16. The formation of 3-alkyl-2-methoxypyrazine (67).

A mutant of *Corynebacterium glutamicum* was found to accumulate 3 g/l of tetramethylpyrazine after 5 days (*68*). Isoleucine, valine, leucine, pantothenate and thiamine were required for growth of the organism.

A strain of *Bacillus cereus* #147 was shown to produce only 0.3 g/l of the same tetramethylpyrazine after 7 days of fermentation. The liquid synthetic medium required 4.0 % glucose and 0.2 % yeast autolysate for maximal yields (*69*).

Aspergillus oryzae grown on soybeans and wheat flour in solid-state fermentations was shown to be responsible for the production of 19 substituted pyrazine compounds. However, pyrazines were not formed during the Maillard reaction (non-enzymatic browning) that occurred in the brined, slightly acidic soybean hydrolysate stored at room temperature. Those pyrazines found in levels exceeding 100 nanomoles/kg in fermented soya cake were: methyl-, 2,5-dimethyl-, 2,6-dimethyl-, 2,3-dimethyl-, trimethyl- and tetramethylpyrazine (*70*). Certain *Aspergillus oryzae* and *Aspergillus sojae* strains were shown to be pyrazine producers under certain conditions. The wide variety of 2-hydroxy-3,6-substituted pyrazines produced by *A. sojae* undoubtedly contributes to the complex flavor qualities of soy sauce (*71-73*). *Bacillus subtilis* and *Bacillus magnetarium* were proven to produce 2,5-dimethylpyrazine (154.8 μg/100 g), 2,3,5-trimethylpyrazine (23.0 μg/100 g) and tetramethylpyrazine (19.8 μg/100

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g) during cocoa bean fermentation (the amounts formed after 5-6 days of fermentation) (*74*).

More recently, the production of 2,5-dimethylpyrazine and tetramethylpyrazine by *Bacillus subtilis* IFO 3013 grown on soybeans was reported (*75*). Solid-state cultivations were carried out either in 100-ml bottles or in a fixed-bed column reactor at 27°C. 2,5-Dimethylpyrazine was obtained using soybeans enriched with 75 g threonine/kg initial dry weight, giving 0.85 g metabolite/kg i.d.w. after 6 days. Tetramethylpyrazine production involved addition of 90 g/kg initial dry weight after 14 days. In this way, solid-state cultivation was shown to be a fermentation method for the production of roasty flavor compounds.

2,5-Dimethylpyrazine and tetramethylpyrazine were also produced by *B. subtilis* IFO 3013 when the organism was grown in solid substrate conditions using ground soybean suspended in water (*76*). Optimisation studies showed that the best way to produce the two aroma compounds involved enrichment of the medium with L-threonine and acetoin. A recovery of 2 g/l total pyrazines from the culture medium was achieved.

Bacillus cereus strains, isolated from cocoa boxes in Bahia (Brazil), were able to produce 2-acetyl-l-pyrroline **3** (*77*). The presence of this flavor compound was reported for the first time to be formed at 35°C, well below the temperatures considered to be required for its thermal formation. *B. cereus* strains ATCC 10702, 27522, 33019, 14737 and some other *B. cereus* strains isolated from cocoa boxes proved to be able to convert precursors such as proline, ornithine, glutamate, glucose, amylose, amylopectin, D-lactose, maltose, ribose, sucrose and *N*-acetyl-D-glucosamine into 2-acetyl-1-pyrroline **3**. An examination of the volatile compounds isolated by simultaneous distillation–solvent extraction showed five *B. cereus* strains to produce 30-75 μg/kg 2-acetyl-1-pyrroline **3** after a 2 day incubation on standard plate count agar. The highest yield was obtained from solid state media (Plate Count Agar) enriched with 1.0 % glucose (458 μg/kg) and 1.0 % amylose (514 μg/kg) after 2 days of incubation. However, the production of 2-acetyl-1-pyrroline by *B. cereus* has only been achieved in solid systems (Petri dishes); up to now the bacterium has not produced 2-acetyl-1-pyrroline in liquid media.

A series of ^{13}C- and ^{15}N-labeling experiments showed *B. cereus* to utilize glucose, glutamic acid, and proline for the formation of 2-acetyl-1-pyrroline.

A great deal of research has been devoted recently to the most important Maillard flavor compounds of bread and cooked rice. This research culminated in several useful syntheses of these important flavor impact compounds in such a way that these compounds now are readily accessible for further research purposes. In addition, new and fundamentel insights have been generated in the mechanism of formation of these most important Maillard flavor compounds.

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Chapter 10

Identification of New Odorous Heterocyclic Compounds in French Blue Cheeses

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A number of nitrogen-containing volatile compounds were found in the medium polarity fractions of vacuum distillates obtained from selected French blue cheeses. Six major compounds found in this fraction, some having interesting odors as determined by a GC-sniffing experiment, were isolated and characterized by GC with FID and NPD detectors, GC/MS in EI and CI modes, GC/FTIR and ^{1}H-NMR including two-dimensional NMR. Owing to the spectroscopic data, the six compounds were tentatively identified as a homologous series of 2-alkyl-2,4,5-trimethyl-2,5-dihydro-oxazoles. Syntheses of the six compounds confirmed the tentative results and also allowed for the identification of other minor components of the same chemical family found in this cheese fraction. These syntheses also allowed us to hypothesize a chemical synthesis of these volatiles in cheese from the precursors acetoin, ammonia, and methyl-ketones, which are abundantly present in blue cheeses.

In Heteroatomic Aroma Compounds; Reineccius, G., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2002.

Introduction

In the study of flavor volatiles of various French blue cheeses (Roquefort, Bleu des Causses, Bleu d'Auvergne) 129 compounds were identified and quantified (*1*). Among them, two methyl-ketones (heptan-2-one and nonan-2-one) were the most abundant and total methyl-ketones represented up to 50 to 75 % of the total odorous profile for all the cheeses (*1*). Secondary alcohols, esters, sulfur compounds, lactones and aldehydes were the other major volatile compounds (*1*). However, at that time, some odorous volatile components detected in a GC-sniffing experiment could not be identified. The present work was undertaken in order to identify and characterize these odorous compounds.

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Experimental

Extraction and Fractionation

The cheese extracts were obtained by vacuum distillation (*1*). Column fractionation was performed on silica activated with 15% water (W/W). Elutions started with pure pentane and continued with solvent systems of increasing polarity up to pure diethyl ether. The fractions with grossly similar composition as determined by gas chromatography were pooled.

Methods

Gas chromatography (GC).

The GC analyses were conducted on a Girdel DI 700 with a 30 m x 0.32 mm DB5 column (J&W Scientific) of 1µm thickness. The carrier gas was hydrogen at a linear velocity of 50 cm/sec at room temperature. The oven temperature was programmed from 40°C to 220°C at a rate of 3°C/min. Injector (splitless) and FID detector temperature were respectively 240 and 250°C. A thermoionic (NPD) detector was used at a temperature of 240°C. A sniffing port was also used in order to characterize the odorous properties of the main compounds. For that, 80% of the column effluent was directed through a T-piece towards the sniffing port, the remaining being directed to the FID. Humidified air was added at the sniffing port maintained at 240°C at a flow rate of 20 ml/min.

Gas Chromatography-Mass Spectrometry (GC-MS).

Electron impact (EI, 70 eV, 150°C source temperature) GC-MS data were obtained with a Nermag R10-10C quadrupole mass spectrometer interfaced to a Girdel 31 gas chromatograph. The GC conditions used were identical to those described above, except helium was used as a carrier gas at a velocity of 35 cm/sec. Chemical ionization data were obtained at 90 eV using methane as reagent gas at a source pressure of 0.3 Torr and a source temperature of 90°C.

Gas Chromatography-Fourier Transform Infrared Spectrometry (GC-FTIR).

Vapor phase infrared spectra were obtained using a Bruker IFS85 Fourier transform infrared spectrometer equipped with a Bruker gold coated light-pipe (20 cm x 0.8 mm i.d.) interfaced to a Carlo Erba 5160 series gas chromatograph. The GC column used was the same as that described above. Helium was used as carrier gas at a velocity of 35 cm/sec, an on-column injector was used and the light-pipe temperature was 200°C. The spectral resolution was 8 cm^{-1} and a narrow-band (4800-600 cm^{-1}) mercury-cadmium-telluride (MCT) detector was used.

Chiral Analyses by Multi-Dimensional Gas Chromatography (MDGC).

The chiral analyses were carried out by MDGC with a Sichromat 2-8 double oven gas chromatograph (Siemens), equipped with a FFAP capillary column (30 m x 0.32 mm) (Quadrex) coupled to a fused silica capillary column (25 m x 0.32 mm) coated with FS Lipodex E (Oktakis 3-O-butyryl-2,6-di-O-pentyl-γ-cyclodextrin) (Macherey&Nagel) or to a capillary column (50 m x 0.25 mm) coated with 1/3 of perethyl-β-cyclodextrin in 2/3 OV 1701.

Preparative Gas Chromatography (Preparative GC).

For preparative GC, a Girdel 30 gas chromatograph fitted with a DB5 capillary column similar to that described above was used with an effluent splitter that allowed only 10% of the effluent to flow to the FID detector. Fractions were collected in U-shaped glass tubes (1 mm i.d.) cooled by liquid nitrogen. The tubes were rinsed with the solvent used for NMR and the purity of the collected fractions was monitored by GC. The quantities were a few µg (ca. 10 µg for the most concentrated compound, #2) for each trapped compound.

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Nuclear Magnetic Resonance (NMR).

^{1}H-NMR spectra were recorded on a Bruker WM 400 instrument operating at 400.13 MHz in 1 mm i.d. coaxial cell contained in 5 mm NMR high quality tubes using 20 µl of benzene-D6 (99.99 % D) as solvent. A two dimensional COSY experiment was conducted for compound 2, a flip angle of 90° for the mixing pulse being used for maximum sensitivity. The signal due to the residual protons of the deuterated solvent was used as internal reference ($\delta = 7.15$ ppm).

Syntheses of 3-Oxazolines

The syntheses were conducted according to a published procedure (*2*). In a typical experiment, compound 2 (2,2,4,5-tetramethyl-3-oxazoline) was obtained by mixing at 0°C a solution of 1.74 g (30 mM) of acetone in 10 ml of water to 10 ml of a 20% ammonia solution in water, and adding in 15 min 1.76g (20 mM) of acetoin. The mixture was stirred overnight at room temperature, then diluted in 100 ml of water and distilled at atmospheric pressure. The distillate (60 ml) was saturated with sodium chloride and extracted three times with 50 ml of diethyl ether. The organic phase was dried with anhydrous sodium sulfate and was then evaporated. The final product was obtained by column chromatography on silica gel 60 (100 g) with the eluent diethyl ether-pentane: 1-1. A yield of 0.6 g (24 %) was obtained. The same procedure was used to synthesize the other 3-oxazolines, compound 7 [2,4,5-trimethyl-2-(1-hydroxyethyl)-3-oxazoline] being obtained by reacting only acetoin with ammonia.

Results and Discussion

The vacuum cheese distillates (*1*) have been fractionated on activated silica. The previously unidentified odorous compounds were found within a fraction of medium polarity (i.e. CH_2Cl_2/diethyl ether: 1/1) for the most ripened cheeses (Roquefort ripened for 210 days and Bleu des Causses ripened for 100 days). Besides some minor components, this fraction contained six major compounds; some of them with interesting odors detected at the GC sniffing port (Table I). As revealed by the response obtained using a NPD detector in the gas chromatographic analysis of the fraction, they were all, including the minor

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ones, nitrogen-containing molecules (Table I). Their EI mass spectra were not found in the common MS data bank and not readily interpreted (see Figure 1 for the EI mass spectrum of compound 2 as an example). The spectra of compounds 3 and 4 on one hand and of 5 and 6 on the other hand were similar, revealing in each case closely related isomers. Chemical ionization GC-MS determined odd molecular weights for all the compounds (Table I), confirming that the molecules contained an odd number of nitrogen atoms.

Table I. Characteristics of the Six Main Unknown Compounds

Compound #	*Odor(GC-Sniffing Port)*	*Average Concentration in Cheese (μg/kg)*	*NPD Detector Response*	*MW (CI-GC/MS)*
1	-	50	+	113
2	Floral, fresh	2,500	+	127
3	Vegetal, thyme	250	+	155
4	Fruity, intense	250	+	155
5	Vegetal	130	+	183
6	-	130	+	183

A common feature to all the mass spectra was the fragment ion observed at m/z 112 (except for compound 1 which has a molecular mass of 113). An exact mass determination on this fragment at m/z 112 for compounds 2 and 4 revealed the ion composition $C_6H_{10}NO^+$ (measured: 112.0762, calculated: 112.07623).

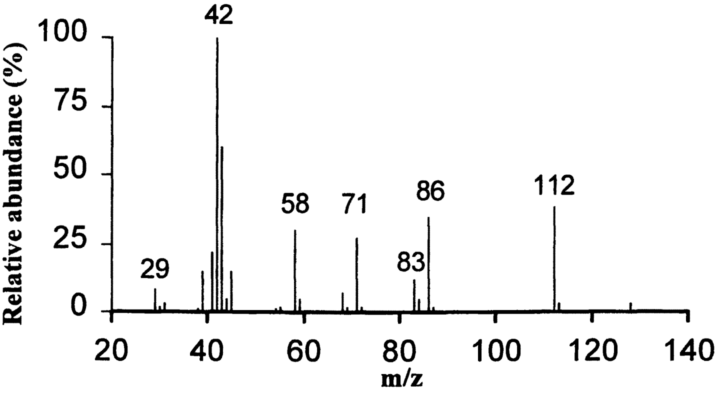

Figure 1. EI mass spectrum of unknown compound 2.

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The determination of the exact mass of the fragment found at m/z 140 for compound 4 revealed the composition $C_8H_{14}NO^+$ (measured: 140.1075, calculated: 140.10754) for this $[M-15]^+$ ion.

The exact mass measurements together with the CI data suggested the formula $C_7H_{13}NO$ for compound 2 and $C_9H_{17}NO$ for compound 4. These formulas implied two unsaturation equivalents (ring + double bond) for the molecules.

All the GC-FTIR spectra obtained for the six major compounds revealed a common feature: a rather intense absorption band found at 1664 cm^{-1}. This band position with such an intensity was not reported in vapor-phase infrared spectra databases. Considering infrared textbooks and other infrared databases, the only plausible hypothesis is that the absorption band is due to the stretching of a carbon-nitrogen double bond ($\nu C{=}N$). This double bond therefore would account for one of the two points of unsaturation.

The six major compounds of the fraction were purified by preparative GC, and only µg quantities of each compound were obtained as needed for micro-sampling for NMR. All the ^1H-NMR spectra (Table II) obtained for these compounds revealed common features.

Thus they all displayed a deshielded methine quadruplet around 4.35 ppm coupled to a methyl doublet around 1.0 ppm, revealing in all the molecules an isolated CH_3-CH- subunit. The other signals revealed the presence of two non-coupled methyl groups and of another alkyl chain (a methyl group for compound 2 found around 1.5 ppm, an alkyl group for compounds 3, 4, 5, and 6 with the terminal methyl group found at ca. 0.9 ppm). The similarities between the spectra of 3 and 4 and between 5 and 6 confirmed they are respectively isomers. Compound 1 appeared also as a mixture of diastereoisomers.

All these spectroscopic data suggest unambiguously a heterocyclic structure, with the ring accounting for the second point of unsaturation. Two hypotheses were formulated for the compounds: either 2-alkyl-2,4,5-trimethyl-3-oxazoline or 5-alkyl-2,4,5-trimethyl-3-oxazoline.

Both isomers were synthesized for compound 2 (i.e. 2,2,4,5-tetramethyl-3-oxazoline and 2,4,5,5-tetramethyl-3-oxazoline). The spectroscopic and retention data of the synthetic compounds identified unambiguously compound 2 as 2,2,4,5-tetramethyl-3-oxazoline (or 2,2,4,5-tetramethyl-2,5-dihydro-oxazole). Therefore, the other major compounds were tentatively identified as a homologous series of 2-alkyl-2,4,5-trimethyl-3-oxazolines. The first in the series (compound 1) was identified as 2,4,5-trimethyl-3-oxazoline. Compounds 3 and 4 were identified as having the alkyl chain as a propyl group, and compounds 5 and 6 with the alkyl chain as a pentyl group. These pairs of compounds (3 and 4, 5 and 6, respectively) were well separated by the GC conditions used and these pairs were also determined to be diastereoisomers.

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Table II. ^{1}H-NMR Data of the Six Unknown Compounds

Compound #	*^{1}H-NMR chemical shifts (multiplicity)*
1	Mixture of two diastereoisomers in 40/60 % ratio 1: 0.93 (3H, d, $C\underline{H}_3$-CH); 1.4 (3H, d, $C\underline{H}_3$-CH); 1.52 (3H, d, $C\underline{H}_3$) 2: 1.0 (3H, d, $C\underline{H}_3$-CH); 1.45 (3H, d, $C\underline{H}_3$-CH); 1.51 (3H, d, $C\underline{H}_3$) and 4.35 (broad multiplet)
2	1.01 (3H, d, $C\underline{H}_3$-CH); 1.45 (3H, s, $C\underline{H}_3$); 1.51 (3H, s, $C\underline{H}_3$); 1.54 (3H, s, $C\underline{H}_3$); 4.35 (1H, q, $C\underline{H}$-CH_3)
3	0.91 (3H, t, $C\underline{H}_3$-CH_2);1.02 (3H, d, $C\underline{H}_3$-CH); 1.52 (3H, s, $C\underline{H}_3$); 1.55 (3H, s, $C\underline{H}_3$); 1.3-1.4 (broad m); 1.75 (2H, t, $C\underline{H}_2$); 4.35 (1H, broad q, $C\underline{H}$-CH_3)
4	0.92 (3H, t, $C\underline{H}_3$-CH_2); 1.03 (3H, d, $C\underline{H}_3$-CH); 1.43 (3H, s, $C\underline{H}_3$); 1.53 (3H, s, $C\underline{H}_3$); 1.45-1.55 (broad m); 1.8 (2H, t, $C\underline{H}_2$); 4.35 (1H, q, $C\underline{H}$-CH_3)
5	0.9 (3H, t, $C\underline{H}_3$-CH_2); 1.02 (3H, d, $C\underline{H}_3$-CH); 1.55 (3H, s, $C\underline{H}_3$); 1.56 (3H, s, $C\underline{H}_3$); 1.2-1.62 (broad m); 1.8 (2H, broad m); 4.35 (1H, broad q, $C\underline{H}$-CH_3)
6	0.87 (3H, t, $C\underline{H}_3$-CH_2); 1.05 (3H, d, $C\underline{H}_3$-CH); 1.45 (3H, s, $C\underline{H}_3$); 1.54 (3H, s, $C\underline{H}_3$); 1.2-1.62 (broad m), 1.84 (2H, broad m); 4.35 (1H, q, $C\underline{H}$-CH_3)

400 MHz, in C_6D_6, 1 mm coaxial cell (20 μl) in 5 mm tube

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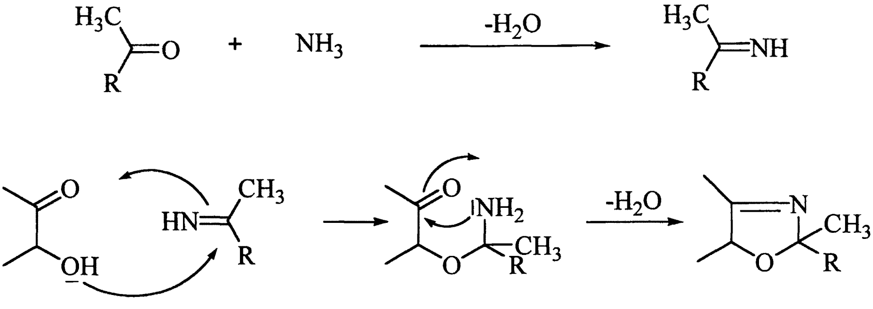
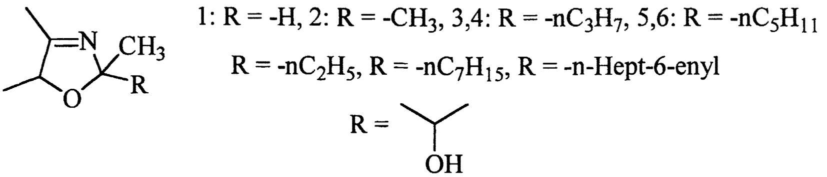

H3C
O
R
+
NH3
-H2O
H3C
NH
R
O
OH
CH3
HN
R
O
NH2
CH3
R
O
-H2O
N
CH3
O
R

Figure 2. Reaction scheme between ammonia, acetoin and methyl-ketones to form 2,5-dihydro-oxazoles.



1: R = -H, 2: R = $-CH_3$, 3,4: R = $-nC_3H_7$, 5,6: R = $-nC_5H_{11}$

R = $-nC_2H_5$, R = $-nC_7H_{15}$, R = -n-Hept-6-enyl

Figure 3. Some 3-oxazolines identified in French blue cheeses.

The first compound of this series (2,4,5-trimethyl-3-oxazoline) has been previously identified in boiled beef (*3*) and in roasted peanuts (*4*). Its published MS spectrum (*3*) is identical to that of compound 1 characterized in this study (see Figure 4). It has also been obtained in the Strecker degradation of DL-alanine with butane-2,3-dione (*5*). Its published perception threshold values found in the ppm range (*6*) hardly makes it a potential flavor-active compound in blue cheeses where it is found at a concentration of 50 ppb (Table I). Moreover it was not detected at the sniffing port (Table I). The EI mass spectra of the other compounds not previously identified in any food are also displayed in Figure 4.

The six major compounds were synthesized according to a published procedure for 3-oxazolines (*2*), allowing confirmation of the structural hypotheses. Basically, this procedure consisted of the reaction at room temperature of ammonia in the presence of acetoin and suitable methyl-ketones. According to the classical scheme depicted in Figure 2, ammonia reacts with a methyl-ketone giving rise to an imine, which reacts immediately with the α,β-hydroxy-keto group of acetoin to cyclize in the 2,5-dihydro-oxazole ring.

These syntheses allowed the confirmation of the structures of the six major compounds found in blue cheeses (i.e. 2-alkyl-2,4,5-trimethyl-3-oxazoline) as well as the structures of minor components of the same family that were found in the same cheese fraction (Figure 3).

As already stated, all but compound 1 (molecular weight is 113) exhibit an intense ion-fragment at m/z 112 corresponding to the trimethyl-3-oxazoline unit. Among the newly identified compounds, 2,4,5-trimethyl-2-(1-hydroxyethyl)-3-oxazoline was confirmed to result by the reaction of ammonia with two acetoin units, as already demonstrated recently (*7*). The compound 2-hept-6-enyl-2,4,5-trimethyl-3-oxazoline clearly resulted from the reactions between ammonia, acetoin, and non-8-en-2-one, which were found in the volatiles of the blue cheeses (*1*).

The six major synthetic compounds 1 to 6 have been characterized sensorially in a GC-sniffing experiment with 5 sniffers (Table III). Compound 1 exhibited roasted and cocoa notes but also green notes confirming previous observations (*4*). The diastereoisomers 3 and 4 imparted interesting and rather intense odors, but their odor thresholds were not determined. Compounds 5 and 6 as well as the 2,4,5-trimethyl-2-(1-hydroxyethyl)-3-oxazoline were not detected at the GC sniffing port in the ppm range by any of the sniffers.

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Table III. Odor and Chiral Properties of the Main 3-Oxazolines Identified

Compound	*Odor Intensity*	*Odor Descriptor*	*Configuration (Synthetic)*	*Configuration (in Cheese)*
1	++	green, then roasted, cocoa	4 peaks (2 racemic diastereoisomers)	Nd
2	+	green, spicy, pungent	racemate	38 % - 62 %
3	+++	green, orange peel, spicy, pleasant	racemate	39 % - 61 %
4	+++	green, cucumber, melon, pleasant	racemate	35 % - 65 %
5	-		racemate	40 % - 60 %
6	-		ill-separated	ill-separated

Concentrations used for the GC sniffing experiment: 1 μg/μl i.e. 1ppm, 1 μl injected

Chiral column: FS Lipodex E (OctaKis 3-O-butyryl-2,6-di-O-pentyl-γ-cyclodextrin)

Nd: non determined

As all these new odorants were chiral molecules, the enantiomeric ratios have been determined for all the major diastereoisomers in a two-dimensional GC experiment using a chiral column of the Lipodex E family (Table III). In cheese they all exhibited ratios in the range 40 to 60%, compound 1 being present in two diastereoisomeric forms in the ratio (40% / 60%) for which we were not able to determine the enantiomeric ratios. These enantiomeric ratios could be explained by the enantiomeric ratio of acetoin found in cheese, as these heterocyclic compounds form probably in cheese in a manner similar to that by which they are synthesized. In fact, the starting precursors ammonia, acetoin, and methyl-ketones are all important constituents of cheese, the last ones being the major volatile compounds of the blue cheeses under study (*1*). Despite the fact that acetoin should be a racemate due to tautomeric equilibrium, the enantiomeric ratios of commercial acetoin and of acetoin found in blue cheese have been measured in a two-dimensional GC experiment using a perethyl-β-cyclodextrin/OV 1701 column as a chiral column where the two enantiomers of acetoin are well separated. As expected, commercial acetoin was a racemate, whereas blue cheese acetoin was never found as a racemate. The enantiomeric ratios varied from 40/60 in one blue cheese under study and up to 20/80 in some

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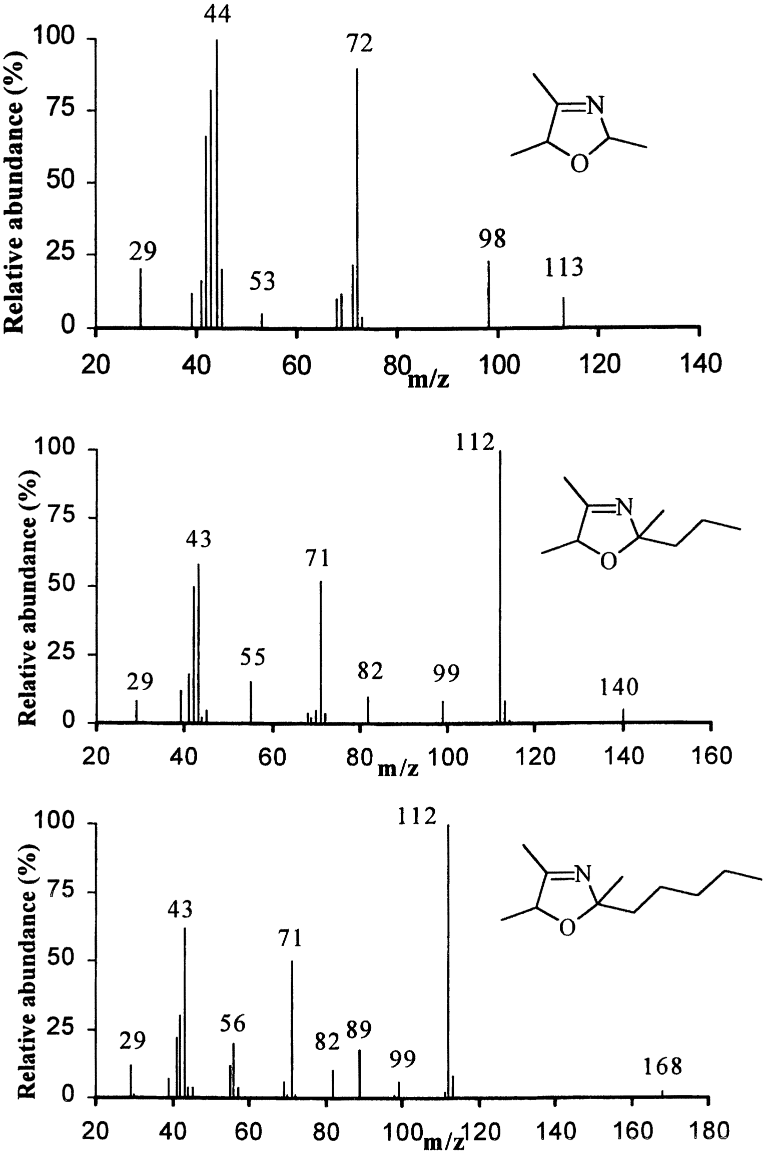

Figure 4. EI mass spectra of 3-oxazolines identified in French blue cheeses.

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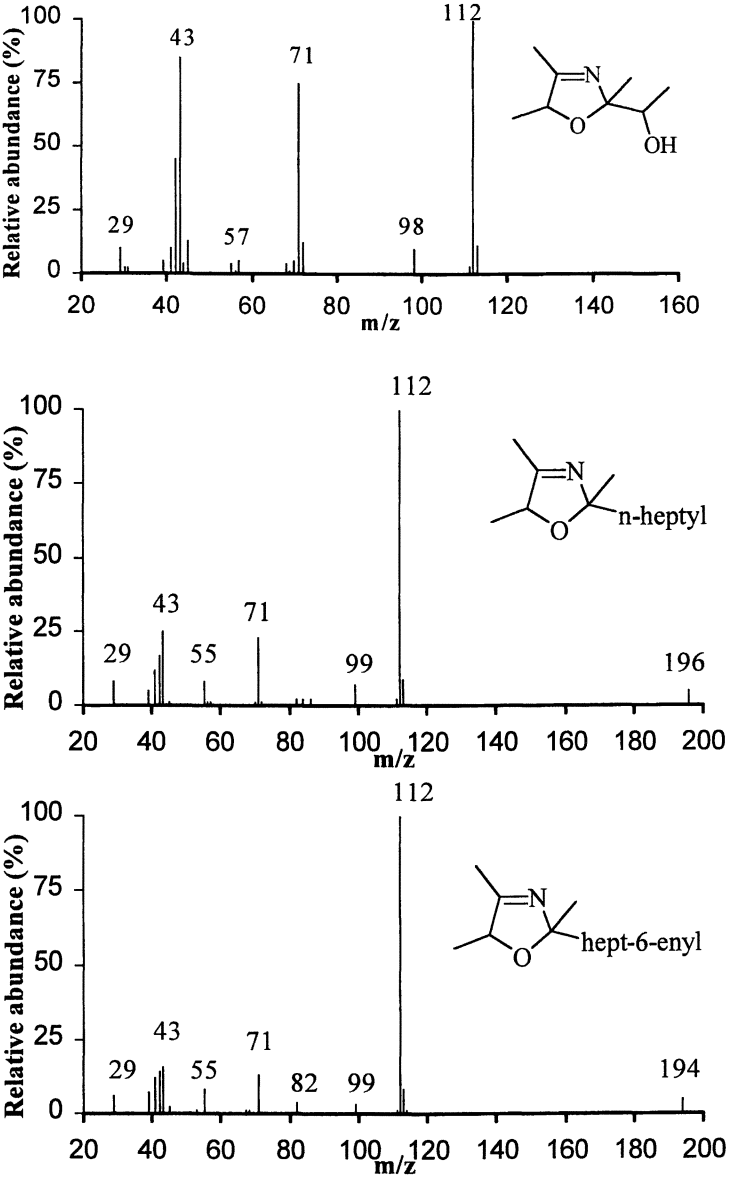

Figure 4 (continued).

other blue cheeses. These unexpected enantiomeric ratios could be only explained by a certain biological specificity and control in cheese. As the enantiomeric ratio for acetoin found in one of the blue cheeses under investigation is similar to the ratios found in the new heterocyclic molecules, it is most probable that acetoin is a precursor of these molecules, confirming the proposed mechanism of formation. However, they are not systematically present in cheese, whereas the precursors are all ways present in cheese, particularly in mould-ripened cheese. In this study these compounds are found in well ripened blue cheeses, but not in young ones. The formation, therefore, could be dependent on such parameters as ammonia concentration, pH, temperature and ripening time. Some of the identified 3-oxazolines have been found in other cheeses: Comté, Camembert (this laboratory, unpublished results) and also characterized in the alkaline volatile fraction of Swiss gruyère (*8*), although at that time the authors were not able to identify these compounds and published only their EI mass spectra (*8*).

Conclusions

New oxazoline derivatives have been characterized within a fraction of medium polarity of some French blue cheeses. Their odorous and chiral properties have been described, although their perception thresholds have not been determined. It appears that they are formed from spontaneous chemical synthesis from precursors present in abundance in cheese. However, as they are not systematically present in cheese, their formation seems to be dependent on many parameters such as ammonia concentration in cheese, cheese pH and ripening temperature and time, although a biological or enzymatic pathway could not be a priori excluded.

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Chapter 11

Biogeneration of Roasted Notes Based on 2-Acetyl-2-thiazoline and the Precursor 2-(1-Hydroxyethyl)-4,5-dihydrothiazole

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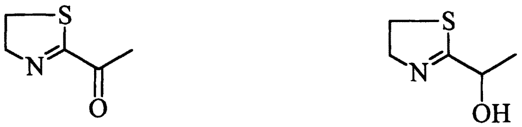
Roasted notes contribute to the flavor of thermally processed foods such as meat and bread. 2-Acetyl-2-thiazoline (2-AT) is one of the key volatile compounds responsible for the roasted and popcorn-like aroma character. We report on the biogeneration of flavoring preparations with intense roasted notes, which are characterized by a high content of 2-AT. Moreover, we found that the addition of 2-(1-hydroxyethyl)-4,5-dihydrothiazole (HDT) as aroma precursor added to pizza dough resulted in an enhancement of the roasted note.

Introduction

Several types of compounds are known to elicit a roasted note, mainly heterocyclic components formed in the course of the Maillard reaction, such as pyrazines, pyrrolines, pyridines, thiazolines and thiazines. These heterocyclic compounds are important constituents in many foods, such as cooked and roasted meat, bread, chocolate, coffee, and beer (*1*). Among these aroma volatiles, thiazolines and thiazoline derivatives play a key role in roasted flavors, particularly in meat products (*2*), and have received increasing research attention (*3*).

One of the most important thiazolines, which exhibits an intense roasted aroma character, is 2-acetyl-2-thiazoline (2-AT), 1 (Figure 1). It was reported for the first time as a volatile constituent of beef broth (*4*) and was later identified as a sensory relevant constituent of roasted beef (*5*). Several methods to generate 2-AT by organic synthesis (*6*) and the Maillard reaction (*7*) have been published. Moreover, Hofmann and Schieberle (*7*) proposed a reaction pathway involving cysteamine and methylglyoxal as substrates to produce 2-(1-hydroxyethyl)-4,5-dihydrothiazole (HDT), **2** (Figure 1), which was then transformed to 2-AT by heat treatment in a model reaction. However, the impact of HDT as a potential precursor of 2-AT in a food model has never been demonstrated.



2-acetyl-2-thiazoline **1**

2-(1-hydroxyethyl)-4,5-dihydrothiazole **2**

Figure 1. Important thiazolines having intense roasted aroma characters.

This paper reports on the generation of roasted notes based on aerobic incubation of cysteamine, ethyl-L-lactate and D-glucose with baker's yeast. Sensory-directed chemical analysis was applied to characterize the key flavor compounds and the reaction intermediates. In addition, we report here on the potential of HDT as aroma precursor to improve the roasted note of pizza dough after baking.

Experimental

Bioconversion

A flavoring preparation was obtained by fermentation of cysteamine and ethyl-L-lactate with baker's yeast (Figure 2). Commercial yeast cream solution (150 ml) was placed in a 500 ml flask equipped with an electrode and a magnetic stirrer (500 rpm). The flask was kept at 35°C using an oil bath and the pH adjusted to 9.8 with 2M sodium hydroxide. The pH was automatically maintained throughout the reaction using a Metrohm pH-stat device (Impulsomat 614). Cysteamine (385 mg, 5 mmol) and ethyl-L-lactate (590 mg, 5 mmol) were then added. Ten and 5 g of D-glucose were added after 4h and 24h of incubation, respectively. A kinetic study was performed and after 48h of reaction, the mixture was centrifuged and the supernatant was further treated.

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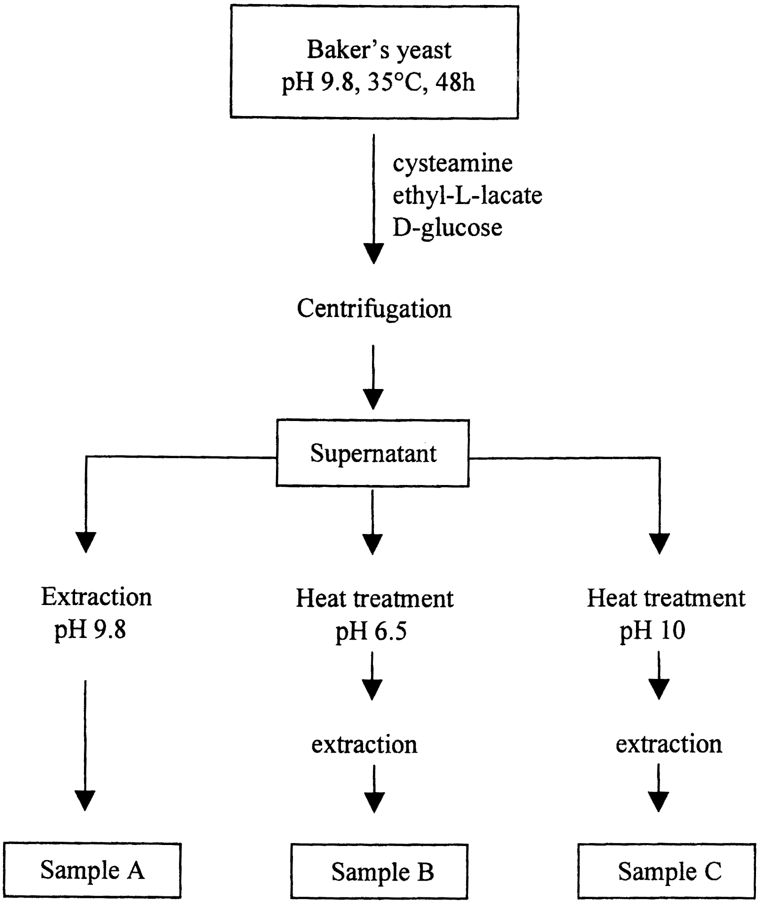
Baker's yeast
pH 9.8, 35°C, 48h
cysteamine
ethyl-L-lacate
D-glucose
Centrifugation
Supernatant
Extraction
pH 9.8
Heat treatment
pH 6.5
Heat treatment
pH 10
extraction
extraction
Sample A
Sample B
Sample C

Figure 2. General procedure for preparation of flavor samples.

Preparation of Flavoring Samples

Thirty ml of the liquid phase (supernatant), obtained as described above (Figure 2), were saturated with sodium chloride and extracted with diethyl ether to give sample A. Another 30 ml of the liquid phase were acidified to pH 6.5 with 15% hydrochloric acid and refluxed for 30 min in a 50 ml flask equipped with a reflux condenser and magnetic stirrer to obtain sample B. A third part of the supernatant (30 ml) was refluxed in the same manner as above but without acidification (pH 10) to obtain sample C. After cooling to room temperature, the aqueous solutions were evaluated sensorially, then saturated with sodium chloride and extracted with diethyl ether overnight using a rotary perforator (liquid-liquid extraction). The organic phases were dried over anhydrous sodium sulfate and purified by high vacuum distillation at 3 x 10^{-3} mbar. The contents of the traps were combined, dried and concentrated to about 1 ml for chromatographic analyses.

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Sensory Evaluation

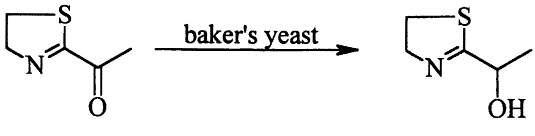
The aroma of each sample was evaluated by sniffing the headspace of the freshly prepared samples (A, B, C). Ten assessors were asked to describe the aroma quality and intensity. For descriptive analysis, a limited number of aroma descriptors were provided to the panel in order to reduce the number of attributes and simplify the aroma characterization. The intensity range of the aroma was scored from 1 (weak) to 3 (intense).

Analyses by Gas Chromatography and Mass Spectrometry

GC and GC-O analyses were performed using a Carlo Erba gas chromatograph (Mega 2, GC 8000) equipped with automatic cold on-column injector, FID, and sniffing port. Fused silica capillary columns (OV-1701 and DB-FFAP) were used, both 30 m x 0.32 mm and film thickness 0.25 μm. The temperature program for the OV-1701 was: 35°C (2 min), 40°C/min to 50°C (1 min), 6°C/min to 240°C (10 min), and for the FFAP was: 50°C (2 min), 6°C/min to 180°C, 10°C/min to 240°C (10 min). GC-MS analyses were carried out using a Finnigan MAT-8430 mass spectrometer using the same GC conditions described above. The MS-EI spectra were generated at 70 eV and MS-CI at 150 eV with ammonia as the reagent gas.

Applications of HDT in Pizza

HDT is commercially unavailable and was prepared by microbial reduction of the carbonyl group of 2-AT using baker's yeast as biocatalyst. This approach



2-AT

1

HDT

2

Figure 3. Biogeneration of 2-(1-hydroxyethyl)-4,5-dihydrothiazole.

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was identified as the most appropriate and easiest way to produce HDT in one step and under mild conditions (Figure 3).

The application of HDT was performed in two ways using pizza dough as a food model. In the first application, a solution of HDT (1,6 mg/ml) was mixed with other ingredients of the dough. Three different concentrations of HDT were tested, ranging from 0.01 to 1.0 g of HDT solution per 50 g of raw dough. In the second application, HDT solution was applied as a surface coating at five different levels ranging from 0.04 to 1.0 g per 50 g of raw dough. The highest amount corresponded to the maximum limit before appearance of white spots during baking.

For the frozen pizza, samples were pre-baked for 8 min at 220 °C, wrapped in plastic bags without modified atmosphere and kept frozen for 2 weeks. For the refrigerated pizza, samples were wrapped in plastic bags with modified atmosphere (50% N_2/ 50% O_2) and kept refrigerated for maximum of 1 week. Frozen and refrigerated pizza samples were baked for 8 and 15 min respectively at 200°C in a rotary convection oven.

Sensory evaluation by triangle test procedures was performed on the baked pizza samples. Samples spiked with HDT were compared to the corresponding reference samples. Tasting sessions were performed under red light to avoid visual identification of the different product.

Panelists (12) were asked to identify which pizza sample out of the three was different. The samples were presented in the following two schemes: 1 reference, 2 HDT spiked samples or 2 references and 1 HDT spiked sample. The assessors were asked to provide comments about the samples on why a sample was selected as different.

Results and Discussion

Biogeneration of Flavoring Preparations

A flavoring preparation of commercial interest was obtained by fermentation of cysteamine and ethyl-L-lactate with baker's yeast. Ten assessors described the aroma quality of the freshly concentrated extract as roasted, dried sausage and sausage skin-like and of high odor intensity.

As shown in Table I, sixteen odor-active volatile compounds were detected by GC-O using two capillary columns of different polarity. Thirteen odorants were identified by matching retention indices, odor qualities and mass spectra with those of reference compounds, if available, or with literature data. 2-Methylthiazolidine, isovaleric acid and 2-acetyl-2-thiazoline the most intensely smelled compounds in the sample. Several other odorants were also identified in this flavoring preparation, i.e., 2-methyl-3-furanthiol (MFT), 3-mercapto-2-pentanone, 2-ethyl-3,5-dimethyl-pyrazine, and 2-AT. These odorants have been cited as characteristic constituents of boiled and roasted meat (*4*,*5*). As shown in this study, these aroma impact compounds can also be generated by fermentation using suitable precursors and without applying any heat treatment.

Several, mainly cyclic, sulfur-containing compounds were also identified such as thiazolines and thiazolidines. Three thiazolines were identified as 2-acetyl-2-thiazoline 1, 2-(1-hydroxyethyl)-4,5-dihydrothiazole (HDT) 2 and 2-methyl-2-thiazoline 3 (Figure 4). 2-AT was the most intensely smelling odorant and is describesd as having a pleasant roasted, popcorn-like odor. This compound was identified by GC-O, GC-MS, and the sensory and chromatographic properties of this compound were identical to those of the reference compound.

In sample A, we identified HDT by matching retention indices and mass spectra with those published in the literature (7). This is the first time that these two compounds (HDT and 2-AT) have been generated via fermentation using baker's yeast as a biocatalyst, and without applying any heat treatment. The predominant volatile compound generated in sample A was identified as N-acetyl cysteamine 4. This compound, which smells burnt, yeasty and musty, is probably the precursor of 2-methyl-2-thiazoline 3. Indeed, this last aroma compound was generated upon storage; most likely via intramolecular cyclisation followed by elimination of water (Figure 4). However, the contribution of this compound to the overall aroma appears to be rather low.

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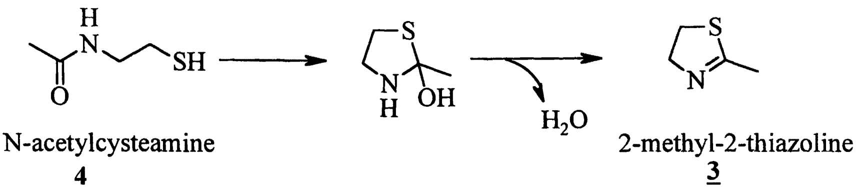

Figure 4. Hypothetical formation of 2-methylthiazoline from N-acetyl cysteamine.

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Table I. Odorants Detected by GC/O in Sample A

N°	*Compound*	*Identification*			*Linear Retention Index*		*Aroma Quality*	*Aroma Intensity*
		RI	*MS*	*Ref*	*FFAP*	*OV-1701*	*(GC/O)*	*Sample A*
1	Isobutanol	X	X	X	1085	725	Malty	2
2	3-Methyl-1-butanol	X	X	X	1203	848	Metallic, musty, malty	2
3	2-Methyl-3-furanthiol	X	-	X	1305	932	Meaty, roasty	2
4	3-Mercapto-2-pentanone	X	-	X	1354	1021	Catty, sulfury	2
5	3-Isopropyl-2-methoxypyrazine	X	-	X	1398	1149	Roasted	1-2
6	**2-Methylthiazolidine**	**X**	**X**	**-**	**1418**	**1025**	**Putrid, amine-like**	**3**
7	2-Ethyl-3,5-dimethylpyrazine	X	X	X	1453	1149	Roasted, earthy	2
8	Butanoic acid	X	X	X	1625	970	Sweaty, yeasty	2
9	Isovaleric acid	X	X	X	1665	1024	Sweaty, rancid, yeasty	2-3
10	**2-Acetyl-2-thiazoline**	**X**	**X**	**X**	**1748**	**1245**	**Roasted, popcorn**	**2-3**
11	2-(1-hydroxyethyl)-4,5-dihydrothiazole	X	X			1270	Roasted, amine-like	1-2
12	Unknown		-		1774		Roasted	1-2
13	Unknown		-		1790		Roasted	1-2
14	2-Phenylethanol	X	X	X	1905	1276	Spicy, almond-like	1-2
15	Unknown		-		2040		Roasted	2
16	N-Acetylcysteamine	X	X	X	2200	1450	Burnt, yeasty, musty	1-2

RI: Retention Index; MS: Mass Spectrometry; Ref: Reference compound available; Aroma Intensity: 1 (weak), 2 (medium), 3 (high).

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A reference sample was obtained using the same incubation conditions as for sample A, but without addition of cysteamine and ethyl-L-lactate. After extraction and concentration as detailed in the methods section, the odor of the sample was described as only yeasty and musty. GC analyses did not show any of the sulfur-containing compounds found in sample A, thus indicating that the sulfur compounds were generated from cysteamine.

The thermal treatment of the supernatant at pH 6.5 and 10 resulted, after extraction, in samples B and C, respectively. The aroma qualities of these samples were described as roasted, popcorn, and bread crust-like, and all were found to have high odor intensities. However, sample B was more intense than C, thus suggesting the importance of the pH to flavor formation during heat treatment.

The aqueous solutions obtained after heat treatment were extracted with diethyl ether. The extracts were then concentrated and analyzed by GC-O on two capillary columns of different polarity (FFAP and OV-1701). As shown in Table II, 2-acetyl-2-thiazoline was the dominant aroma compound in both samples B and C. This result is in good agreement with the sensory evaluation of the two samples which were clearly described as roasted and popcorn-like.

The amount of 2-AT in sample B was higher than in sample C (Figure 5). However, sample C contained more 2-methylthiazolidine, 2-ethyl-3,5-dimethylpyrazine and trimethypyrazine than sample B. This observation explains the difference in aroma intensity and quality between the two samples, and shows the influence of the pH on the formation of some aroma impact components.

Impact of HDT as an Aroma Precursor

2-(1-hydroxyethyl)-4,5-dihydrothiazole has been proposed as a potential precursor of 2-AT in a model reaction. A possible formation mechanism of 2-AT was proposed by Hofmann and Schieberle (*7*) as shown in Figure 6.

However, the impact of HDT as a precursor to improve the roasted notes of baked goods has never been demonstrated in food models. As shown in Table III, HDT improved the roasted note of pizza dough. When this aroma precursor was incorporated into the dough, panelists described samples as roasted, toasted and popcorn-like. However, when HDT was applied as surface coating, these aroma notes were lost by release into the oven during baking; which led to a nice smelling odor in the kitchen, but less aroma remaining in the baked dough.

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Table II. Odorants Detected by GC/Olfactometry in Samples B and C

N°	*Compound*	*Identification*			*Linear Retention Index*		*Aroma Quality*	*Aroma Intensity*	
		RI	*MS*	*Ref*	*FFAP*	*OV-1701*	*(GC/O)*	*Sample B*	*Sample C*
1	Diacetyl	X	-	X	990	680	Buttery, sweet	2	-
2	Isobutanol	X	X	X	1084	725	Malty	1	1
3	3-Methyl-1-butanol	X	X	X	1201	845	Metallic, musty, malty	2	1-2
4	2-Acetyl-1-pyrroline	X	-	X	1328	1013	Roasted	1-2	1
5	Trimethylpyrazine	X	-	X	1397	1078	Roasty, earthy	-	2
6	2-Methylthiazolidine	X	X	-	1415	1025	Putrid, amine-like	1	2
7	Unknown		-		1437		Roasted, earthy	-	1-2
8	Unknown		-		1448		Savoury	1-2	-
9	2-Ethyl-3,5-dimethylpyrazine	X	X	X	1451	1149	Roasted, earthy	1-2	2
10	Unknown		-		1473		Pyridine-like	-	1
11	(E)-2-Nonenal	X	-	X	1528	1272	Fatty	-	1-2
12	Isovaleric acid	X	X	X	1664	1024	Sweaty, rancid	1	1
13	**2-Acetyl-2-thiazoline**	**X**	**X**	**X**	**1748**	**1255**	**Roasted, popcorn**	**3**	**3**
14	2-Phenylethanol	X	X	X	1905	1275	Spicy, almond-like	1	2
15	Furaneol	X	-	X	2025	1250	Caramel-like	-	1-2

RI: Retention Index; MS: Mass Spectrometry; Ref: Reference compound available; Aroma Intensity: 1 (weak), 2 (medium), 3 (high).

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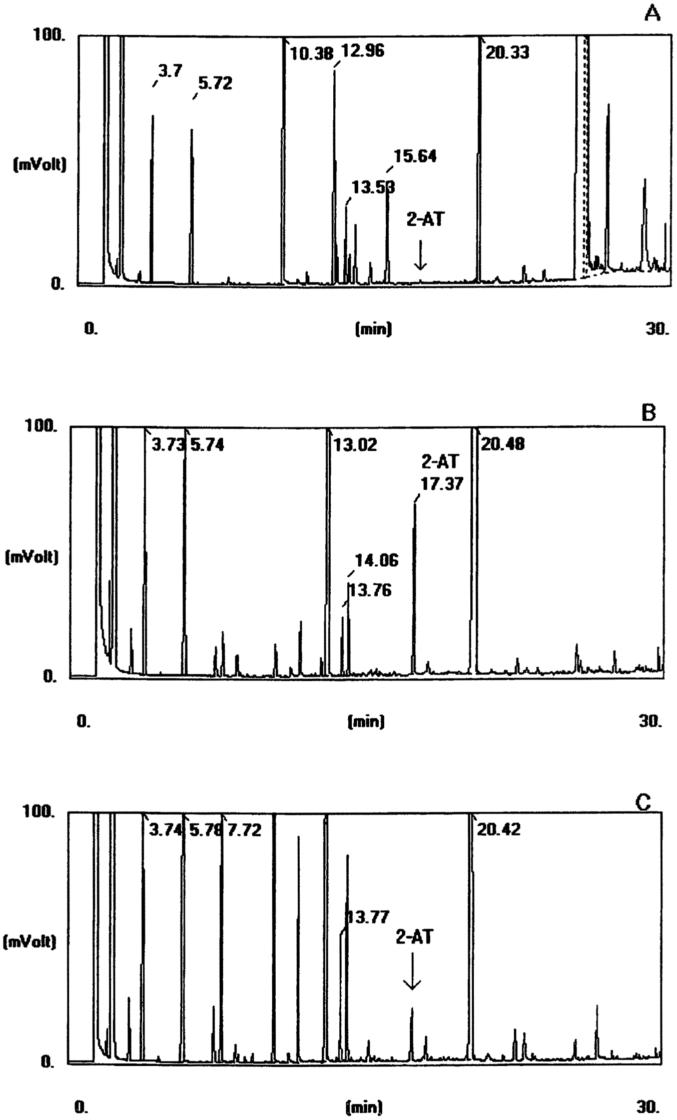

Figure 5. Gas chromatograms of the samples A, B, and C indicating the amounts of 2-acetyl-2-thiazoline (2-AT) generated by bioconversion and combined bio/thermal processing.

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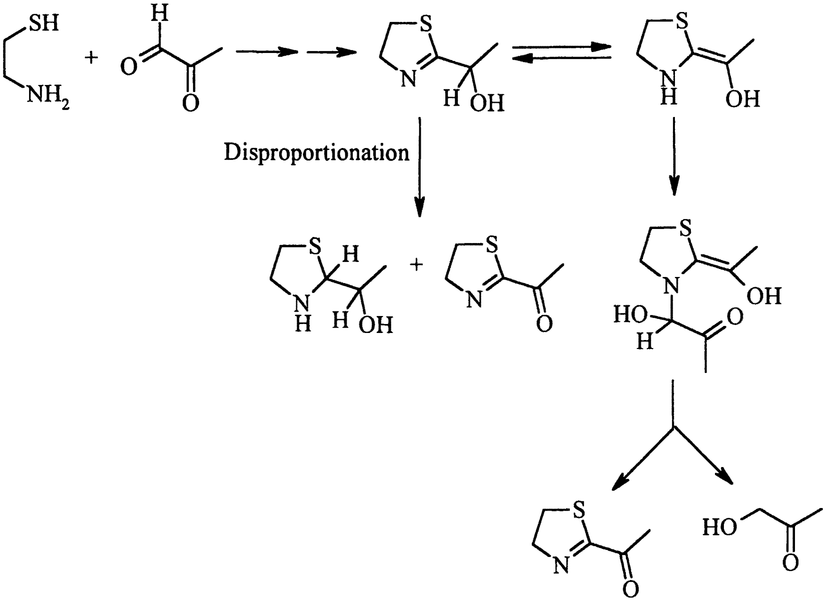

Figure 6. Hypothetical formation of 2-AT proposed by Hofmann and Schieberle. (Reproduced from reference 7. Copyright 1995 American Chemical Society.)

Table III. Sensory Evaluation of Spiked Samples Compared to the Reference

	Frozen Pizza	*Refrigerated Pizza*
HDT mixed into the dough	No difference p-value = 0.14	Significantly different p-value = 0.001
HDT as surface coating	Significantly different p-value = 0.01	Significantly different p-value = 0.002

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Conclusions

Bioconversion of cysteamine and ethyl-L-lactate with baker's yeast resulted in a natural flavoring which was described as dried sausage-like. When this reaction mixture was heated under acidic or alkaline conditions, the resulting samples B and C, respectively, exhibited attractive and intense roasted, popcorn and bread crust-like notes. High amounts of 2-AT were detected in these samples by different chromatographic techniques. Moreover, HDT seems to be a promising precursor to improve roasted note of baked goods.

Acknowledgements

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Chapter 12

A Review of Methods for the Analysis of Oxygen-Containing Aroma Compounds

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A review of historical and current techniques used to isolate and analyze oxygen-containing aroma compounds is provided. Various isolation techniques that have been applied in the analysis of oxygen-containing aroma compounds are discussed as are methods of detection.

Several oxygen-containing functional groups are important to aroma, either to elicit odor on a molecule or serve as a precursor to other odor-active molecules. Oxygenated functional groups include acids, alcohols, esters, lactones (cyclic esters), epoxides, aldehydes and ketones. A general rule is that as a molecule becomes more polar, it becomes less odor-active. For example, measuring odor-active levels of relatively non-polar unsaturated aldehydes requires lower detection limits than does that of the more polar molecules furfural or eugenol (*1*). Many important aroma-producing reactions such as oxidation and browning directly involve the oxygen atom. These reactions may include molecules such as fatty acids, amides (amino acids), and poly-alcohols (carbohydrates), which many have little to no aroma in the initial state but upon decomposition or reacting with other materials form oxygenated aroma compounds.

In Heteroatomic Aroma Compounds; Reineccius, G., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2002.

As a result of the variety and abundance of oxygen reactive precursors, a large number of oxygen-containing aroma compounds exist which collectively exhibit a broad range of physical and sensorial properties.

Isolation Techniques

Headspace

Solubility (polarity) and boiling point are physical properties commonly used in the isolation of aroma compounds from foods. Table I lists these properties for several oxygen-containing aroma compounds. Collectively, these compounds exhibit a broad range of properties that can present challenges for isolation and quantitation. For example, acetaldehyde and vanillin have boiling temperatures of 20°C and 285 °C, respectively, thus would require very different headspace methodologies for isolation.

Table I. Boiling Temperature and Solubility of Some Oxygenated Volatiles

Compound	*BP (°C)*	*Soluble In*
Acetaldehyde	20	water, ethanol, oil
Acetic acid	118	water, ethanol, propylene glycol
Carvone	230	ethanol, oil
Delta decalactone	281	ethanol, oil
Diacetyl	88	propylene glycol, glycerin
Ethanol	78	used as a solvent
Furaneol		ethanol, oil
2,6-Nonadienal	187	ethanol, oil
Propylene glycol	189	used as a solvent
Vanillin	285	ethanol, oil

Source: (*113, 114, 115*)

Dynamic headspace (DHS) has been commonly employed in the isolation of aroma compounds from foods (*2, 3*). Several studies suggest optimized DHS parameters for the analysis of oxygen-containing desirable odorants or off-odors in food products, including, wine (*4*), cheese (*5*), fish (*6*), honey (*7*), apples (*8*), milk (*9*), milk and fermented dairy products (*10*), chicken breast meat (*11*), orange juice (*12*) and cheese (*13*). DHS has been used to monitor aroma

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compounds in foods exposed to oxidative processes; such as, dry cured hams (*14*), wheat germ (*15*), olive oil (*16*) milk (*17*) and vegetable oils (*18*).

One study investigated the oxidative effect of using hydrogen as the purging gas on the recovery of unsaturated aldehydes (*19*). Partially and fully hydrogenated artifacts were formed from 2-alkenals and 2,4-alkadienals when hydrogen was used in place of helium. The losses of unsaturated aldehydes are illustrated for 2,4-decadienal where only 31% was recovered when hydrogen was used as the desorption and carrier gas (*19*). No hydrogenated compounds were observed when helium was used as the GC carrier gas and the authors recommend that helium be used for accurate GC analysis of unsaturated aldehydes.

Hartvigsen et. al suggested a means of removing interfering acids from purge and trap analyses (*20).* They installed an S-shaped glass tube trap containing crushed KOH pellets to remove acetic acid and 2,4-hexadienoic acid from the volatile sampling of fishy and rancid off-odors in fish oil enriched mayonnaise. Without the use of the KOH trap, an 8 min solvent delay had to be used to prevent damage of the MS filament by the relatively high concentrations of these acids (*20*). This solvent delay resulted in a loss of information on low molecular weight volatiles. Also, the high levels of acetic acid shifted retention times and the high level of ions from acetic and 2,4-hexadienoic acid made identification of low level volatiles difficult. Use of the KOH trap removed these acids without the formation of artifacts or alteration in the volatile profiles except for removal of the two acid odors.

Particularly problematic oxygenated compounds have been isolated using high flow dynamic headspace methodology combined with liquid-liquid solvent extraction (*21,22*). This technique has proven very useful to isolate labile compounds such as 2,5-dimethyl–4-hydroxy-3(2H)-furanone, an important odorant that is often difficult to isolate by conventional headspace techniques. Closed-loop dynamic headspace methods that employ longer purge times and minimize losses of low boiling volatiles (*23*) have been used to isolate volatiles from corn tortilla chips (*24*) and tomatoes (*25*). The closed-loop technique addresses the limitation of breakthrough during the purging stage of the dynamic headspace method.

Generally, SPME has utility as a rapid and inexpensive aroma isolation technique from matrices that present difficulties for other isolation techniques. For quantitative purposes, one must pay close attention to sample temperature, type of fiber, fiber exposure time, headspace volume, stirring rate, ionic strength, and medium composition. However, several studies referenced below found limitations that one must be aware of. Problems noted included fiber to fiber variation, low storage stability of fibers, and a dependence of extraction efficiency on relative humidity demonstrated that in order to maintain high sampling precision, you must use the same sampling fiber. Also, highly variable

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results were obtained when fibers were used repeatedly over time (>150 injections) which raises concern for the utility of SPME for shelf-life studies.

SPME has been used to determine ester formation during grape juice fermentation (*27*), quantitation of wine aroma components (*28,29*), formation of branched esters during banana ripening (*30*), quantitation of pentanal and hexanal in cooked turkey (*31*), quantitation of menthone and menthol in various peppermint flavored products (*32*), volatile aliphatic aldehydes in sunflower oil (*33*), some alcohols and esters in beer (*34*) and the headspace of orange juice (*35*). SPME has been used to determine 2-acetyl-1-pyrroline in the headspace of rice (*36*), 2-methylisoborneol and geosmin in catfish (*37*), 2,4,6-trichloranisole in wine (*38*) and isotopic quantitation of diacetyl in wine (*39*). SPME has also found application in determination of origin and quality of vanilla extracts (*40*), origin of olive oils (*41*), varietal differentiation of strawberries (*42*), classification of wines based on concentration of nerol oxide and alpha terpineol (*43*), characterization of Muscat wines by chiral analysis (*44*) and shelf-life prediction of milk (*45*).

SPME has been compared to dynamic headspace with cola (*46*), broccoli (*47*) and milk (*48*), and compared to Freon liquid-liquid extraction of brandy (*49*). In summary, these authors founds that conventional dynamic headspace sampling methods proved more sensitive for highly volatile compounds, while SPME headspace methods extracted more of the intermediate and lesser volatile compounds. SPME proved less suitable for accurate quantitative analysis because certain SPME fibers (PDMS) strongly discriminate against more polar and highly volatile compounds. Liquid-liquid sampling methods proved overall more sensitive and reproducible. The use of SPME fibers also produced artifact peaks, primarily siloxanes from the PDMS fiber, and these artifacts may impose limitations on its use *(46)*.

Solvent Extraction

Solvent extraction methods are typically preferred over most other isolation techniques since they can be relatively simple while generally producing extracts of the highest aroma quality. For example, quantitation of 2-acetyl-1-pyrroline in brown rice has been simplified by using a traditional solvent extraction procedure (*50*). Previously, the authors had used a labor intensive indirect steam distillation method that resulted in thermal losses of the component of interest. By using a direct solvent extraction and subsequent solvent concentration procedure, 2-acetyl-1-pyrroline was detected by GC-MS from small (3.0 g) samples of brown rice more simply and with improved recoveries. Alvarez et. al (*51*) reported the successful replacement of the carcinogenic solvent CS_2 with the less hazardous hexanol as an extracting solvent for free fatty acids, fusel alcohols and esters in beer.

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Pino et. al (*52*) compared pentane, pentane:diethyl ether (2:1) and methylene chloride solvents for the extraction of 3-methyl butanol, butanol, 2-phenylethanol, octanal, benzaldehyde, ethyl propionate, ethyl butanoate, ethyl isopentanoate, ethyl octanoate, ethy decanoate, eugenol and beta ionone in a model system designed to mimic a distilled alcoholic beverage. The authors concluded that the use of pentane solvent provided the best results probably since it extracted the least ethanol.

Ferreira et. al (*1*) proposed a solvent extraction method to quantify oxygenated compounds responsible for the stale flavor of oxidized wines. The technique involves salting out an organic layer from the wine with H_2NaPO_4 and $(NH_4)_2SO_4$, adding diethyl ether:pentane (10:90) to this layer, diluting this organic mixture with saline solution, followed by shaking and centrifugation. The resulting organic phase was directly injected into a GC interfaced to an ion trap mass spectrometer. The oxygenated aroma compounds evaluated were hexanal, *E*-2-hexanal, *E*-2-octenal, *E*-2-nonenal, furfural, 5-methylfurfural, benzaldehyde, 4-hydroxy-4-methyl-2-pentanone, 2-nonanone, 2-butoxyethanol, 1-octen-3-ol, furfurol and eugenol. The method was found to be free from matrix effects, and linearity was satisfactory, global method reproducibility ranged from 3 to 7% for most of the analytes, and detection limits were reported from 10 ng/L to 600 ng/L.

Solvent extraction for the isolation of oxygen-containing aroma compounds has been used either preceding or follow other isolation steps. For example, Soxhlet extraction of bread has been followed by high vacuum distillation (*53*), or solvent extraction of lavender and eucalyptus honeys has been followed by simultaneous distillation/solvent extraction (*54*). This multi-isolation approach is useful in applications where the matrix presents several challenges to a single isolation step. As noted above, solvent extraction can also be used to follow an isolation technique. An example is when an acid/base fractionation scheme is used to simplify an aroma isolate. Acid/base fractionation is commonly used on diluted extracts obtained from solvent, Likens-Nikerson and high vacuum distillation extraction techniques. Acid/base fractionation can be done for a variety of reasons; such as, to optimize quantitation, to simplify a complex extract, or to enhance the subsequent chromatography.

Derivatives

Historically, derivatives have been utilized for the quantification of oxygen-containing aroma compounds. Knapp (*55*) suggests several reasons why derivatives can be useful: to enhance or suppress volatility, enhance chromatography, suppress thermal decomposition in the GC, increase detector sensitivity, or to obtain a total measurement of a compound or compounds that have formed complexes.

Volatile carbonyl compounds have been measured by first reacting them with 2,4-dinitrophenyl hydrazine to form the corresponding 2,4-dinitrohydrazone (DNP) and then analysis of the derivative by GC or HPLC. This technique was applied to identify formaldehyde, acetaldehyde, furfural, 2-acetyl furan and 5-methylfurfural (*56*); quantify and isolate alkan-2-ones, n-alkanals, alk-2-enals and alk-2,4-dienals from casein and milk powders (*57*); isolate and identify monocarbonyl components in whole milk powders (*58*), monitor the quantity of 2-furaldehyde and 5-hydroxymethyl-2-furaldehyde in processed citrus juices (*59*), determine the total carbonyl compounds in aqueous media (*60*) and to analyze carbonyl compounds present in ambient air (*61*). Koivusalmi et. al (*62*) developed a method to quantify aldehydes using DNP and a reversed-phased HPLC method. The authors evaluated methanal, propanal, 2-methylpropanal, butanal and 2-ethylhexanal and reported detection limits of 4.3-21.0 ug/L. Lo Coco et. al (*59*) found detection limits of 10 ug/kg using DNP derivatives of 2-furaldehyde and 5-hydroxymethyl-2-furaldehyde in honey. Hydrazine reagents used as derivatizing agents in environmental analysis have been recently reviewed (*63*).

Shibamoto (*64*) developed a simple and sensitive derivatization method for analysis of formaldehyde, acrolein and malonaldehyde in air or food samples. Formaldehyde is determined based on a reaction with cysteamine to form thiazolidine whereas the acrolein and malonaldehyde are reacted with n-methylhydrazine to form 1-methyl-2-pyrazoline and 1-methylpyrazone, respectively. These derivatives were then analyzed by GC-nitrogen phosphorus detection. For formaldehyde, this method overcomes the disadvantage of the most common prior method which prepared 2,4-DNPH derivatives of formaldehyde under strongly acidic conditions. This reaction environment may cause undesirable reactions such as artifact formation, and decomposition of carbohydrates ands proteins in foods and beverages. According to the authors, this method also greatly simplifies the analysis of acrolein. Most methods for acrolein analysis involve tedious derivatization with 2,4-DNPH or hexylresocinol and then quantification by non-specific colormetric techniques. This method involves specific one-step reactions of acrolein with either morpholine or N-methyl hydrazine to form 3-morpholinopropanal or 1-methyl-2-pyrazoline, respectively, which are then easily analyzed by GC-NPD. The detection limit of the derivative 1-methyl-2-pyrazoline was reported as 5.9 pg acrolein in air, with recovery efficiencies of 98% and 100% for 150 ug and 15 ug, respectively. In addition to formaldehyde and acrolein, the he authors demonstrated the utility of this methodology to measure aldehydes and ketones in coffee, tea, cocoa, nonfat dry milk, soy sauce, air, beef and pork fat, and grapefruit.

Solid phase microextraction (SPME) and derivatization have been combined in the analysis of oxygen-containing aroma compounds. Martos and

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Pawliszyn (*65*) reported on a procedure where they absorbed o-(2,3,4,5,6-pentafluorabenzyl)-hydroxyl amine (PFBA) onto a poly-(dimethyl-siloxane)/divinyl benzene (PDMS/DVB) fiber, which was utilized for the study of the headspace volatiles of deciduous leaf and coffee grounds. The derivatization occurs when a carbonyl compound absorbs onto the PDMS/DVB fiber with PFBA. The carbonyl reacts to form the corresponding oxime that is subsequently desorbed for analysis. This technique can be useful for field studies since desorption does not need to be done immediately after derivatization. The authors demonstrated that sensitivity could be influenced by exposure time and temperature, though the quantity of available derivatizing reagent can also be a limiting factor with extended periods of exposure time.

Ebeler and Spaulding (*66*) developed a thiazolidine derivatization technique to study a variety of aldehydes in wine. The objective of their work was to develop a single assay that would include specificity, sensitivity, cover a broad range of free aldehydes and include the aldehydes that had formed complexes with sulfur dioxide and phenolics in wine. The aldehydes they included were methanal, ethanal, propanal, 2-methylpropanal, butanal, 3-methylbutanal, 2-methylbutanal, pentanal, hexanal, heptanal, octanal and nonanal. The thiazolidine derivatives were found to have detection limits of 1-2 ug/mL and about 0.1 ug/mL for GC/MS and nitrogen-phosphorous detector (NPD), respectively. Though the NPD provided approximately ten-fold greater sensitivity, the authors did not find a linear response at concentrations below 1 ug/mL.

Traditionally, fatty acid profiles of oil are obtained by derivatizing them to the methyl ester. However, when the short chain acids are converted into volatile methyl esters, they are often lost prior to injection on a gas chromatograph. This can be an issue in the analysis of dairy products. Formation of butyl esters has been used as an alternative to methyl esters to decrease volatility (*67*).

Instrumentation

Detectors

While many different non-specific GC detectors can be used to detect oxygenated aroma compounds, research on oxygen selective detectors has not attracted the same level of activity as other elements such as sulfur. Oxygen-containing aroma compounds may be detected using other specific detectors through derivatization, i.e. adding an element that can be specifically detected. Detectors such as the atomic emission (AED) and infrared (IR) can be specific for oxygen although the detector most commonly employed for qualitative and

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quantitative evaluation of oxygenated aroma compounds is the mass spectrometer (MS). It should also be noted that the human nose also has been successfully coupled with the gas chromatograph in GC-effluent sniffing techniques. Oxygenated compounds with extremely low sensory thresholds (below instrument detection thresholds) may be tentatively identified or concentrations crudely estimated using a combination of sensory and GC methodology (gas chromatography/olfactometry).

IR detectors have been previously discussed for flavor analysis (*68*). Recently Kallio et. al (*69*) compared Fourier transform IR to other techniques for aroma analysis of strawberries. Chang et. al (*70*) and Seitz et. al (*71*) used GC coupled with MS and IR to identify 2-acetyl-2,3-dihydropyridine and 5-ethyl-1-cyclopentene-carboxaldehyde, respectively, in bread. The IR detector has advantages of specificity, especially for carbonyl carbons that exhibit strong absorbance, the ability to scan with selective wavelengths, and provide qualitative information. The principle limitations are that the IR detectors lack sensitivity and are not routinely coupled with a GC in most analytical flavor laboratories.

The AED is very specific for oxygen, particularly at the emission wavelength of 171 nm (*72*) and the factors influencing oxygen selectivity have been reported (*73*). Measuring oxygen at 171 nm has specifications for minimum detection level of 150 pg/sec, whereas the specifications for carbon at 193 nm, nitrogen at 174 nm and sulfur at 181 nm are 1, 30 and 2 pg/sec, respectively (*71*). The AED has application for quantitative analysis of pesticide residues (*74-78*), methyl mercury in fish (*79*), chloropyrifos in fruits and vegetables (*80,81*), selenium in some vegetables (*82,83*), organotin in seawater (*84*), biological samples (*85*) and food packaging (*86*). Published accounts utilizing the AED for flavor analysis have primarily been for sulfur analysis (*87-90*). Since most, if not all, oxygenated flavor compounds contain carbon, there is little advantage of using the AED for the sole purpose of oxygen analysis as greater sensitivity can be achieved by using the AED to measure carbon at 193 nm, or by use of other traditional GC detectors.

However, the AED could be utilized for compound independent calibration (CIC), a technique that could have application for quantitation of oxygenated aroma compounds. CIC is a unique quantitative tool based on the concept that the AED response to each element has no dependence on the compound from which it originates; therefore calibration response factors are based on each element and not on the compound. Utilizing CIC, the AED performs quantitative analysis of each element and the summation of all elements is converted to quantitation of the known compound. CIC provides the advantages of quick and simple calibration, provides total element analysis and provides quantitation in the absence of identical standards; the latter can be useful when standards are unavailable, toxic, or unstable (*91*).

The oxygen sensitive FID operates by disintegrating oxygenated compounds (that also contain carbon) into carbon monoxide (CO), then converting CO into methane which is subsequently measured by conventional FID (92). Gokeler (92) suggests that sensitivity, measured by signal to noise ratio, is influenced by the reactor tube temperature, the oxygen residue in the carrier and is proportional to the amount of oxygen in the molecule relative to the molecular weight; the lower the molecular weight the greater the molar sensitivity to the molecule. For example, Gokeler (*92*) reports sensitivities of 12 and 49 ppm for methanol and 1-octanol, respectively, as each solution contains roughly 6 ppm of oxygen. Sensitivity can be increased 10 fold by increasing the injection volume and venting all but the known oxygenated compounds.

Woelfel and Hartmen (*93*) investigated the formation of acetals by GC-MS. The principle objective of their work was to present mass spectral fragmentation patterns and retention indices of common acetal derivatives as the published literature is relatively weak on this topic. It is common for the flavor industry to use ethanol, propylene glycol and glycerol as major or minor components of the solvent system of a finished flavor. Under certain conditions, aldehydes that comprise the flavor may react with the hydroxy solvents and form acetals. As a result, the formation of acetals may alter the profile of the flavor over time, reducing the shelf-life of the flavor. Examples of common aldehydes used in flavors include vanillin, benzaldehyde, cis-3-hexenal, and cinnamic aldehyde.

The MS has also been used for the quantification of aroma active compounds by the use of isotopes. In the mid 1960's, Unilever used radiolabeled isotopes to quantify lactones in butter (*94*). More recently, the use of stable isotopes in flavor analysis has been employed by Schieberle and Grosch (*95*) and the technique has been recently reviewed (*96*). Essentially the known odorant of interest is synthesized with the objective of replacing two or more hydrogen or ^{12}C atoms with deuterium or ^{13}C atoms, respectively, such that the synthetic odorant is increased in molecular weight by at least two units. The labeled compound is then used as an internal standard for quantitation. The advantages of using stable isotopes for quantitation is that this technique is highly accurate, provides increased sensitivity and validates the identity of the components of interest. This method proves very useful for the detection and quantitation of many oxygen-containing compounds that are very odor active, labile, difficult to recover, and exist at very low levels in foods or beverages (e.g. n-nonenal, n-octanone, n-hexenoic acid, sotolone, furaneol and β-damascenone). The principle limitations are that the labeled standards are generally not commercially available (therefore they must be synthesized), the specific synthesis might not be published and the reagents can be expensive.

The human nose has also been employed as a detector coupled with a gas chromatograph, primarily for discrimination of compounds in an extract to decipher which contribute to an aroma. There currently are four different GC-

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effluent sniffing techniques: aroma extract dilution analysis (AEDA)(*97*) and Charm (*98*) are based on detection thresholds, Osme (*99*) is based on intensity and the method proposed by Pollien et. al (*100*) is based on detection frequency among six to ten assessors. AEDA has been evaluated (*101*) to suggest it delivers qualitative information that is representative of the foodstuff from which the aroma was extracted. AEDA and Charm have been compared to each other (*102*) though the authors did not recommend one technique over the other. While Osme has not received as much attention in the literature as Charm or AEDA, it has been shown to be sensitive and reproducible (*99, 103*). GC-effluent sniffing techniques can determine which oxygenated compounds have aroma and can also detect aroma compounds that are present below detection levels of the instrumentation. In short, the GC-effluent sniffing techniques provide direction as to which parts of the extract or chromatogram merit further attention.

Stable Isotope of Oxygen (^{18}O)

The stable isotope of oxygen, ^{18}O, has been used to detect adulteration, determine origin (botanical, chemically reacted botanical precursors, or synthetic), and elucidate chemical pathways of oxygenated compounds. Specific detection of ^{18}O is easily achieved by MS and NMR (*104-112*).

Isotopic ^{18}O techniques for beverage authenticity have been employed for many years *(104-107)*. This method, which utilizes knowledge of delta ^{2}H and delta ^{18}O values, is used to detect the addition of low levels of sugars to juices and sugars and must enrichment in wines, thus providing an accurate means to detect adulteration. This method of analysis can be used to differentiate a natural flavor from the synthetic counterpart. For example, assertion of the chemical origin of the valuable natural flavoring, raspberry ketone (4-(4-hydroxyphenyl)butan-2-one) has been demonstrated *(108)*. By analyzing delta ^{18}O and delta ^{13}C values the authors were able to determine if the compound was all natural, a natural botanical precursor further chemically reacted, or the synthetic component. The unquestionable reliability of stable isotope analyses makes this method useful for food and flavor industries to insure they have received the valuable and authentic natural components they have purchased, and comply with legal labeling requirements.

Stable isotopic analysis of ^{18}O has been used to determine both the regional and chemical origin of highly valued and certified food products such as butter and wine *(109, 110)*. Multielement stable isotope ratios of C, N, O, S and Sr determined in butters produced in the EU were able to prove regional origin, which conventional chemical methods cannot *(109)*. The application of delta ^{18}O analysis to wine water is used in the EU to determine wine origin and adulteration by watering down. Compiled delta ^{18}O coupled with delta ^{13}C values are being used to form a data bank of authentic values for wines from Italy, France and Germany *(110)*.

By incorporating ^{18}O into chemical precursors or by plant feeding studies using ^{18}O labeled precursors, the chemical pathways of flavor compounds can be determined. Two examples are presented that demonstrate this approach. Use of ^{18}O proved that trans-2-nonenal in bottled beer (indicated as responsible for cardboard off-flavor) does not occur via lipid oxidation as was previously believed *(111)*. Feeding Pelargonium graveolens with labeled [1,1-2H2, 18O]citronellol, demonstrated that this botanical is able to convert the fed precursor into cis-/trans-rose oxide *(112)*. The labeling pattern and diastereomeric ratio of the resulting cis-/trans-rose oxides were in agreement with a proposed enzymatic oxidation mechanism of citronellol in the allylic position and subsequent cyclization of the resulting diol to cis-/trans-rose oxide with retention of the oxygen of [1,1-2H2, 18O]citronellol.

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Conclusions

Clearly oxygenated compounds are an important class of aroma compounds. In general, sensitivity is not an issue as the majority of these compounds are known and detectable by current instrumentation. Notable exceptions are the hydroxyfuranones, damasenone and geosmin. It appears that most of the recent research on oxygenated aroma compounds is focused on improved isolation and quantitative techniques.

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Chapter 13

Flavor Contribution and Formation of Heterocyclic Oxygen-Containing Key Aroma Compounds in Thermally Processed Foods

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The biogeneration of oxygen-containing compounds is mainly based on the introduction of carbon dioxide into organic molecules by photo oxidation in plants. In addition, water and molecular oxygen can be enzymatically introduced. Although oxygen is also inserted into unsaturated compounds by auto oxidation, the generation of aroma compounds during food processing and storage is mainly characterized by losses of oxygen from oxygen rich molecules, primarily as water. In particular, carbohydrates are important precursors of oxygen-containing heterocyclic compounds, such as furanones and pyranones. In the present paper, the contribution of compounds like 4-hydroxy-2,5-dimethyl-3(2H)-furanone or 3-hydroxy-4,5-dimethyl-2(5H)-furanone to the flavors of processed foods are briefly discussed. New results on the formation pathways of 3(2H)- and 2(5H)-furanones and Maltol are presented.

In Heteroatomic Aroma Compounds; Reineccius, G., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2002.

Introduction

General Considerations

Besides the 10^{15} tons of molecular oxygen present on earth, oxygen is also the 3most abundant chemical element found in the inorganic material that forms the earth's crust, e.g., in oxides and carbonates. Furthermore, about 10^{18} tons of oxygen are bound in water and about 2.3 x 10^{12} tons occur in free carbon dioxide.

Carbon dioxide, water and molecular oxygen are the main sources in the biosyntheses of oxygen-containing molecules, and the use of carbon dioxide in plant photosynthesis yielding cellulose and starch is certainly one of the most important reactions in nature. Just to give an impression on how oxygen may be introduced into bio-organic molecules, four simplified reactions catalyzed by enzymes such as carboxylases (A), hydratases/oxido-reductases (B), oxygenases/ hydratases (C), and dioxygenases/oxido-reductases (D) are shown in Figure 1. The importance of these reactions to the formation of food aroma compounds is discussed in more detail in another chapter of this book.

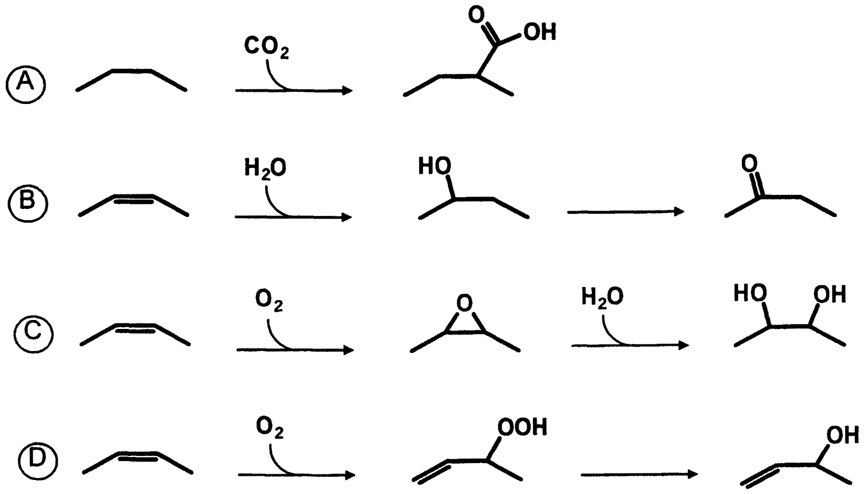

Figure 1. Reactions by enzymes such as carboxylases (A), hydratases/ oxido-reductases (B), oxygenases/ hydratases (C), and dioxygenases/oxido-reductases (D) catalyze the insertion of oxygen into biomolecules.

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Although hydrolysis and oxidation by air oxygen also play an important role in non-enzymatic reactions that occur during food processing, elimination of water or carbon dioxide as well as substitution reactions are more abundant when foods are heated (Figure 2). As shown in A (Figure 2) loss of three molecules of water generates furfural from a pentose and the loss of carbon dioxide from ferulic acid yields 4-ethenyl-3-methoxy-phenol.

The elimination of water or carbon dioxide generally increases the volatility of the compounds and, therefore, the potential for being flavor-active. The influence of the number and the position of oxygen atoms on the volatility of six carbon compounds is shown in Figure 3. The boiling point is increased significantly from ethyl butylether via 2-hexanone to 2-hexanol. However, in this series, introduction of a second oxygen atom does not increase volatility as much. Esters and α-dicarbonyls show quite low boiling points, whereas the presence of a second hydroxy group significantly increases the volatility.

Classification of Oxygen-containing Food Aroma Compounds

During food processing, the following reactions may lead to the generation of oxygen-containing aroma compounds:

- The peroxidation of unsaturated compounds (e.g., fatty acids and terpenes) mainly yielding alcohols, ketones and aldehydes.
- The degradation of phenyl propanoic acids, such as ferulic acid, giving rise to aromatic aldehydes, ethers and alcohols.
- The degradation of carbohydrates forming furanaldehydes, furanones and other Maillard-type compounds.
- The degradation of free amino acids yielding *Strecker* aldehydes.

Because many reactions are possible in a complex food matrix at elevated temperatures, during processing normally hundreds of volatiles are generated, e.g., more than 800 compounds have been identified in roasted coffee *(1)*. However, from the flavor point of view it has to be taken into account that volatility is a necessary but not a sufficient criterion of a compound to act as a flavor molecule. The limitations are caused by the human odor receptors, which are very selective. One important attribute of a volatile compound with respect to flavor perception by humans is the odor threshold. Odor threshold is defined as the lowest concentration of a volatile in a given matrix which can be detected by the human odor receptors, normally assayed by a sensory panel. This influence of the structure of a compound on odor threshold is exemplified in Figure 4 for the three well-known oxygen-containing food odorants diacetyl, (E)-ß-damascenone, and 3-hydroxy-2-ethyl-5-methyl-2(5H)furanone (3-HEMF; Abhexon).

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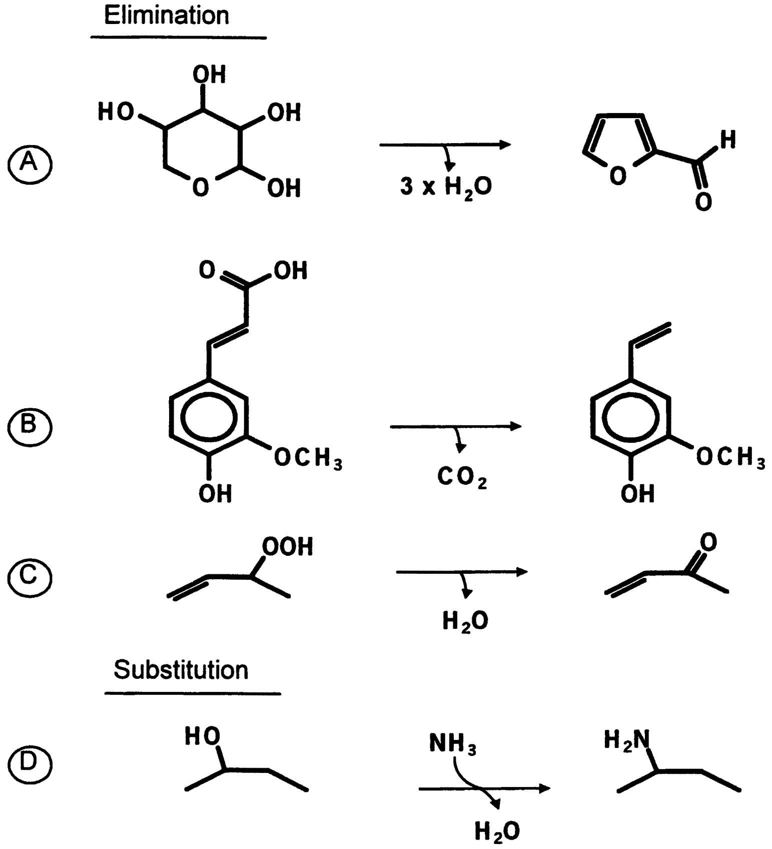
Elimination
A
HO
OH
OH
O
OH
3 x H2O
O
H
O
B
O
OH
OCH3
OH
CO2
OCH3
OH
C
OOH
H2O
O
Substitution
D
HO
NH3
H2O
H2N

Figure 2. Simplified reactions leading to a loss of oxygen from biomolecules during food processing.

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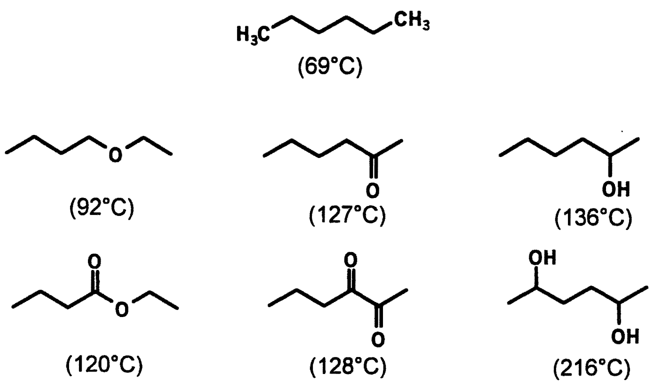
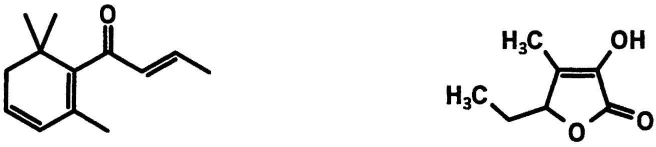
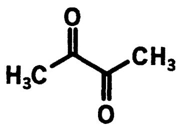

H3C
CH3
(69°C)
O
(92°C)
O
(127°C)
OH
(136°C)
O
O
(120°C)
O
O
(128°C)
OH
OH
(216°C)

Figure 3. Influence of the introduction of an oxygen function into n-hexane on the boiling points of the volatiles formed.


O
H3C
OH
H3C
O
O

β-Damascenone (0.003)

3-Hydroxy-5-ethyl-4methyl-2(5H)-furanone (0.003)



2,3-Butandione (15)

Figure 4. Examples of potent oxygen-containing food odorants (odor threshold in ng/L air).

Although 2,3-butandione is the most volatile among the three compounds, the odor threshold is 10^4 higher than that of (E)-ß-damascenone, a carbon-13 compound. Even 3-HEMF, which contains three oxygen atoms, exhibits a much lower odor threshold in air. This example clearly shows that the successful identification of key aroma compounds in foods cannot be based on the volatility of compounds alone, but will require a combination of instrumental techniques that include use of the human nose as a biosensor.

Screening techniques, such as the CHARM analysis *(2)*, Aroma Extract Dilution Analysis (AEDA) *(3)*, or the finger span cross modality matching technique *(4)*, followed by quantitative experiments, and, finally, a recombination of the food aroma using reference compounds, have been proven useful tools to find key flavor compounds in foods *(5)*.

Since the number of oxygen-containing volatiles in foods is very high, the present paper focuses on cyclic oxygen-containing aroma compounds generated from carbohydrate degradation. After a brief presentation of the molecules generated in such reactions and the contribution to different food flavors, special emphasis will be given to formation pathways. Many reaction schemes were previously proposed in the literature; in this paper only results of quantitative studies are included in which model studies with reaction intermediates were performed.

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Summary of Results

Flavor Contribution of Carbohydrate Derived Oxygen-Containing Compounds

In an investigation on barley malt, Brand in 1894 *(6)* detected a caramel-like smelling compound that was later on identified as Maltol (Figure 5, no. 1) by Peratoner and Tamburello *(7)*. The flavor contribution of Maltol to rye bread crust *(8)* and Karamalt *(9)* was recently established by application of Aroma Extract Dilution Analysis (AEDA).

A "reduced" derivative of Maltol, the so-called dihydromaltol (no. 2 in Figure 5), which also has a caramel-like odor, was reported for the first time by Mills and Hodge *(10)* in a processed fructose-proline mixture. The flavor contribution of this compound to foods was recently reported for Karamalt *(9)*, as well as for dried paprika powder *(11)*. Severin and Seilmeier *(12)* have reported Norfuraneol (no. 3 in Figure 5) as a pentose degradation product. Although Norfuraneol has been detected in many volatile fractions of foods, the flavor contribution has not yet been established. A flavor contribution was, however, recently confirmed in a processed ribose-cysteine mixture *(13)*.

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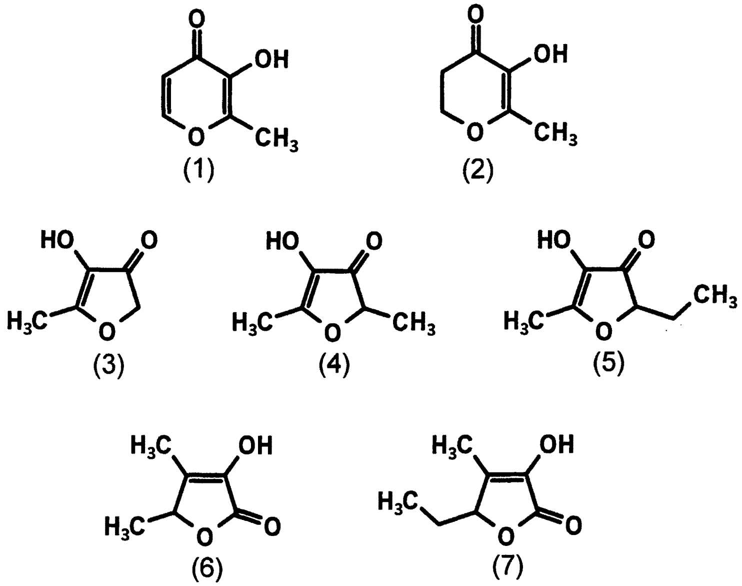

Figure 5. Structures of cyclic carbohydrate derived oxygen-containing odorants (1-5): caramel-like aroma, (6-7): seasoning-like aroma.

4-hydroxy-2,5-dimethyl-3(2H)-furanone (4-HDF, Furaneol), which was identified for the first time by Hodge et al. *(14)* in processed mixtures containing hexoses, is a well known aroma compound of wheat bread crust *(15)*, popcorn *(16)*, roasted coffee *(17)*, roasted beef *(18)* and French fries *(19)*. However, the homologous compound, 4-hydroxy-2-ethyl-3-methyl-3(2H)-furanone(4-HEMF, Homofuraneol, no. 5 in Figure 5), which was identified for the first time by Sulser et al. *(20)*, only contributes significantly to the flavor of roasted coffee *(17)*. A similar situation with respect to the flavor contribution of homologous compounds can be observed for the two seasoning-like smelling compounds, 3-hydroxy-4,5-dimethyl-2(5H)furanone (3-HMF; Sotolon; no. 6 in Figure 5) and 3-HEMF (Abhexon; no. 7 in Figure 5). The latter has been known since 1967 *(20)*, however, the flavor contribution to foods has been established for only a few foods, e.g. roasted coffee *(17)*. On the other hand, 3-HMF, which was identified later *(21)* has since then been confirmed as a key aroma compound in many foods, e.g., roasted coffee *(17)*, roasted chicken *(22)*, stewed beef *(23)*, French fries *(19)*, Karamalt *(9)* and dried paprika powder *(11)*.

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By application of Aroma Extract Dilution Analysis, odorants are ranked according to the relative odor contribution based on Flavor Dilution (FD) factors *(5)*. If one assigns the highest FD-factor reported for a food extract as 100, the second highest as 50, the third highest as 25, and so on, it is possible to compare results of AEDAs reported for different foods. However, it has to be stated that this procedure is a rough estimate of sensory importance because AEDA experiments are typically based on different amounts of foods. In Table I, AEDA results reported in the literature for six different processed foods are summarized. The data clearly suggest an important role of 4-HDF and, to a lesser extent of 3-HMF, in all of the foods considered. In comparision, the homologous compounds, 4-HEMF (Homofuraneol) and 3-HEMF (Abhexon), were only important in roasted coffee. Norfuraneol and dihydromaltol were not detected by the GCO technique and, therefore, are regarded to have no importance in these food flavors.

The Odor Activity Value concept offers a simple explanation for the different flavor contributions of the seven cyclic aroma compounds to the foods considered. The odor activity value (also called odor unit, aroma value etc., Equation 1) of a compound is defined as the ratio between concentration and odor threshold in a given matrix *(5)*:

$$\text{Odor Activity Value} = \frac{\text{Concentration}}{\text{Odor threshold (Matrix)}} \qquad (1)$$

To contribute to a food flavor, an aroma compound has either to be present at high concentrations, must have a low odor threshold, or both. A comparison of odor thresholds for the six oxygen heterocyclics in water (Figure 6) clearly reveals significant differences in odor thresholds. While Maltol and Norfuraneol show quite high odor thresholds, 3-HMF (Sotolon) and, in particular, 3-HEMF (Abhexon) exhibit very low odor thresholds.

Quantitative data reported in the literature reveal a predominance of 4-hydroxy-2,5-dimethyl-3(2H)-furanone (Table II) compared to the other six odorants. Although Sotolon is always present at much lower concentrations, the importance to flavor is due to the lower odor threshold of this compound. The results presented in Table II suggest that in many foods, 4-HDF is always generated to a significant extent, whereas the other heterocyclics need specific precursors and reaction conditions. For example, the formation of high amounts of Maltol seems possible only in cereal products.

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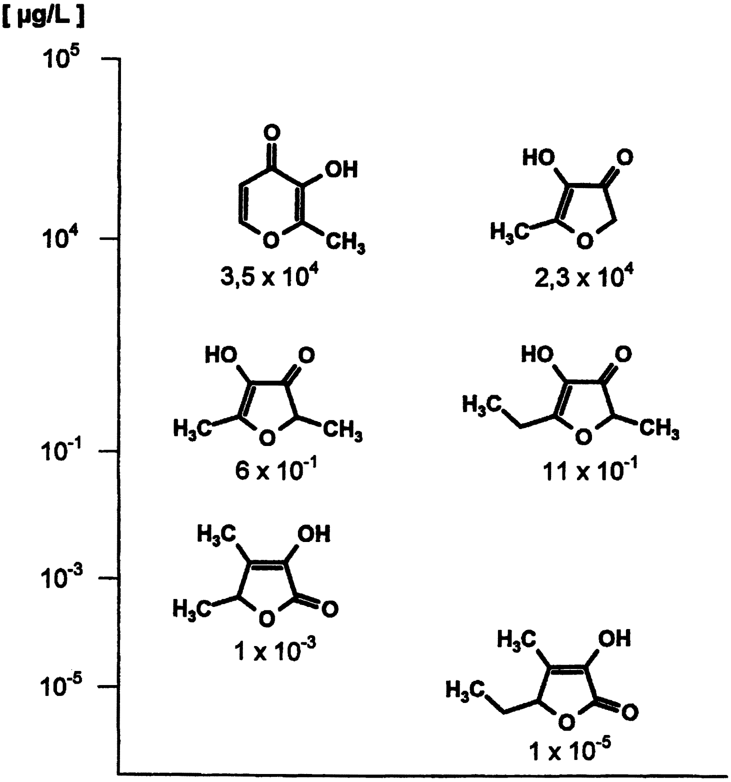

Figure 6. Oxygen-containing sugar degradation products ranked according to odor thresholds in water.

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Table I. Relative Flavor Dilution (Rfd) Factors Determined for Cyclic Oxygen-Containing Carbohydrate Degradation Products in Several Processed Foods[a]

	rFD					
Odorants	*Stewed Beef*	*Wheat Bread Crust*	*French Fries*	*Roasted Chicken*	*Coffee Powder*	*Kara-malt*
4-Hydroxy-2,5-dimethyl-3(2H)-furanone (4-HDF; Furaneol)	**100**	**100**	**100**	**100**	12.5	50
3-Hydroxy-4,5-dimethyl-2(5H)-furanone (3-HDF; Sotolon)	3.2	>0.2	**12.5**	6.3	**25**	**12.5**
4-Hydroxy-2(5)-ethyl-2(5)-methyl-3(2H)-furanone (4-HEMF; Homofuraneol)	>0.2	>0.2	>0.1	>0.2	**6.3**	>0.2
3-Hydroxy-5-ethyl-4-methyl-2(5H)-furanone (3-HEMF; Abhexon)	>0.2	>0.2	>0.1	>0.2	**12.5**	0.8
3-Hydroxy-2-methyl-pyrane-4-one (Maltol)	>0.2	**6.3**	>0.1	>0.2	>0.2	**1.6**

[a] 4-Hydroxy-5-methyl-3(2H)-furanone (Norfuraneol) and 2,3-dihydro-5-hydroxy-6-methylpyran-4-one (Dihydromaltol) did not exceed a relative FD > 0.4 in these foods.

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Table II. Concentrations of Cyclic Oxygen-Containing Carbohydrate Degradation Products in Various Foods[a]

Compound	*Conc. (μg/kg)*				
	Wheat Bread Crust	*Karamalt*	*French Fries*	*Roasted Beef*	*Coffee Powder*
4-Hydroxy-2,5-dimethyl-3(2H)-furanone	6040	8800	2778	1108	112000
4-Hydroxy-2-ethyl-5-methyl-3(2H)-furanone	n.a.	n.a.	n.a.	n.a.	16800
3-Hydroxy-4,5-dimethyl-2(5H)-furanone	n.a.	17	5	9	1360
3-Hydroxy-5-ethyl-4-methyl-2(5H)-furanone	n.a.	n.a.	n.a.	n.a.	104
3-Hydroxy-2-methylpyran-4-one	n.a.	61000	n.a.	n.a.	n.a.

[a] Quantitation was performed by means of stable isotope dilution assays. n.a.: Not analyzed because of very low FD-factors found during AEDA.

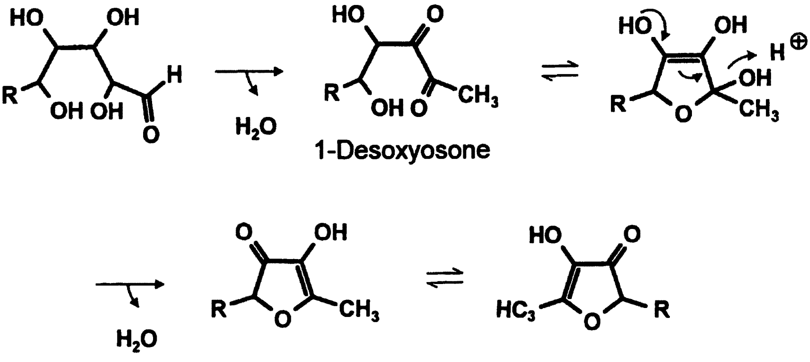

Figure 7. Formation of 4-hydroxy-5-methyl-(Norfuraneol; R = H) or 4-hydroxy-2,5-dimethyl-3(2H)-furanone (4-HDF; R = -CH_3) from ribose or rhamnose, respectively.

Formation of Oxygen-Containing Heterocyclic Aroma Compounds

3(2H)-Furanones

In the literature, pentoses are reported as important precursors of Norfuraneol *(24)*, whereas rhamnose is known to generate significant amounts of 4-HDF *(25)*. However, in most foods, hexoses, in particular, glucose and fructose, are the most abundant monomeric carbohydrates. To better understand the efficacy of ribose, glucose and rhamnose in 4-HDF formation, the three sugars were reacted in the presence of the amino acid cysteine to induce the formation of the 3-furanones. Results of quantitative studies *(26)* confirmed data in the literature on the specific formation of Norfuraneol from ribose and 4-HDF from rhamnose (Table III). 4-HEMF was formed in small amounts from all carbohydrates with a slight preference from rhamnose.

The formation of Norfuraneol from ribose or 4-HDF from rhamnose can be readily explained by the reaction scheme shown in Figure 7. Formation of the 1-desoxyosone from the respective aldose, enolization process and cyclization, followed by elimination of the hydrogen group at C-1 yields the target compounds. This reaction explains the much higher amounts of end product formed from the respective carbohydrates (cf. Table III). However, the results in Table III generate the following two important questions:

- How can 4-HDF be formed from glucose, because according to Figure 7 the R-group would then be a hydroxymethyl group?

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- How can either Norfuraneol (5 carbons) or 4-HEMF (7 carbons) be formed from sugars having different numbers of carbon atoms?

Table III. Amounts of 4-Hydroxy-5-methyl- (Norfuraneol), 4-Hydroxy-2,5-dimethyl- (4-HDF; Furaneol) and 4-Hydroxy-2(5)-ethyl-5(2)-methyl-3(2H)-furanone (4-HEMF; Homofuraneol) from Different Carbohydrates[a]

Carbohydrate	*Amount (μg)*		
	Norfuraneol	*4-HDF*	*4-HEMF*
Ribose	54530	13.5	0.6
Glucose	13.5	79.4	0.1
Rhamnose	19.1	19800	2.1

[a] The carbohydrate (10 mmol) and cysteine (3.3 mmol) were dissolved in phosphate buffer (100 mL; 0.5 mol/L; pH 5.0) and reacted for 20 min at 145°C in an autoclave.

An explanation for the formation of 4-HDF from glucose is the generation of 4-HDF via acetylformoin by a reductive step as previously proven *(27)*. Hofmann and Schieberle *(28)* recently synthesized acetylformoin and reacted it either alone or in the presence of reducing agents such as methylene reductinic acid and ascorbic acid. The results (Table IV) confirm that AF is an effective intermediate in 4-HDF formation and support the view *(27)* that reducing compounds, such as ascorbic acid, are needed to generate 4-HDF from hexoses. The significant amounts formed from AF alone can be explained by the reductone moiety acting either as an electron acceptor or donor.

The generation of 4-HDF from a pentose can only be explained by cleavage of the carbohydrate skeleton followed by subsequent *Aldol* addition of the cleaved compounds. Compounds that may be involved in this reaction are 2-oxopropanal and 2-oxopropanol. By *Aldol* addition, the symmetric 3,4-dihydroxy-2,5-dioxo pentane may be formed, which after enolization and cyclization eliminates water to yield 4-HDF (Figure 8).

Table IV. Amounts of 4-Hydroxy-2,5-Dimethyl-3(2H)-Furanone (4-HDF) Formed from Acetylformoin (AF) under Various Conditions

Acetylformoin *(1 mmol) Reacted with[a]:*	*4-HDF (μg)*
No additions	748
Methylene reductinic acid	1485
Ascorbic acid	1959

[a] Precursors (1 mmol each) were mixed with silica gel (1 g), then heated for 10 min at 160°C in sealed glass tubes (10 mL total volume).

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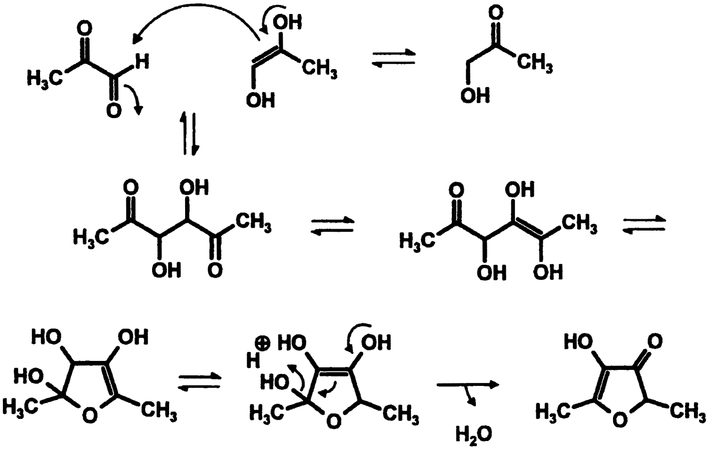

Figure 8. Formation of 4-hydroxy-2,5-dimethyl-3(2H)-furanone by an Aldol reaction of 2-oxopropanal and 2-oxopropanol.

Table V. Influence of pH on the Amounts of 4-Hydroxy-2,5-dimethyl-3(2H)-furanone (4-HDF) formed from 2-Oxopropanal and 2-Oxopropanol[a]

pH	*4-HDF*	
	µg	*mol-%*
3.0	59.6	0.05
5.0	364.5	0.28
7.0	1450.5	1.10

[a] 2-Oxopropanal (1 mmol) and 2-oxopropanol (1 mmol) were reacted for 20 min at 145°C in phosphate buffer (50 mL; 0.5 mol/L).

Table VI. Influence of pH on the Formation of 4-Hydroxy-2,5-dimethyl-3(2H)-furanone (4-HDF) from Ribose, Glucose and Rhamnose[a]

Carbohydrate	*Amount (µg) Formed at pH*		
	pH 3	*pH 5*	*pH 7*
Ribose	0.8	19	208
Glucose	2.3	80	788
Rhamnose	1140	19800	79860

[a] The carbohydrate (10 mmol) and cysteine (3.3 mmol) were reacted at 145°C for 20 min in phosphate buffer (100 mL; 0.5 mol/L; pH 5.0).

Such Aldol-type reactions should occur favorably at higher pH values. To study 4-HDF formation from 2-oxopropanal and 2-oxopropanol, both were reacted with increasing the pH, and the 4-HDF formed was quantified by a stable isotope dilution analysis *(26)*. The results (Table V) clearly indicate that the two carbon-3 compounds are key intermediates in 4-HDF formation in aqueous conditions. Similar results were obtained in studies on the formation of Norfuraneol by replacing 2-oxopropanol with hydroxy-acetaldehyde and of 4-HEMF by replacing 2-oxopropanal by 2-oxobutanal *(26)*.

Prior to the *Aldol* addition, both compounds may be formed by a retro-*Aldol* cleavage of either the 1- or the 3-desoxyosone *(26)*. Retro-Aldol reactions are also favored under alkaline conditions. So, increasing the pH in the model described in Table III should increase the yields of 4-HDF. This proposal is clearly corroborated by the results displayed in Table VI.

2(5H)-Furanones

Due to the branched skeleton (at carbon 4), neither 3-HDF (Sotolon) nor 3-HEMF (Abhexon) can be obtained simply by dehydration of carbohydrates. Theoretically, various mechanisms have been proposed to explain the formation of Sotolon from carbohydrate cleavage products. As an example, the formation by an Aldol reaction of 2,3-butandione and hydroxyacetaldehyde is proposed in Figure 9. Enolization of the 2,3-dihydroxy-4-oxo-3-methylpentanal formed directly yields 3-HDF after elimination of water. In model reactions using four different precursor systems *(26)* the reaction between diacetyl and hydroxyacetaldehyde was found to be most effective in generating 3-HDF. Similar results were obtained for the formation of the homologous compound 4-HEMF from 2,3-pentanedione and hydroxyacetaldehyde *(26)*.

Maltol

Maltol is often cited as formed by dehydration of glucose or fructose via 1-desoxyosone as the key intermediate (Figure 10). However, if this is true, it is unclear why Maltol is normally not present in foods containing high amounts of these free sugars, e.g., processed fruit juices. Literature data suggest *(7, 8)* that Maltol is preferentially formed during thermal treatment of fermented cereals, such as malt or rye bread crust. Because these normally contain higher concentrations of maltose, it seems reasonable that this disaccharide is a potential precursor of Maltol. To compare the efficacy of maltose, glucose, and fructose in Maltol formation, the three carbohydrates were reacted singly in the presence of the amino acid proline *(29)*. The Maltol formed was quantified by a newly developed stable isotope dilution assay *(29)*. In an aqueous reaction system, maltose proved to be ca. 16 times more effective in Maltol generation

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than glucose or fructose, respectively (Table VII). Under nearly anhydrous conditions (Model B; Table VII), ca. 93 times more Maltol was formed from maltose than from glucose, even with no amino acid added. These results confirm earlier research that showed the important role of the disaccharide maltose in Maltol formation *(30)*.

3,5-Dihydroxy-2-methyl-5,6-dihydropyran-4-one (Figure 10) is often reported as key reaction product of glucose degradation *(30)*. Obviously the 4-glucosyl-1-desoxyosone (**1**; Figure 10) favors the elimination of water at C-5, whereas the 1-desoxyglucosone preferentially eliminates water at C-1 to yield **2**.

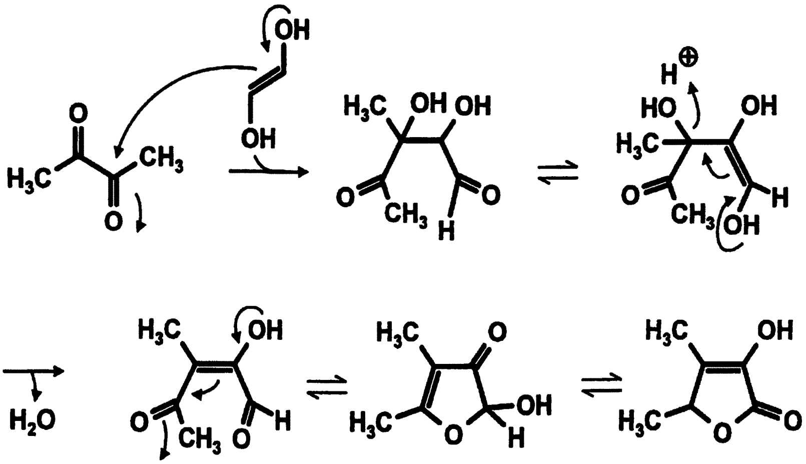

Figure 9. Formation of 3-hydroxy-4,5-dimethyl-2(5H)-furanone from 2,3-butandione and hydroxyacetaldehyde.

Table VII. Formation of 3-Hydroxy-2-methyl-pyran-4-one (Maltol) from Different Carbohydrates

Carbohydrate	*Maltol (µg) Formed in*	
	Model A[a]	*Model B*[b]
Glucose	7	15
Fructose	8	<0.1
Maltose	120	1400

[a] The carbohydrate (10 mmol) was reacted in the presence of proline (3.3 mmol) for 20 min at 145°C in phosphate buffer (50 mL; 0.1 mol/L; pH 7.0).

[b] The carbohydrate (10 mmol) was mixed with silica (6 g) and reacted for 10 min at 160°C without amino acid addition.

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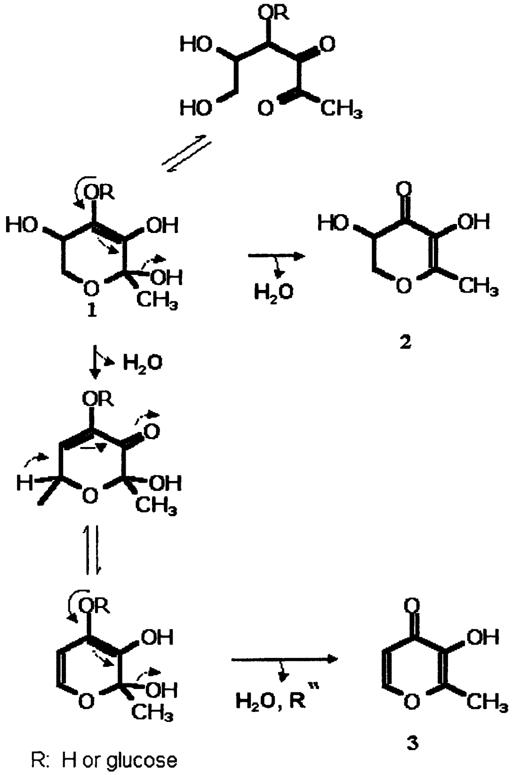
OR
HO
O
HO
O
CH₃
OR
HO
OH
OH
O
CH₃
1
H₂O
HO
O
OH
O
CH₃
2
H₂O
OR
O
H
O
OH
CH₃
OR
OH
OH
O
CH₃
H₂O, R"
O
OH
O
CH₃
3
R: H or glucose

Figure 10. Formation of Maltol (3) and 3,5-dihydroxy-2-methyl-5-6-dihydropyran-4-one (2) from the 1-desoxyosone of glucose (R = H) or maltose (R = Glucose), respectively.

Conclusions

The oxygen rich carbohydrates are well-known precursors of caramel- and seasoning-like smelling aroma compounds in foods. Among the reaction products thermally generated by elimination of water and/or Aldol-type reactions 3(2H)- and 2(5H)-furanones still bearing three of the carbohydrate oxygens of the sugars are among the most potent.

In particular, 4-hydroxy-2,5-dimethyl-3(2H)-furanone (4-HDF) with a caramel-like aroma note and 3-hydroxy-4,5-dimethyl-5(2H)-furanone (3-HDF) eliciting a seasoning-like odor contribute to the flavors of many foods such as roasted coffee, roasted meat, chocolate or karamalt. Although both are alternatively formed from specific precursors biochemically synthesized in plants, in processed foods; monosaccharides also contribute significantly to the amounts formed. The results suggest that during heat treatment different pathways occur leading to the same flavor compound: 4-HDF can be formed either from the intact hexoses by water elimination/reduction, but also its generation from carbohydrate cleavage products is important. Cleavage of carbohydrates and subsequent *Aldol*-type additions were confirmed as important ways to generate the homologous 4-HEMF as well as 3-HDF (Sotolon) and 3-HEMF. When the yields of the two 3-furanones (4-HDF; 4-HEMF) and the two 2-furanones (3-HDF; 3-HEMF) formed in a Maillard type reaction from ribose, glucose and rhamnose are compared, 4-HDF was the predominant oxygen-containing aroma compound formed (Table VIII). 3-HDF was next in rank, followed by 4-HEMF. This result is well in line with quantitative data reported for processed foods (Table II) corroborating the general role of hexoses in the formation of the cyclic furanones.

Table VIII. Comparison of the amounts of carbohydrate degradation products formed from several carbohydrates[a]

Compound	*Amount (µg) formed from*		
	Ribose	*Glucose*	*Rhamnose*
4-Hydroxy-2,5-dimethyl-3(2H)-furanone	18.5	79.4	19800
4-Hydroxy-2(5)-ethyl-5(2)-methyl-3(2H)-furanone	0.6	0.1	2.1
3-Hydroxy-4,5-dimethyl-2(5H)-furanone	1.6	1.4	4.5
3-Hydroxy-5-ethyl-4-methyl-2(5H)-furanone	<0.1	<0.1	<0.1

[a] The carbohydrate (10 mmol) and cysteine (3.3 mmol) were reacted at 145°C for 20 min in phosphate buffer (100 mL; 0.5 mol/L; pH 5.0).

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Chapter 14

Biological Pathways for the Formation of Oxygen-Containing Aroma Compounds

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While sulfur- and nitrogen-containing aroma compounds are often considered to be more potent than oxygen-containing aroma compounds, oxygen-containing aroma compounds are widely distributed in foods and often make a very significant contribution to aroma. This is true across food categories including raw plant materials, fermented, and thermally processed foods. Wide arrays of oxygenated aroma compounds (e.g. acids, alcohols, aldehydes, furans, pyrans, lactones, etc.) have sufficiently low sensory thresholds and defined characters to be important aroma contributors. This paper will discuss some of the biological pathways leading to both the most abundant and some of the more potent oxygenated aroma compounds.

Oxygen-containing aroma compounds are ubiquitous in food systems. The prevalence of these compounds in food aromas can be associated with two factors; oxygen is the most abundant element on the surface of earth, and oxygen is a reactive atom that readily participates in chemical reactions to form innumerable compounds (*1*). While oxygen-containing aroma compounds are typically not considered as potent as sulfur- or nitrogen-containing compounds,

In Heteroatomic Aroma Compounds; Reineccius, G., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2002.

these compounds have been found to make a major contribution to numerous foods (e.g. fruits, beer, cheeses, etc.).

Much of the early flavor research focused on oxygen-containing aroma compounds. This is primarily because of the abundance of oxygenated compounds in the volatile isolates. If one considers the Maillard reaction, the Strecker aldehydes typically quantitatively dominate the aroma isolate. While in this case, they may not be the most sensorially important volatiles in the profile; they generally are the most abundant. The same might be said for oxygen-containing volatiles in the profile of fermented foods (e.g. acids) and many plant aroma profiles (e.g. esters). These compounds again are significant in making a contribution, albeit negative, to storage off- flavors due to lipid oxidation. There is little question that oxygen-containing volatiles are widely found in foods and often play a key role in defining aroma.

Since other sections of this text consider the formation of oxygen-containing aroma compounds by non-biological pathways, this manuscript will focus on biological pathways. We will illustrate the typical pathways for the formation of some of the more abundant oxygenated volatiles (e.g. carbonyls and esters) in plants. We will also present how five of the more potent oxygen-containing aroma compounds (3-hydroxy-4,5-dimethyl-2(5H)-furanone, β-ionone, β-damascenone, 1-octen-3-one, and (E,Z)-2,6-nonadienal; thresholds $\leq$ 0.01μg/L) are formed in nature. We will not go back to the origin of the precursors of these example compounds (e.g. fatty acids, carotenoids or amino acids) since pathways for their biosynthesis can be found in most college texts.

Abundant Oxygenated Aroma Compounds

Fatty Acid Precursors

Several different pathways for the formation of volatiles may involve fatty acids as precursors. One pathway involves the enzyme-catalyzed oxidation of unsaturated fatty acids (*2*). This mechanism yields large quantities of carbonyls, some of which become reduced to alcohols and potentially esterified to yield esters. A second pathway is normal fatty acid metabolism. Fatty acid metabolism provides a pool of volatiles serving both as native aroma compounds or as precursors in further reactions. While later in this chapter we present specific pathways for fatty acid oxidation leading to the formation of 1-octen-3-ol and 2,6-nonadienol, we will introduce these two pathways in a more general sense here.

In terms of lipid oxidation mechanisms, lipoxygenase is the first enzyme involved in this process forming a peroxide of an unsaturated fatty acid by adding molecular oxygen. Isomerases may then rearrange this peroxide which

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ultimately affords more choices in degradation products. The peroxide is subsequently broken down by additional enzymes to form a wide variety of carbonyls. Many of the alcohols and acids are enzymatically converted to esters, which provide the basis of many fruit flavors. The cleavage points and some resultant degradation products of linoleic and linolenic acid oxidation are shown in Figure 1.

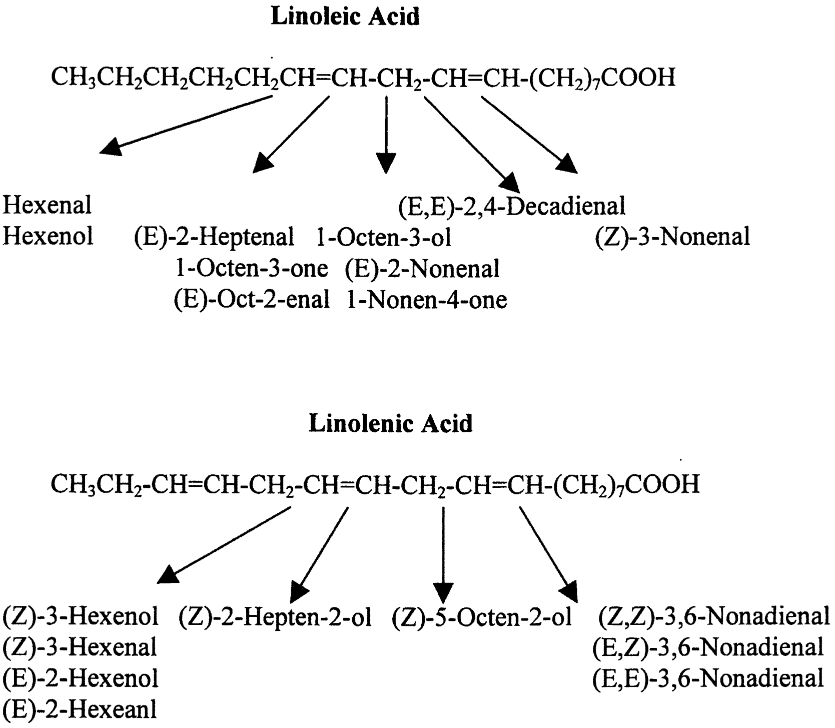
Linoleic Acid
$CH_3CH_2CH_2CH_2CH_2CH=CH-CH_2-CH=CH-(CH_2)_7COOH$
Hexenal
Hexenol
(E)-2-Heptenal
1-Octen-3-one
(E)-Oct-2-enal
1-Octen-3-ol
(E)-2-Nonenal
1-Nonen-4-one
(E,E)-2,4-Decadienal
(Z)-3-Nonenal
Linolenic Acid
$CH_3CH_2-CH=CH-CH_2-CH=CH-CH_2-CH=CH-(CH_2)_7COOH$
(Z)-3-Hexenol
(Z)-3-Hexenal
(E)-2-Hexenol
(E)-2-Hexeanl
(Z)-2-Hepten-2-ol
(Z)-5-Octen-2-ol
(Z,Z)-3,6-Nonadienal
(E,Z)-3,6-Nonadienal
(E,E)-3,6-Nonadienal

Figure 1. Aroma compounds from the oxidative degradation of linoleic and linolenic acids (2).

Considering that each plant has its unique distribution of unsaturated fatty acids, enzymes and environment (e.g. pH), it is easy to understand that each plant will have similarities but yet differences in aroma.

Normal fatty acid metabolism also provides a host of oxygenated aroma

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compounds. This pathway provides a wide range of fatty acids, alcohols, aldehydes, methyl ketones and lactones and is illustrated in Figure 2 (*3*). The products of this pathway are generally even numbered in carbon atoms (except for the methyl ketones).

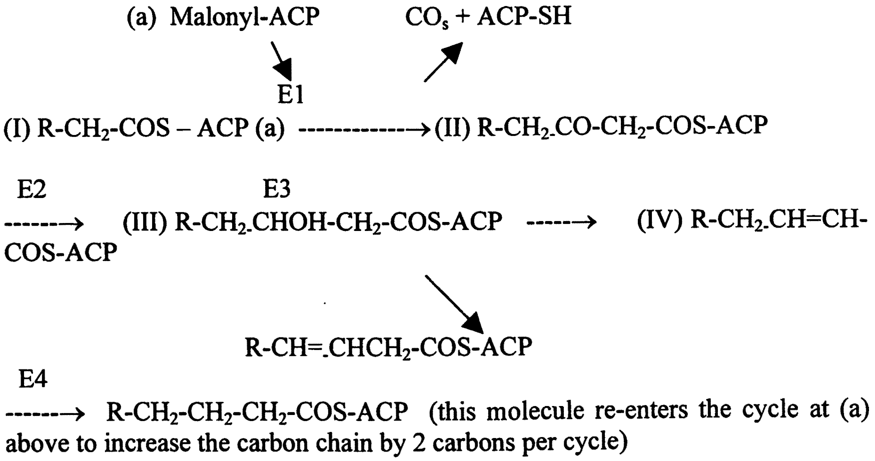
(a) Malonyl-ACP
CO_s + ACP-SH
E1
(I) $R\text{-}CH_2\text{-}COS - ACP$ (a)
(II) $R\text{-}CH_2.CO\text{-}CH_2\text{-}COS\text{-}ACP$
E2
E3
(III) $R\text{-}CH_2.CHOH\text{-}CH_2\text{-}COS\text{-}ACP$
(IV) $R\text{-}CH_2.CH{=}CH\text{-}COS\text{-}ACP$
$R\text{-}CH{=}.CHCH_2\text{-}COS\text{-}ACP$
E4
$R\text{-}CH_2\text{-}CH_2\text{-}CH_2\text{-}COS\text{-}ACP$ (this molecule re-enters the cycle at (a) above to increase the carbon chain by 2 carbons per cycle)

Figure 2. Fatty acid biosynthesis in plants (3). Compound I gives saturated fatty acids; II and III give methyl ketones and secondary alcohols; and IV gives unsaturated fatty acids, unsaturated aldehydes unsaturated alcohols and γ-lactones. E1 acyl ACP, malonyl-ACP ligase; E2 β-oxoacetyl-ACP:NADH oxidoreductase; E3 D-β-hydoxyacyl-ACP:NAD(P) oxidoreductase; and E4 α,β-dehydroacyl-ACP:NADH(NADPH) oxidoreductase.

Amino Acid Precursors

It is obvious that the above mentioned pathways yield straight chain compounds. Straight, branched chain and aromatic volatiles are produced from amino acids. The pathways proposed for the formation of branched chain oxygen-containing volatiles in banana are represented by the mechanism shown in Figure 3. This involves the decarboxylation and deamination of the base amino acid to yield an aldehyde. The aldehyde can be oxidized to an acid or reduced to an alcohol. These acids and alcohols are potentially esterified to form esters.

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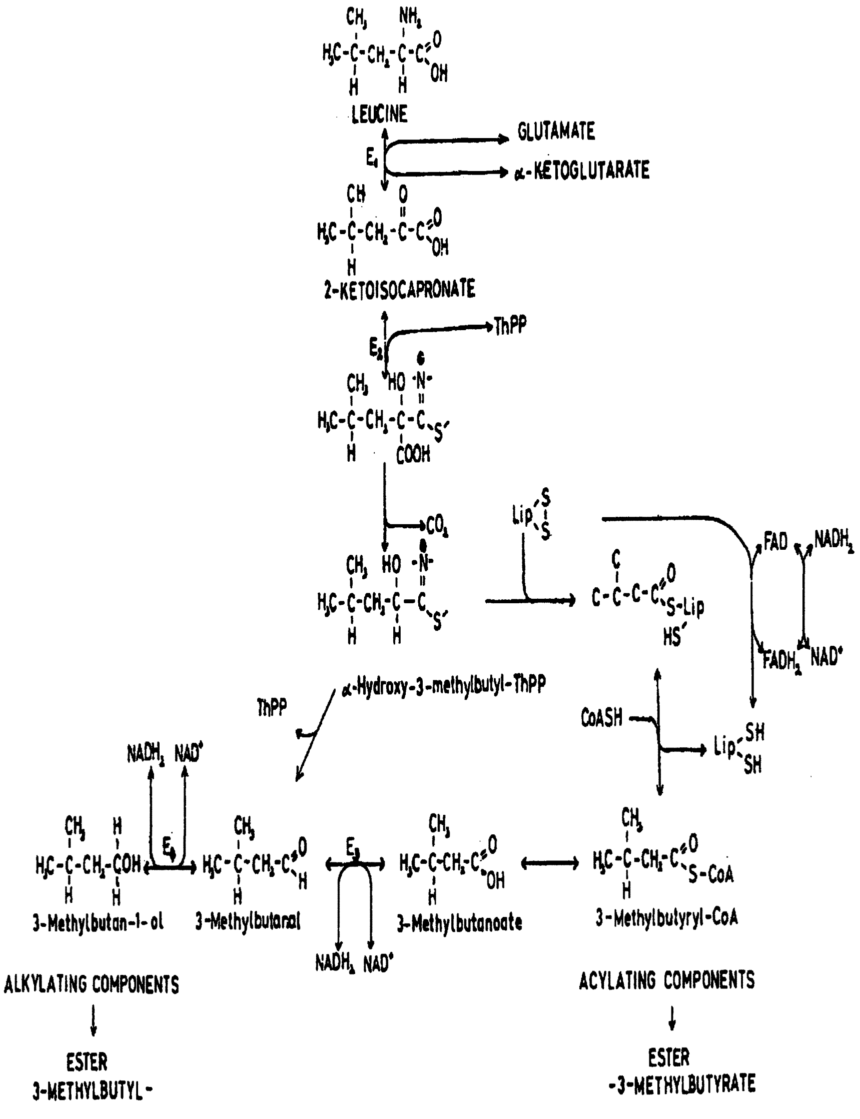
LEUCINE
E1
GLUTAMATE
α-KETOGLUTARATE
2-KETOISOCAPRONATE
E2
ThPP
CO2
α-Hydroxy-3-methylbutyl-ThPP
ThPP
Lip S S
C-C-C-C S-Lip HS
FAD
NADH2
FADH2
NAD+
CoASH
Lip SH SH
NADH2 NAD+
E4
E3
NADH2 NAD+
3-Methylbutan-1-ol
3-Methylbutanal
3-Methylbutanoate
3-Methylbutyryl-CoA
ALKYLATING COMPONENTS
ACYLATING COMPONENTS
ESTER
3-METHYLBUTYL-
ESTER
-3-METHYLBUTYRATE

Figure 3. Conversion of amino acids into the aroma compunds of banana as ilustrated by leucine: E1 L-leucine aminotransferase; E2 pyruvate decarboxylase; E3 aldehyde dehydrogenase; E4 alcolho dehydrogenase; ThPP thiamin pyrophosphate; LipSS oxidized lipid; reduced lipoic acid; FAD flavin-ademine dinucleotide; NAD$^+$ oxidized nicotinamide-adenine dinucleotide; CoA-SH, coenyme A (3).

Lignin Precursors

The aroma compounds related to lignin do not arise from lignin degradation but are related to intermediates in the biosynthesis. Compounds such as vanillin (vanilla), eugenol (clove), guaiacol and vinyl guaiacol are examples of such compounds.

Biosynthesis of Potent Aroma Compounds

The remainder of this chapter will present biosynthetic pathways illustrating the mechanisms of formation for some of the more potent oxygenated aroma compounds.

3-Hydroxy-4,5-dimethyl-2(5H)-furanone

Commonly known as sotolon or caramel furanone, this compound is a flavor impact compound in aged sage (*4*), raw sugar cane (*5*), fenugreek (*6,7*), lovage (*8*), flor sherry (*9*), botrytized wines (*10,11*) and sweet fortified wines (*12*). The odor has been described as caramel, curry or nutty and it is extremely potent with an odor threshold in water of 0.001µg/L (*13*). Different pathways for the formation of sotolon have been proposed in the literature. Figure 4 shows a proposed biogeneration pathway for the formation of sotolon in French flor-sherry, Vin Jaune (*14*). The precursor, threonine is released during autolysis of yeast during maturation and is converted enzymatically into α-ketobutyric acid via desaminase (from Saccharomyces). The subsequent steps are not enzyme catalyzed but chemical reactions. Initially, an aldol condensation occurs between α-ketobutyric acid and acetaldehyde which then cyclizes and loses water. During wine maturation, changes in temperature, alcohol content and pH have been shown to have a dramatic impact on the amount of sotolon produced (see Figures 5 and 6). As would be anticipated, temperature had a positive effect while alcohol content and pH had a negative effect on sotolon production. These results suggest that seasonal changes in the cellar temperature or normal variations in fermentation (pH or alcohol content) could result in different wine flavor profiles.

An alternative pathway for the formation of sotolon is illustrated in Figure 7 (*15*). The precursor, 4-hydroxyisoleucine (HIL) is an unusual amino acid that occurs in relatively high concentrations in fenugreek. HIL can cyclize (intranucleophilic attack) and it is proposed that the amine reacts with a α-diketone (internucleophilic attack) in which the adduct forms a Schiff's base. Strecker degradation results in a deamination step forming the heterocyclic, α-diketone, which tautomerizes with the enol form (sotolon). Although this reaction is thermally catalyzed, formation could also occur at ambient temperature.

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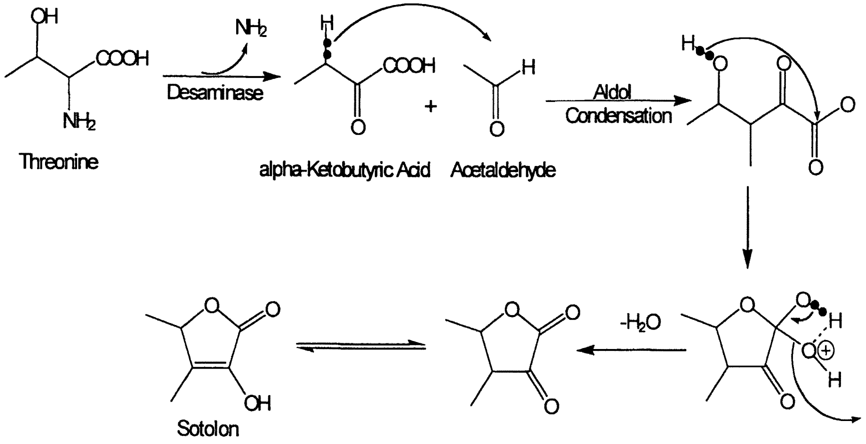
OH
COOH
NH2
Threonine
NH2
Desaminase
H
COOH
O
+
H
O
alpha-Ketobutyric Acid
Acetaldehyde
Aldol
Condensation
H
O
O
O
O
O
O
O
O
O
H
O
H
-H2O
O
O
O
O
OH
Sotolon

Figure 4. Proposed biogeneration of sotolon in French wine (Reproduced with permission from reference 14. Copyright 1995 American Chemical Society).

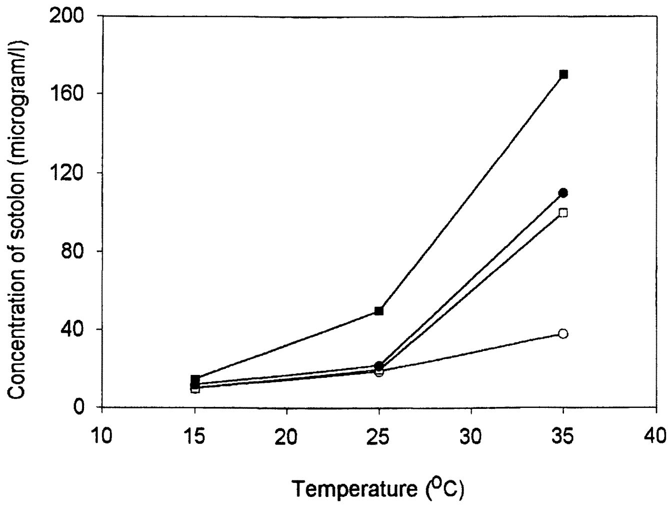

Figure 5. Formation of sotolon as a function of temperature and alcohol content. Key: at 6 days, (●) 12°GL, (○) 15°GL; and at 10 days, (■) 12°GL, (□) 15°GL. (Reproduced with permission from reference 14. Copyright 1995 American Chemical Society.)

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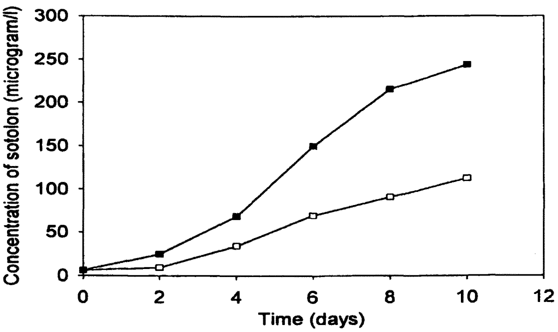

Figure 6. Kinetics of formation of sotolon, α-Ketobutyric acid (100mg/L) and acetaldehyde (260mg/L) heated at 35°C. Key: (■) pH 3; (□) pH 3.5. (Reproduced with permission from reference 14. Copyright 1995 American Chemical Society.)

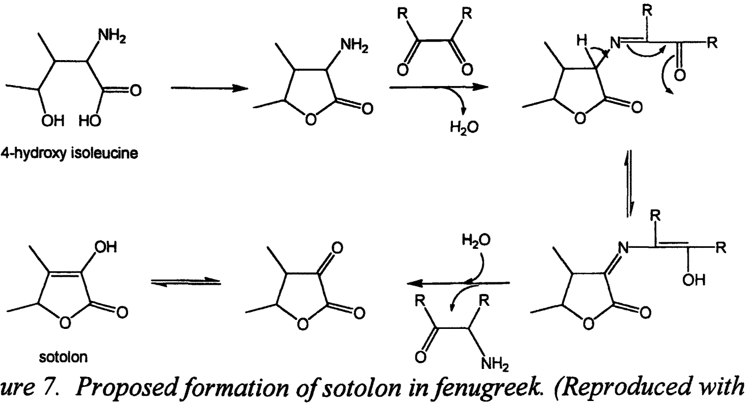
NH_2
O
OH
HO
4-hydroxy isoleucine
NH_2
O
O
R
R
O
O
H_2O
R
H
N
R
O
O
O
R
N
R
OH
O
O
H_2O
R
R
O
NH_2
O
O
O
OH
O
O
sotolon

Figure 7. Proposed formation of sotolon in fenugreek. (Reproduced with permission from reference 15. Copyright 1995 INRA.)

A biotechnological process for the production of sotolon has also been proposed using this unique amino acid HIL (Figure 8). In this process HIL from fenugreek seeds is converted to the corresponding 2-keto acid by oxidative deamination. The bacterium *Morganella morganii* can accomplish this biotransformation step (L-amino acid oxidase). The 2-keto acid can subsequently cyclize into sotolon (enhanced at lower pH). The stereochemistry of this unusual amino acid is also shown in Figure 8 and illustrates that the chiral center of C4 (4S) is maintained in the final product, sotolon (5S). Consequently, the sotolon produced consists of one enantiomeric form. HIL has also been reported in the flowers of the Mexican tree *Quararibea funebris* (*16*), which is used as part of a chocolate drink. Surprisingly, this amino acid had the opposite configuration at C4 (4R), which in this case sotolon would consist of the 5R enantiomer.

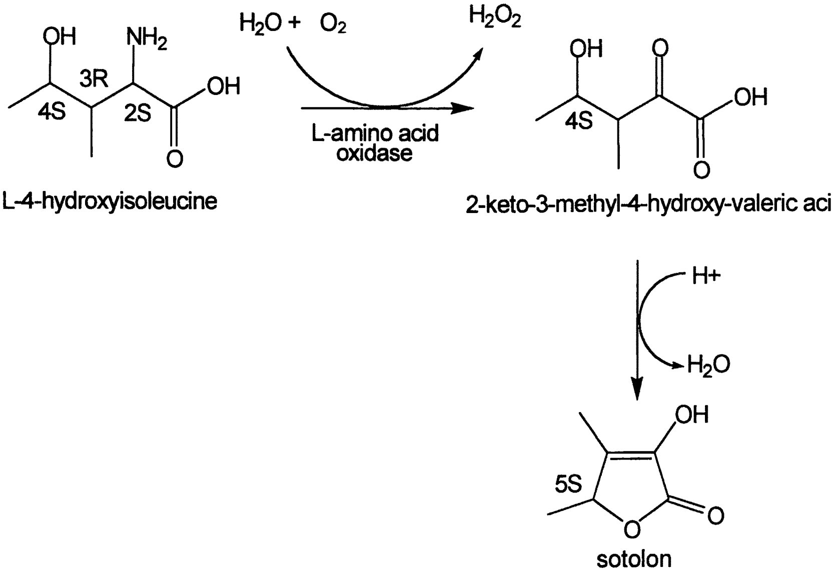

Figure 8. Biotechnological production of sotolon. (Reproduced with permission from 17. Copyright 1995 INRA.)

β-Ionone

Ionones are widely distributed in nature and are important constituents of many essential oils (*18*). The odor character of β-ionone has been described as

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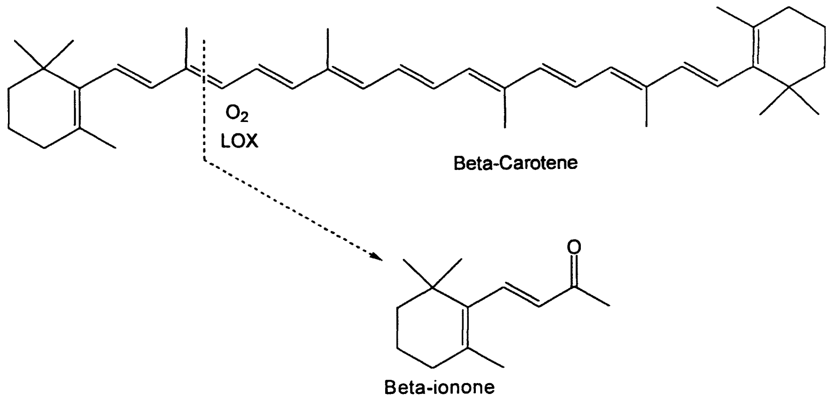
O2
LOX
Beta-Carotene
O
Beta-ionone

Figure 9. Biogeneration of β-ionone from β-carotene.

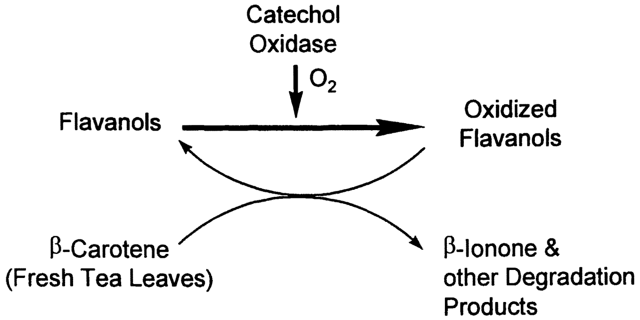
Catechol
Oxidase
O2
Flavanols
Oxidized
Flavanols
β-Carotene
(Fresh Tea Leaves)
β-Ionone &
other Degradation
Products

Figure 10. Biogeneration of β-ionone during black tea manufacture. (Adapted with permission from reference 23. Copyright 1973 American Chemical Society.)

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woody, sweet, fruity, berry-like with a green berry background and this compound has an extremely low odor threshold (in water-0.007μg/L, (*19*). β-ionones are thought to originate in nature from β-carotene and can occur by direct oxygenation (singlet oxygen, free radicals) during photosensitive reactions (*20,21*), aerobic fermentations (*22,23*) or by storage in air (*24*). Figure 9 illustrates the formation of β-ionone from the oxidation of β-carotene. During tea fermentation β-ionone is produced from a co-oxidized coupled reaction that is enzyme catalyzed (Figure 10). Oxidized flavanols are formed during the aerobic fermentation process via catechol oxidase and act as strong oxidizing agents. The oxidized flavanols subsequently cause the oxidative degradation of β-carotene to β-ionone, a compound that contributes to the aroma of tea.

An example of a biosynthetic pathway for the formation of β-ionone is presented in Figure 11. This system involves lipoxygenase (LOX) in a co-oxidation process generally considered an enzymatically born radical system. During this process LOX converts β-carotene into a peroxy radical. It is assumed not to be immediately transformed into hydroperoxides but requires a labile "H" (activated by double bonds) and can then undergo a hemolytic cleavage step forming the β-ionone compound. Aldehydes are proposed as the liable "H" source.

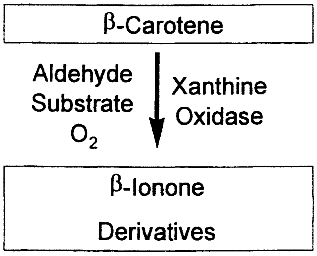

Figure 11. Biosynthetic pathway for the formation of β-ionone from β-carotene (25).

β-Damascenone

First discovered in Bulgarian rose oil (*26*), β-damascenone has become a valuable flavor and fragrance compound. The odor character of β-damascenone has been described as woody, sweet, fruity, and earthy with green floral nuances. It has an extremely low odor threshold (in water - 0.002μg/L (*19*). It is a C-13 nor-isoprenoid compound (similar to β-ionone) oxygenated at the C-7 position instead of the more common C-9 position. Because of this difference in the oxygenated position, simple carotenoid degradation alone cannot produce this compound. Instead, β-damascenone is believed to be formed via biodegradation of allenic and acetylenic carotenoid intermediates (see Figure 9). This process explains the oxygen transposition from C-9 to C-7. Xanthophyll (neoxanthin) is believed to oxidize to the allenic carotenoid structure and therefore, is thought to be a precursor of β-damascenone.

Both of these intermediates (allenic and acetylenic) structures have also been isolated as glycosides and therefore, could be considered as natural substrates or β-damascenone precursors. Enzymatic (glycosidase), thermal or acidic conditions have likewise been shown to release free β-damascenone from this non-volatile form (*27, 28*).

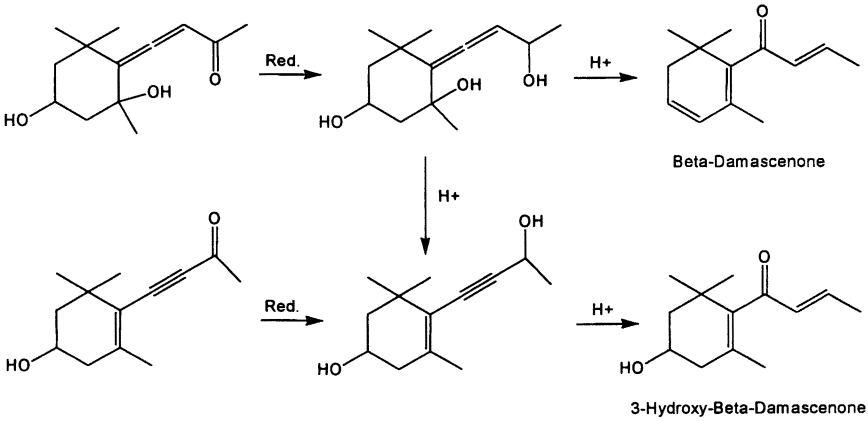

Figure 12. Biogeneration of β-damascenone from allenic and acetylenic carotenoids. (Adapted with permission from reference 29. Copyright 1992 American Chemical Society.)

1-Octen-3-one

A lipid derived compound, 1-octen-3-one is a key aroma character impact constituent in mushrooms (*30*). It is also an off-odorant formed during lipid oxidation (*31,32*). The odor character of 1-octen-3-one has been described as powerful, harsh metallic, mushroom-like odor and it is extremely potent with an odor threshold in water of 0.005μg/L (*33*). A proposed pathway for the biogeneration of 1-octen-3-one in mushrooms is illustrated in Figure 13. The

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substrate, linoleic acid, is converted into the 10-hydroperoxide isomer presumably as a result of lipoxygenase activity. An intrinsic lyase enzyme specific only to the 10-position cleaves the hydroperoxide resulting in the formation of 1-octen-3-ol (*34*). The vinyl alcohol can be transformed into 1-octen-3-one via auto-oxidization (*35*). Even though 1-octen-3-ol also has a mushroom-like aroma and is present in much larger amounts than 1-octen-3-one (*36*), the ketone has a much lower odor threshold value (*37*) generally making it the more important aroma volatile.

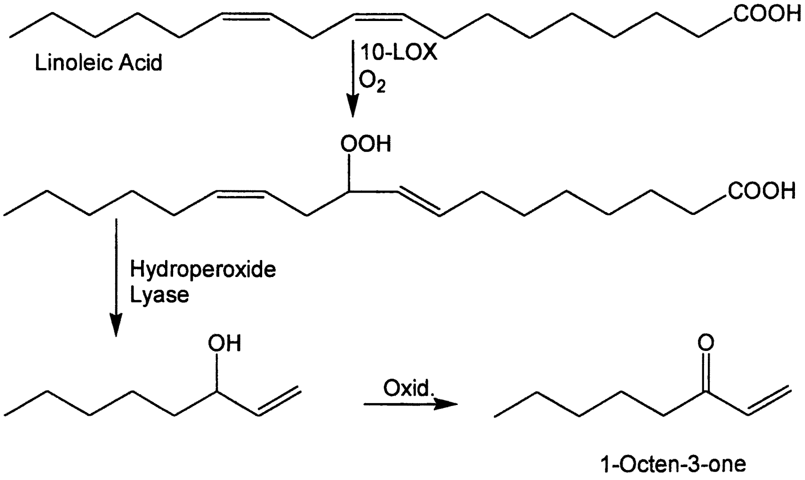

Figure 13. Biogeneration of 1-octen-3-one from linoleic acid in mushrooms.

(E,Z)-2,6-Nonadienal

An important flavor impact compound in cucumbers (*38, 39*) and oysters (*40*), (E,Z)-2,6-nonadienal like 1-octen-3-one is a lipid-derived aroma compound. The odor character of (E,Z)-2,6-nonadienal has been described as melon, sweet, green, fatty, aldehydic, with a fresh vegetable note. It is extremely potent having an odor threshold in water of 0.01µg/L (*41*). A proposed biosynthetic pathway for the formation of (E,Z)-2,6-nonadienal in cucumbers is shown in Figure 14. Linolenic acid, liberated from endogenous lipid via acyl hydrolases, is converted to the hydroperoxide isomer by 9-lipoxygenase. Subsequently, cleavage of the hydroperoxide by lyases forms (Z,Z)-2,4-nonadienal which is then converted by an isomerase enzyme to (E,Z)-2,6-nonadienal. It is interesting to note that this aroma compound is not present

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in the intact cucumber tissue but is only liberated upon cutting or chewing the tissue (*39*).

In oysters (seafood), this aroma compound is generated via a similar enzymatic pathway with the exception that the lipid substrate is eicosapentaenoic acid (C20:5, n-3) and this pathway uses a unique enzyme 12-LOX (*40,42*).

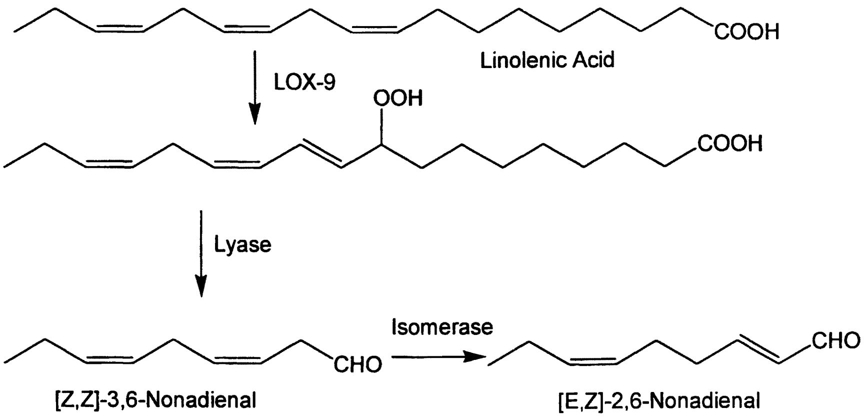

Figure 14. Biogeneration of (E,Z)-2,6-nonadienal from linolenic acid in cucumbers. (Adapted with permission from reference 43. Copyright 1992 American Chemical Society.)

Summary

Oxygen-containing volatiles are extremely abundant in nature. They often have sensory significance either due to high concentrations, or low sensory thresholds. In this review we have attempted to illustrate how some of the more abundant (e.g. carbonyls and esters) are formed in nature. We have also selectively chosen five of the more "potent" oxygen-containing aroma components to illustrate more detailed biosynthesis pathways.

The precursors of these volatiles may be major components of the foods, e.g. amino acids and lipids, or more minor components such as the carotenoids. The pathways largely depend upon enzymes for one or more steps. For example, an enzyme may start the reaction or form highly reactive intermediates that chemically react to form the final product. It is quite common that the oxygen enters the molecule as a result of direct oxidation of a substrate.

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Chapter 15

Importance of Free Fatty Acids in Parmesan Cheese

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The contribution of free fatty acids (FFAs) to Parmesan cheese aroma was evaluated using aroma extract dilution analysis (AEDA) and odor activity value (OAV) methodologies. FFAs with high flavor dilution (FD) values were isolated with an amino solid phase extraction column and quantified using gas chromatography. Both AEDA and OAV analyses suggest that acetic, butanoic, hexanoic, octanoic and decanoic acids are extremely important to the flavor of Parmesan cheese due to the very high FD and OAV values obtained. Butanoic acid is the most important as measured by OAV, both nasally and retronasally. These FFAs are the major contributors to the lipolyzed flavor of this type of cheese. Branched chain FFAs, such as 2-methylpropanoic and 3-methylbutanoic acids, were identified by AEDA but were not found to be important aroma compounds by this approach. FFAs levels in various brands of Parmesan cheeses are also reported in this paper.

In Heteroatomic Aroma Compounds; Reineccius, G., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2002.

Introduction

Parmesan cheese is widely used as a table cheese as well as a food ingredient due to its delicate flavor and flavor compatibility. By definition, Parmesan cheese has to be aged for ten months *(1)*. This long aging process results in a cheese with a very rich aroma and excellent taste. However, little has been published about the aroma chemistry of this cheese. It has been assumed that FFAs are important to the aroma but there has been little data to support this assumption.

FFAs have been reported to be important to many varieties of cheeses. It was found that high concentrations of butanoic, hexanoic and octanoic acids contribute to the sharp flavor of Ras cheese (*2*). The addition of lipase during cheese making increases FFA levels and improves the flavor of this cheese (*3*). Cheddar cheese flavor is also related to the concentration of butanoic and hexanoic acid (*4*). Cheddar flavor intensity increases and then decreases as the concentrations of butanoic and hexanoic acid increase (*4*). The best Cheddar cheese flavor was observed at 45-50 ppm butanoic acid and 20-25 ppm hexanoic acid: a higher degree of lipolysis causes an off-flavor in this cheese (*4*). It was found that butanoic, hexanoic, octanoic and decanoic acids all contribute to the flavor of a Cheddar-like goat cheese (*5*). In addition, 4-methyloctanoic acid and 4-ethyloctanoic acid contribute to the unique "goaty" aroma in this cheese (*5*).

FFA distribution in Parmigiano Reggiano cheese has been studied (*6-11*). It was suggested that the short chain FFAs and some branched chain fatty acids are important to the aroma of Parmesan cheese (*8-11*). However, the contribution of FFAs to the aroma of Parmesan cheese is still not well understood.

AEDA and OAV calculations are very useful methodologies to study the aroma contribution of compounds to food (*12-15*). It has been used to study the flavor contribution of FFAs in Camembert cheese (*16-18*), Emmemtaler cheese (*19,20*) and Cheddar Cheese (*21,22*). This technique has not been used to investigate the importance of FFAs in Parmesan cheese.

Our objective was to investigate the aroma importance of FFAs in Parmigiano Reggiano cheese by GC/O techniques and determine the odor potencies FFAs by AEDA and OAV methodologies.

Materials and Methods

Parmesan Cheese

A wheel (70 lb.) of Parmigiano Reggiano cheese was imported from Italy directly. Several experienced cheese experts judged the cheese as typical of this product. The cheese was manufactured in May of 1996 and had been aged for 24 months. A portion of the cheese was ground to a fine powder under liquid

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nitrogen and frozen at –20 °C until further analysis. The cheese contained 30.6% fat, 32.5% protein and 29.2% moisture. Other Parmesan cheeses were obtained from a local store. These cheeses were also ground and stored at –20 °C for further analysis.

Extraction

One kg of Parmigiano Reggiano cheese was ground with an Osterizer and transferred into a continuous liquid-liquid extractor (Kontes, cat #584550, Kontes, Vineland, NJ). A 500 mL round-bottom flask filled with diethyl ether (CMS Chempure, GC grade, Houston, TX) was attached at the side arm of the extractor. A small amount of silanized glass wool was inserted into the bottom opening of the chamber to retain the cheese solids in the extraction chamber. The diethyl ether in the receiving flask was distilled and condensed back to the extraction chamber with a water bath maintained at 50°C. After one hr of extraction, the solution was transferred to another flask. Another 250 mL of freshly distilled diethyl ether was added to the extracted cheese and the extraction was continued for three more hrs. At the end of extraction, the diethyl ether in the extraction chamber was colorless. All of the extraction solutions were combined.

High Vacuum Distillation

The diethyl ether extract was placed into a funnel designed to introduce the extract into the high vacuum apparatus. The extract was then slowly dripped into a falling film high vacuum evaporation device, which was heated at 50°C. Volatiles were draw by vacuum into a 500 mL round bottom flask immersed in liquid nitrogen. Four additional liquid nitrogen cold traps were connected in series. The second and the third traps were used to further condense the volatile compounds while the last trap was used to prevent potential contamination from the back flush of the vacuum pump. Small amounts of high vacuum grease were carefully applied to the joints of the traps. A diffusion pump (Edwards, model # B30207110, Edwards High Vacuum International, Wilmington, MA01887, USA) and a two-stage vacuum pump (model 1405, Welch vacuum, Thomas Industries Inc., Skokie, IL) were used to provide an ultimate vacuum of 10^{-6} torr for the distillation system.

After distillation, all of the distillate fractions were combined. To increase aroma recovery, the extracted lipid was passed through the high vacuum distillation device two more times. The traps were carefully rinsed with 10 mL freshly distilled diethyl ether, and then the water and all rinses were combined with the distillate.

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Fractionation

The distillate was concentrated to 200 mL in a 40°C water bath with a strip-silvered distillation column (28 mm x 752 mm, cat #505050-2820, Kontes, Vineland, NJ). Water (100 mL) was added and mixed with the distillate. The distillate was slowly adjusted to pH 11 with 10% NaOH and then 60 g of sodium chloride was added to improve the partitioning of aroma compounds into the organic phase. The aqueous phase was then separated from the organic phase using a separatory funnel. Both phases were saved for further analysis.

The aqueous phase was adjusted to pH 1.7 with 2 N H_2SO_4, and extracted three times with 100 mL portions of re-distilled diethyl ether. The ether extracts were combined and dried overnight with 10 g of anhydrous $MgSO_4$. The dried ether extract was then filtered through a Whatman 1PS filter paper. For subsequent GC/O analysis, the filtrate was slowly concentrated to 4 mL in a 40°C water bath with a strip-silvered distillation column as described above and was further concentrated to 0.5 mL with a small Vigreux column.

Analysis by GC/O-Mass Spectrometry (GC/O-MS)

GC/O-MS analysis was performed simultaneously on a Hewlett Packard Model 6890 GC and 5972 MS equipped with an olfactometry device. An MS open splitter was used at the end of the GC column to split the GC effluents into two parts. One part of the effluent was directed to the MS with a deactivated fused silica column (50 cm, 0.1 mm ID), while the other part of the effluent was directed to a sniff-port. A column flow rate of 2 mL/min was used for chromatographic separation, and 0.8 mL/min of column flow was directed to the MS while 1.2 mL/min of column flow was directed to the sniff-port. The portion of analytes directed to the MS was controlled by adjusting the dimension (length and diameter) of the capillary columns. The MS temperature was set at 280°C. The instrument settings of maximum sensitivity by auto-tune were used. The mass scan range was from 29 to 300 mass units. A Hewlett Packard MSD Chemstation (version 3.00A) was used to control the instrument and analyze the data. To achieve simultaneous GC/O and MS analysis, an additional signal board was installed in the GC-MS instrument. An on-off switch box was used to trigger a voltage applied to the signal board. The Chemstation software acquired two channel signals simultaneously: one channel from the total ion chromatogram of the MS, and the other channel from the olfactometer signal board. Thus, a GC/O chromatogram and MS total ion chromatogram were obtained simultaneously.

To obtain FD values, the concentrate was sequentially diluted at a ratio of 1:1 with diethyl ether. An Hewlett Packard FFAP column (25m, 0.32 mm ID, 0.52 μm film thickness, Hewlett Packard #19091F-112, San Fernando, CA) was

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used for chromatographic separation. A 0.5 μL sample was introduced to the GC column with an on-column injection technique. The carrier gas flow rate was set at 2 mL/min. The oven temperature was held at 60 °C for 1 min, 60°C to 240°C at a rate of 5°C/min, and held at 240°C for 5 min. One person was used for the sniffing test, and duplicate runs were performed on all samples. Odor-active aroma compounds were identified by MS, and confirmed by retention index and aroma quality using commercial FFA standards as references.

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FFA Quantification

The FFAs were quantified based on Jong and Badings' (*23*) work with some modifications. All FFA standards and internal standards were purchased from Nu Chek Prep, Inc. (Elysian, MN) and had purities greater than 99%. Multiple internal standards were used for calibration. The internal standard solution was prepared by dissolving 100 mg each of pentanoic acid, heptanoic acid and nonanoic acid in hexane-isopropanol (1:1) and diluting to 100 mL with the same solvent. Pentanoic acid was used as internal standard for butanoic acid and hexanoic acid, heptanoic acid was used as internal standard for octanoic acid, and nonanoic acid was used as the internal standard for decanoic acid. A standard solution was prepared by dissolving 100 mg of each FFA of interest in hexane-isopropanol (1:1) and then diluting to the desired concentration with the same solvent. Standard solutions with concentrations of 5 to 150 μg/mL of FFAs were prepared.

Cheese (2 g) was used for FFA analysis. The sample was mixed well with 1 mL of 2N H_2SO_4, 1.0 mL of internal standard solution and 5 g of anhydrous Na_2SO_4. Twenty mL of hexane-isopropanol (1:1) was added to the mixture and then ground for two minutes to dissolve the lipids. The sample was then centrifuged and the supernatant saved.

The FFAs were purified with a Solid Phase Extraction (SPE) column containing 500 mg amino resin (J&W, cat # 188-1056, Folsom, CA). The column was conditioned with 10 mL of heptane. Five mL of extract was applied to the column. After the solution had passed through, the column was rinsed with 5 mL of chloroform/isopropyl alcohol (2:1) to remove the non-FFA fraction. The FFAs were then eluted with 5 mL of diethyl ether-formic acid (98:2).

The FFAs were analyzed directly with a Hewlett Packard 6890 GC system. One μl of sample was injected in a pulse-splitless mode with pulse pressure at 75 psi for 1 min. A Hewlett Packard FFA (25m X 0.32mm X 0.52μm) column was used for separation. The carrier gas was helium with a constant flow rate of 1mL/min. The GC oven temperature was set to 90 °C for 2 min, raised to 240 °C at a rate of 8 °C/min. and held at 240 °C for 10 min. The injector temperature was set to 250 °C. A flame ionization detector (FID) was used to detect the analytes. Data was collected and analyzed with the MSD Chemstation.

OAV

OAV were calculated by dividing the concentration of the compounds in the sample by the sensory thresholds obtained from published literature.

Results and Discussion

AEDA

FFAs can be derived from lipolysis, proteolysis and lactose fermentation during cheese ripening. Both esterase and lipase have lipolytic activities, and can hydrolyze milk triglycerides to FFAs. Most of the FFAs with carbon chain lengths from C4 to C20 come from triglyceride hydrolysis by milk and microbial lipases during cheese aging. A lower proportion of FFAs with carbon chain length from C2 to C6 can come from lactose fermentation and amino acid degradation (*24*). A small amount of FFAs can also be generated from the oxidation of alcohols, aldehydes, ketones, and esters.

Straight short chain FFAs were the major components in the acidic fraction. The AEDA results revealed that acetic, butanoic, hexanoic, octanoic, and decanoic acids were likely the important compounds due to high FD values (Figure1). Among them, acetic and octanoic acids have the highest FD values, followed by butanoic and hexanoic acids. However, this small difference is probably not significant. Propanoic, pentanoic, 3-methylbutanoic and heptanoic acids have much smaller FD values. 2-Methylpropanoic and 2-methylbutanoic acids have FD values less than 8 and they are not shown in the figure. Thus, it appears that acetic, butanoic, hexanoic and octanoic acids are most likely key FFAs contributing to the flavor of this cheese.

Both acetic acid and propionic acid have pungent, typical vinegar odors. Butanoic and hexanoic acid contribute to sweaty, cheesy, lipolyzed aroma notes. At low concentrations, these acids contribute to the typical cheesy notes. Octanoic acid contributes a waxy flavor at 5 ppm, and a soapy, rancid heavy aroma when the concentration is above 50 ppm (*11*). It has been considered as the main goaty compound in dairy products. Decanoic acid contributes to a soapy aroma.

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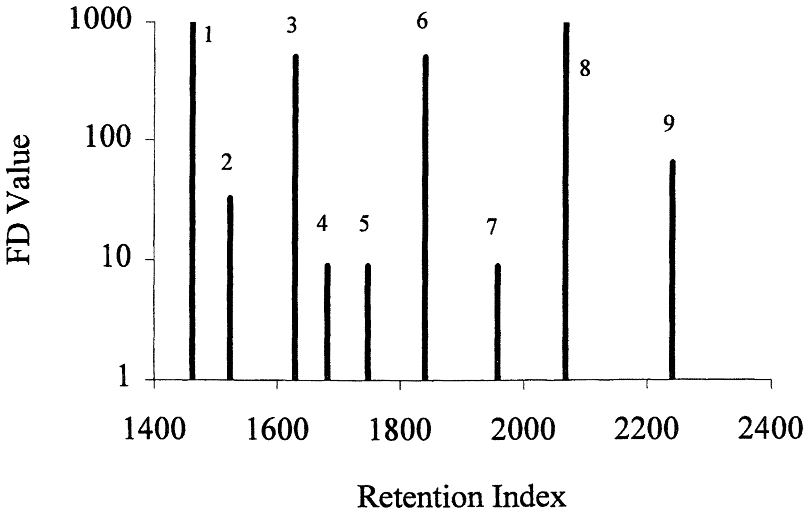

Figure 1. FD values of the acidic fraction of Parmigiano Reggiano cheese. 1, Acetic acid (FD 1024); 2, Propionic acid (FD 32); 3, Butanoic acid (FD 512); 4, 3-Methylbutanoic acid (FD 8); 5, Pentanoic acid (FD 8); 6, Hexanoic acid (FD 512); 7, Heptanoic acid (FD 8); 8, Octanoic acid (FD 1024); 9, Decanoic acid (FD 64).

Some branched chain fatty acids may also make a significant contribution to the overall cheese flavor (*9,10*). 2-Methylpropanoic acid and 3-methylbutanoic acid have been reported to be important in Cheddar cheeses (*22*) and Camembert cheese (*16,17*) by AEDA methods. Branched chain fatty acids are generally derived from lipid biosynthesis of some ruminants and are minor components. Branched chain FFA can also generated from microbial pathways. The degradation of certain amino acids such as leucine, isoleucine and, valine through transamination and decarboxylation can also result in branched chain fatty acids (*25,26*).

Compared to straight chain FFAs, it appears that substitution at the 2-position on shorter chain (≤C6) FFAs increased thresholds. For example, 2-methylbutyric acid and 2-ethylbutyric acids have slightly higher thresholds than butyric acid. On the other hand, substitutions at 3- or 4-positions generally had a lowering effect on the threshold of FFAs, e.g. 3-methylbutyric acid has a much lower sensory threshold than butyric acid. This effect is most pronounced for the 4-methyl and 4-ethyl substitution on octanoic acid. 4-Ethyloctanoic acid has the lowest threshold of 2 ppb and has a very potent goat-like aroma.

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Branched chain fatty acids have been identified in Parmesan cheese. 2-Methylpropanoic, 2-methylbutanoic, 3-methylbutanoic, 2-ethylbutanoic, 2-methylhexanoic, 2-ethylhexanoic and 2-ethyloctanoic acids have all been identified in Parmigiano Reggiano cheese (*6, 11*), but the aroma contributions to this cheese have not previously been investigated.

2-Methylpropanoic, 2-methylbutanoic and 3-methylbutanoic acids were also identified by GC/O-MS in this experiment. However, AEDA results showed that these three compounds were likely not as important as the other straight chain FFAs because of the lower FD values. In this cheese, the degree of lipolysis was very high, and the sweaty, cheesy aromas were primarily derived from straight short chain FFAs.

Benzoic and phenylacetic acid were also identified in this cheese, but were not found to be important aroma contributors. Previous studies reported that 2-methylbutanoic acid and 3-methylbutanoic acid were the only branched chain fatty acids present at concentrations slightly above sensory thresholds in this cheese (*11*). Our work confirms that branched chain and aromatic FFAs make limited aroma contributions to Parmesan cheese flavor.

OAV

In AEDA, only compounds present at concentrations higher than the sensory thresholds in the vapor phase are detected. So the resulting FD value can only be used as a screening criterion because this method does not take into account the interactions between the aroma compounds and the food matrix. OAV is the ratio of absolute concentration of the aroma component in the food to its sensory detection threshold value that has been determined in a like food matrix. By considering the food matrix, OAV is a better methodology to evaluate the aroma compounds in a food.

To extract FFAs from cheese, a hexane-isopropyl alcohol solvent system was used because this system gives good extraction recovery of FFAs with minimum esterification. The FFAs were then isolated using an ion exchange solid phase extraction column and directly analyzed on a FFAP GC column using multiple internal standards. The concentrations of the FFAs determined in the experiment were in agreement with literature values (*11*).

In order for an aroma compound to be perceived, the concentration in the vapor phase above the matrix must exceed the sensory detection threshold in the vapor phase. When calculating the OAV, it is very important to use the sensory threshold value obtained in the same matrix (or a comparable one) as the food system containing the key aroma compounds. The sensory detection threshold of a compound depends greatly upon the matrix. Threshold values of aroma compounds determined in air or simple solutions are not the same as those observed in a food matrix. The aroma compounds can interact with the water,

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carbohydrates, proteins, and fat present in a food. The interactions, in turn, influence the odor perception of a compound. Consequently, the vapor pressure of the compound in the matrix directly affects the sensory threshold in the matrix.

The vapor pressure of a compound in the matrix depends upon volatility and solubility. Fat is a good solvent for many hydrophobic flavor compounds. Hydrophobic compounds have a greater tendency to be dissolve in the fat phase and exhibit a lower vapor pressure in the fat phase versus an aqueous phase. Thus, sensory thresholds of hydrophobic compounds are much higher in a fat phase than in an aqueous phase. The opposite is also true for hydrophilic compounds. Thus, a change in the fat content will affect the odor thresholds of odor active compounds in a food. Protein and carbohydrate also influence the flavor threshold by binding to the flavor compounds through hydrogen bonding or other types of molecular interactions (*27,28*).

The sensory detection thresholds of FFAs have been investigated in both water and oil by many authors (*29-35*). The values vary widely due to different methods used in these studies. In general, short chain FFAs have higher threshold values in water than in fat, possibly due to higher solubility in water (the vapor pressure is lower in the aqueous media than the fat media). Long chain FFAs have much lower sensory detection thresholds in water than in fat because of t poor water solubility.

The pH also exerts a considerable influence on the flavor impact of FFAs. The pH of the media will affect the ratio of protonated fatty acids to ionized fatty acids; it is the protonated FFAs that contribute aroma. At a pH of 5.2, considerable portions of FFAs exist in a salt form, which reduces the aroma impact of these compounds.

Since no sensory threshold data have been obtained for compounds in a cheese matrix, threshold values from the literature, in water or oil phases, are used in this study. The OAVs are calculated both nasally (by smelling) and retronasally (by tasting) because the sensory threshold values are different by each means.

Table I shows that all volatile FFAs have very high OAVs. Among these FFAs, butanoic acid has the highest OAV both by nasal and retronasal methods, followed by acetic acid. Generally, the sensory detection threshold by retronasal is lower than nasal. This trend is most dramatic for butanoic acid. Butanoic acid has a much higher OAV retronasally than nasally due to the lower sensory detection threshold obtained retronasally. Even though the perceived flavor intensity is not linearly related to concentration, the OAV data shown that the short straight chain FFAs are present at concentrations many times greater than the sensory thresholds, thus these compounds are very important flavor contributors to this cheese.

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Table I. OAVs of Selected Aroma Compounds

	Compounds	Conc. (ppm)	Odor Threshold (ppm) Nasal	Retronasal	OAV* Nasal	Retronasal
1	Acetic acid	1424	32.3	22	44	65
2	Butanoic acid	482	2.7	0.9	179	536
3	Hexanoic acid	260	9.2	27.1	28	10
4	Octanoic acid	147	19	11.3	8	13
5	Decanoic acid	283	22	16	13	18

*OAV= concentration/threshold. The threshold values were found in the following sources: Compound 1) Nasal (*34*), Retronasal (*32*); Compound 2) Nasal (*34*), Retronasal (*35*); Compounds 3, 4, 5) Nasal (*36*), Retronasal (*35*).

FFAs of several brands of Parmesan cheeses were also studied (Table II). Even though the values vary tremendously from brand to brand, all Parmesan cheeses have very high levels of volatile FFAs, and subsequently high OAVs for these components (Table III). The results further show that short chain FFAs are very important to Parmesan cheese flavor.

Table II. FFAs (ppm) in Various Brands of Parmesan Cheese

Brand	Butanoic Acid	Hexanoic Acid	Octanoic Acid	Decanoic Acid
A-Reggiano	397	249	146	374
B-Italian	678	363	234	355
C-Sbrinz	110	56	35	92
D-BelGioioso	99	44	25	83
E-Grana Padano	346	181	116	246
F-American	95	50	50	87
G-American	432	183	111	231
H-American	1890	447	219	410

Most of the Parmesan cheeses have FFA distributions (percentage of total FFAs) similar to that of milk-fat (Table IV). One cheese sample, however, has over 20% butanoic acid as a percentage of total FFAs. This indicates that the enzymes used in the manufacturing of this cheese are highly selective towards short chain fatty acids.

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Table III. OAVs of Various Brands of Parmesan Cheeses

	Butanoic Acid		*Hexanoic Acid*		*Octanoic Acid*		*Decanoic Acid*	
Brand	*nasal*	*retro nasal*	*nasal*	*retro nasal*	*nasal*	*retro nasal*	*nasal*	*retro nasal*
A-Reggiano	147	441	27	9	8	13	17	23
B-Italian	251	753	40	13	12	21	16	22
C-Sbrinz	41	122	6	2	2	3.1	4	5.8
D-BelGioioso	37	110	5	1.6	1	2.2	4	5.2
E-Grana Padano	128	384	20	6.7	6	10	11	15
F-American	35	106	5	1.8	3	4.4	4	5.4
G-American	160	480	20	6.8	6	9.8	10	14
H-American	700	2100	49	16	12	19	19	26

Table IV. FFA Profiles (Percentages of Total FFAs) of Various Brands of Parmesan Cheese

Brand	*Butanoic Acid*	*Hexanoic Acid*	*Octanoic Acid*	*Decanoic Acid*
Milk-fat	3.6	2.2	1.2	2.5
A-Reggiano	4.3	2.7	1.6	4.1
B-Italian	5.3	2.9	1.8	2.8
C-Sbrinz	3.9	2.0	1.2	3.3
D-BelGioioso	5.0	2.2	1.3	4.2
E-Grana Padano	5.6	2.9	1.9	4.0
F-American,WI	4.5	2.4	1.8	4.1
G-American	7.1	3.0	1.8	3.9
H-American	22.3	5.3	2.6	4.8

Conclusions

AEDA and OAV show that short chain FFAs are extremely important to the flavor of Parmesan cheese. AEDA revealed that the FFAs; acetic, butanoic, hexanoic and octanoic, have the highest FD values. OAV calculations found butanoic, hexanoic, octanoic and decanoic acid all have high OAVs and that these compounds are the major contributors to the lipolyzed flavor of this type of cheese. Among the FFAs, butanoic acid is the most important as measured by OAV, both nasally and retronasally. Branched chain FFAs, such as 2-methylpropanoic and 3-methylbutanoic acids, exhibited lower FD values are not as important to Parmesan cheese aroma.

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Chapter 16

Classical and Modern Methods for the Determination of Halogenated Aroma Compounds in Foods: An Overview

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This paper provides a review of methods for the analysis of halogenated volatiles in foods. The analytical procedure is composed of three main steps-extraction from the sample matrix, separation using gas chromatography (GC), and identification. Specific methods reported in the literature for the analysis of halogens are compared and discussed. The advantages and disadvantages of each method are also included.

Off-flavors in foods and water have often been linked to the presence of volatile halogenated compounds. Trihalomethanes (THMs), such as chloroform, bromodichloromethane, and bromoform, have been found in drinking water and beverages (*1-10*). Halogenated (chlorinated and/or brominated) phenols/anisols have been reported in fish, meats, dairy products, wine, coffee, beverages, fruits, and grains (*11-24*), and fumigants (e.g. ethylene dibromide and

In Heteroatomic Aroma Compounds; Reineccius, G., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2002.

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dichloropropene) have been found as residues in cereals, grains, grain-based products, and fruits (*25-36*). These compounds generally have very low sensory thresholds and are offensive. Thus, contamination of a food typically renders the food unacceptable. The detection, identification and quantification of these compounds in foods is, therefore, of considerable economic importance.

This review provides an overview of the methods reported in the literature for the analysis of volatile halogens. It includes a discussion of the methods used in aroma isolation, separation, and identification. While quantification is often also an imporant step in this process, the approaches used in quantification is not unique to halogens and thus, is not discussed in this review.

Aroma Isolation

The choice of method for halogen isolation from foods or beverages is likely the most critical decision made in this analysis. Every isolation method introduces biases in the volatile profile of the isolate ultimately obtained and thus, this choice is critical to the success of the analysis. The techniques used in the isolation of halogenated volatiles are identical to those used in obtaining any volatile isolate: there is nothing unique about halogens that dictates a particular aroma isolation procedure. If there is any unique consideration, it is that the halogens responsible for off-flavors typically have extremely low sensory thresholds and thus, large sample sizes and substantial concentration are required particularly for lipid-containing foods.

This section of this paper will discuss the methods that have appeared in the literature for halogen analysis. They inlude direct injection, static headspace extraction, dynamic headspace extraction, liquid-liquid extraction, distillation, and other miscellaneous extraction methods.

Direct Injection

Due to the simplicity, reproducibility, and lack of bias during sample preparation, direct injection methods have been used for aqueous samples (such as drinking water) (*1, 37-40*). Direct injection of water does, however, result in significantly shortened gas chromatographic column life and will reduce the sensitivity of electron-capture detectors (ECD). Due to both limitations in sample injection size and the negative effect on detector sensitivity, detection limits of 90-150 ppb for dichloroethane and 500 ppb for dichlorobenzene have been reported in the literature (*1*). While sensitivity is good, the extremely low sensory thresholds of halogenated compounds often require lower detection limits. Thus, direct injection has found limited use in the laboratory.

Static Headspace Extraction

Static headspace is simple, rapid and well suited to the analysis of highly volatile compounds. Volatile fumigants including methyl bromide (*32, 35*), carbon tetrachloride, ethylene dichloride, and ethylene dibromide (*27*) have been determined by headspace methods in grapefruit, wheat berries, flour, bread, cocoa, and peanuts. Determination of trihalomethanes (THMs) formed in drinking water during water treatment have also been analyzed by this method (*2, 3, 6, 10*). In addition, static headspace has been widely used to analyze other volatile halocarbons (VHCs), such as 1,1,1-trichloroethane, carbon tetrachloride, trichloroethylene, tetrachloroethylene, and bromodichloromethane, in diverse foods as well as drinking water (*41-43*). These volatiles have boiling points up to 150 °C. This method basically requires no solvent and introduces only volatiles in the gas phase, which can minimize the entrance of any impurities into the GC system. Headspace methodologies used with an ECD detector for the analysis of samples such as drinking water and beverages have typically yielded detection limits in the low to sub-ppb level (*6, 10, 41*). Standard deviations were in the range of one unit (*6, 10*).

As was noted above, water vapor will affect detector response and even though the amount of water vapor injected by this technique is small, it has been reported to have a negative effect on headspace analysis using ECD (*44, 45*) . To reduce this effect, Vitenberg et al. (*44*) used a phosphoric acid pre-column to absorb moisture.

In the analysis of high-lipid content foods (e.g. some dairy products, oils, fats, shortening, meats, and fish) (*42, 43*), there is seldom enough water vapor in the headspace to affect sensitivity. Detection limits were usually in the range of 10-50 ppb by ECD (*42*). Some authors have attempted to improve on sensitivity by digesting the food with concentrated H_2SO_4 (if necessary) to release matrix-bound volatiles. They also used very high equilibration temperatures (90 °C) to enrich volatiles in the headspace. However, the concentrations of HCVs in the gas phase were still too low for headspace analysis of some foods (*42*).

Dynamic Headspace Concentration

This method has been employed for the analysis of volatile halogenated compounds (mostly THMs) in foods and drinking water (*2, 7-9, 46*). It offers greatly enhanced sensitivity compared to static headspace methods. Detection thresholds (as low as 10 ppt) (*7, 9*) were obtained by using this method. Dynamic headspace methods improve on sensitivity by concentrating the headspace volatiles on an adsorbent material. Tenax is the most common adsorbent for THMs because it has relatively little affinity for water and good adsorption/desorption properties for the THMs (*7, 9*). Despite the low affinity

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of Tenax for water, water can still be a problem and this is dealt with by passing dry purge gas through the trap before desorption (*7*). Lepine and Archambault (*7*) noted that extraction efficiency is very sensitive to the purge time. They found that their most volatile compound, chloroform, was completely purged only after 4 min whereas the least volatile compound, bromoform, took 10 min to reach its maximum value. Wang et al. (*9*) demonstrated that purge gas purity is an important consideration in this method. Since large amounts of purge gas are used, even extremely low levels of gas contaminants can lead to a serious contamination problems.

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Liquid-Liquid Extraction

The main advantages of liquid-liquid extraction are good recovery, precision and simplicity. Various solvents (such as pentane, hexane, dichloromethane, methyl cyclohexane, petroleum ether, ethyl acetate, acetone, acetonitrile, ethanol and methanol) have been reported in the literature for extraction (*4, 5, 13, 16, 17, 20, 25, 28, 30, 34, 47-49*). Among the solvents, acetonitrile has been reported to be suitable for the extraction of halogenated compounds in foods such as vegetable oils (*13*), fish (*47*), cereals (*25*), and crops (*28*). Recovery was very high (86-108 % for chlorinated trifluorotoluenes) even from fatty foods such as fish (*47*). Acetonitrile was also found to have a lower response by electron capture and β-ionization detectors than other solvents such as ethanol and acetone. This can minimize solvent interference with some early eluting components (such as 1,3-dichloropropene and 1,2-dibromoethane) (*25, 28*). Furthermore, acetonitrile elutes later from polar GC columns than some of the more commonly used solvents and this may be a benefit when studying very volatile compounds. However, a study of extracting solvents showed that nonpolar solvents (particularly pentane) are more effective in extracting THMs from water than the polar solvents tested (*4, 49*). The extraction of polyhalogenated residues (which are more polar and hydrophilic, e.g. pentachlorophenol) is aided by extracting a food with 0.1 M potassium hydroxide solution, adjusting the solution pH into the acidic range and then partitioning the polyhalogenated compounds from the acidic solution into toluene (*11*).

One of the most critical factors to consider in solvent extraction is the purity of solvents used. Halides can react with organic olefins during sample preparation and storage to yield halogenated artifacts. Halogens (mostly chlorine and/or bromide) and organic olefins can come from solvent impurities and thereby contribute to artifact formation. To prevent the formation of halogenated artifacts, sodium thiosulfate, sodium sulfite, or ascorbic acid may be added to the sample prior to extraction (*5, 49*). Sodium thiosulfate and sodium sulfite will quench any reactive halogens in the extract (*49*). Ascorbic

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acid also is an effective preservative in the extraction of THMs because it will stabilize trichloroacetone (an intermediate in the haloform reaction) by lowering the pH in addition to removing residual chlorine (*5*).

In some studies, the addition of salts has been reported to increase the extraction efficiency of halogenated compounds from aqueous samples (*28, 49*). This "salting out" effect was also found beneficial in the extraction of THMs from drinking water by dynamic headspace methodology.

When using solvent extraction to isolate halogenated compounds from fatty samples (such as vegetable oils, fish, and oysters), an additional clean-up step is required. Unfortunately, lipids are coextracted with the halogenated organics by organic solvents (*13, 16, 28, 47*). The halogenated organics can be isolated from extracted lipid by column chromatography with Alumina or Florisil-type columns (*13, 17, 23, 28, 47, 50, 51*) or C-18 solid phase extraction (*23*). The sample extract is passed through a bed of adsorbent and the subsequent elution scheme permits the separation of lipid from the halogens of interest.

Distillation

Simple co-distillation with steam (*20, 26, 31, 33*) or simultaneous steam distillation/solvent extraction (SDE) using a Likens-Nickerson (L-N) apparatus (*14, 15, 18, 22, 52*) has found substantial use for the isolation of halogenated compounds from foods. Authors have reported excellent precision and recovery for these methods.

The stability of halogenated compounds during distillation allows the isolation of halogens from a wide range of sample types. Steam distillation with or without solvent was used to extract halogenated fumigants from cereal grains, grain-based products (*26, 33*), and grapefruit (*31*) and also 4-bromo-2-chlorophenol (a contaminant responsible for taints) from melons (*20*). Simultaneous steam distillation using a L-N apparatus has been more widely used in diverse samples such as chicken (*14, 15*), pork (*52*), dried fruits, packaging materials (*18*), coffee (*19*), salmon, and seafoods (*22*). This method is especially well suited for the isolation of chlorinated and brominated phenols/anisols. These volatiles are quite heat stable, have low volatility (thereby require rigorous extraction methods) and readily partition into the extracting solvent.

Other Extraction Methods

Supercritical fluid extraction (SFE) methods have also been used in the analysis of halogenated volatiles in foods. Seitz et al. (*24*) coupled supercritical carbon dioxide extraction with a purge-and-trap instrument to analyze volatiles in grains. Supercritical fluid extraction can be used to efficiently extract and concentrate the volatiles from a fatty food but some of the fat in the sample will also be coextracted and thus, the SFE isolate may not be directly injected into a gas chromatograph. A purge and trap method can be applied to the SFE extract thereby separating the volatiles from fat. The SFE procedure is used to obtain a concentrated sample to purge and then the purge-and-trap method is used to isolate the volatiles from the fat.

Supercritical carbon dioxide possess similar solvent properties as pentane and hexane which have been found to have excellent extraction efficiencies for halogenated compounds (*24*). SFE methods, accordingly, have good extraction efficiencies. After extraction, the supercritical solvent can easily be removed from the extract with minimal loss of volatile organic compounds since they have low boiling points. Also, the optimization of density and solvent strength of supercritical carbon dioxide can be simply attained by adjusting extraction pressures and temperatures.

Solid-phase microextraction (SPME) is a final isolation technique to be mentioned in this review. Page and Lacroix (*53*) reported on applying SPME to the headspace analysis of 33 halogenated volatiles in model aqueous solutions. SPME, with a poly(dimethylsiloxane) (PDMS)-coated fiber, showed a detection limit in the low ppb to low ppt (depending on analytes) levels when coupled to an electrolytic conductivity detector. The authors found headspace extraction by SPME was significantly affected by the lipid content in the samples. The same research group recently found that porous polymers with large surface areas (Carboxen-PDMS and divinylbenzene (DVB)-Carboxen-PDMS) were much more effective than the previously tested PDMS fiber for the analysis of 37 contaminants (including 31 halogenated compounds) in vegetable oils (*53*).

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Separation by Gas Chromatography (GC)

Once one has obtained an aroma isolate by one of the previously described methods, the isolate needs to be separated into individual compounds and then identified and quantified. The separation of volatile halogenated compounds is done by gas chromatography. Due to the diversity of compounds being separated, many different GC column phases have been reported in the literature. As one would expect, capillary columns are widely used (*5, 7, 8, 17, 10, 18, 20-23, 33, 35, 43, 48, 49, 50, 54-57*) and offer many advantages over packed columns (*1, 3, 4, 6, 8, 9, 11, 13, 25, 27-32, 34, 36, 41-44, 47, 51, 58-60*) in resolution and sensitivity but sacrifice loading capacity. When using capillary columns, non-polar columns are generaly preferred over polar columns, particularily for the analysis of extracts containing a considerable amount of water (*5, 7, 21, 49*).

Farrell (*55*) separated 19 chloroanisols (including tetrachloroanisols which were difficult to resolve using other columns) on a capillary column coated with a hydrophobic liquid phase ($C_{87}H_{176}$) by optimizing the temperature program and carrier gas flow rate. To increase the resolution of packed columns, some authors have connected columns in series (*3, 58*) or homogeneously mixed columns (*44, 58*) with polar and non-polar liquid phases. Of these two configurations, the serial arrangement of polar and non-polar liquid stationary phases was found to yield better resolution and were easier to prepare (*58*).

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Detection and Identification

Electron Capture Detector

The most widely used GC detector for the analysis of halogenated volatiles is the electron capture detector (ECD) using either nickel-63 or tritium β-emitter (*1-3, 5-7, 9, 10, 16, 25-28, 30-35, 41-44, 47-49, 61*). The ECD is highly sensitive toward halogenated molecules due to their electronegative nature. The detection limit using ECD has been determined to be as low as ppt levels (*1, 7*). Greater responses were obtained for bromine-containing compounds than chlorinated volatiles (*7*). In some studies, a linear dynamic range covering over 4 orders of magnitude was reported (nickel-63 ECD) (*7, 61*). However, as was mentioned, the ECD cannot tolerate water which may come in with the extraction method (such as direct injection). Thus, various procedures have been developed to remove water from the sample system. These methods inlude a solvent bypass during injection (*62*), dehydration of the extract by the addition of anhydrous salts (such as NaCl and $CaCl_2$) (*25, 30*) and incorporation of a precolumn with a moisture adsorbent (*44*). The use of a dry purge for purge and trap methods have also been adapted (*7*).

Hall (Electrolytic Conductivity) Detector

While electron capture offers some selectivity, the Hall detector (in the halogen mode) responds specifically to halogenated compounds. Therefore, the Hall detector (with or without other detectors such as ECD) has been used in many studies of halogenated volatiles when high selectivity was required (*2, 4, 53, 25, 34, 43, 50, 63*). The Hall detector loses sensitivity following the injection of lipids or contaminated samples (*64*). Thus, clean-up procedures such as column chromatography are beneficial when using this detector.

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Mass Spectrometer (MS)

MS detectors have been widely used for the detection and identification of halogenated compounds in foods (*8, 13, 16, 17, 20, 22, 24, 43, 47-49, 52*). Mass spectral data offer the advantage of being able to both detect and elucidate molecular structure. The use of MS in the single ion monitoring (SIM) mode offers selectivity and sensitivity with detection thresholds being as low as 10 ppt for polychlorinated-anisoles (*18*). The characteristic isotopic composition of chlorine and bromine ($_{35}Cl$:$_{37}Cl$ = 100:32.5 and $_{79}Br$:$_{81}Br$ = 100:98, respectively) (*65*) is a useful property for the identification and quantification of halogenated compounds containing chlorine and bromine. Despite the good sensitivity of this instrument, it is not as sensitive as ECD and is often inadequate. Also, one may not get a strong enough spectrum to do an identification. This problem is often bridged by monitoring multiple ions, e.g. M^+ and $(M+2)^+$, which can facilitate identification (*18*).

Other Detectors

Electron capture, Hall, and mass spectral detectors are the primary detectors used for the analysis of halogenated volatiles. However, flame ionization (FID), infrared (IR), and sniffing have also been used for this purpose. The FID is seldom used alone but typically in combination with other detection methods such as an ECD, Hall or MS detector (*17, 21, 25, 51, 66*). The IR detector has been used to supplement MS data to assist in the identification of halogens (*24*). GC-sniffing has not broadly been applied to the detection of halogenated compounds due to health concerns. However, halogens involved in food taints, such as 2,4,6-trichloroanisole (medical, phenolic, or iodine-like off-flavor), have been detected in foods by GC-sniffing (*17, 21*). The very low sensory thresholds and characteristic odors of the chlorinated anisoles and phenols make GC-olfactometry an ideal choice.

Derivatization

Derivatization is occasionally used to change the physical or chemical properties of halogens that will aid in their analysis. A halogen exchange reaction has been used to improve the detector response of halogenated compounds. The response of an ECD was increased by converting methyl bromide stoichiometrically into methyl iodide through reaction with sodium iodide (*29*). Derivatization can also be used to enhance the volatility and therefore, the chromatographic separation of halogenated compounds. Chlorinated carboxylic acids and polychlorinated (tri, tetra, and penta) phenols have been esterified by diazomethane or diazoethane for this purpose (*11, 14, 15, 19, 67*).

Conclusions

Various isolation techniques have been used to determine halogenated aroma compounds in foods. Analogous to the determination of aroma compounds in general, each isolation method has its strengths and weaknesses. When considering the analysis of very volatile halogens, static headspace or direct injection may be used for aroma isolation depending upon the concentration of halogen in the water or food matrix. The less volatile halogens (chlorophenols or chloranisoles) are typically present in very low quantities (both in the food and in the headspace) and thus, their isolation is more problematic. These food taints are typically isolated by distillation methods. The most unique aspect of halogen analysis is their strong response to selective detectors (ECD, Hall or MS). Halogen analysis virtually always depends upon these detectors for selectivity and sensitivity.

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Chapter 17

The Contribution of Halogenated Aroma Compounds to Food Flavor

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Halogenated compounds are often associated with unnatural contamination due to their use in biocides and sanitizers; however they are quite prevalent in nature. These compounds contribute to the aroma of foods and originate from both natural and pollutant sources. Chlorinated and brominated compounds are the most commonly reported halogenated aroma compounds, but this may simply be due to the difficulty associated with analyzing and identifying other halogenated compounds. The reported halogenated aroma compounds are very potent with very low sensory thresholds, thus minor reactions and slight contaminations can contribute to an aroma.

Halogenated organic compounds have been found naturally occurring with bioactivity in bacteria, fungi, marine organisms, plants, and mammals. Fluorine is the most abundant halogen on land, while chlorine is the most abundant in the oceans (*1*). Halogens and halogenated compounds, especially chlorinated compounds, are extensively used in industry for cleaning, in pesticides, and as fungicides. This paper will cover only halogenated compounds which have been shown to contribute to the aroma of foods. In other words, there has been some sort of sensory analysis including gas chromatography olfactometry, to support that a compound contributes to the aroma of that food. This narrows the

In Heteroatomic Aroma Compounds; Reineccius, G., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2002.

discussion of halogenated compounds to only those found in foods with aroma activity. Three classes of halogenated compounds have been shown to be important in food flavor: brominated phenols, chlorinated phenols, and chloroanisoles. The bromine is primarily of natural origin while the chlorine of the chloroanisoles and chlorophenols results from man's use of chemicals.

Chlorinated Aroma Compounds

Chlorine is found to contribute to aroma primarily in the form of chloroanisoles. All of the chloroanisoles, except the monochloroanisoles, have been described as musty and sometimes as musty with solvent-like notes. Monochloroanisoles are wintergreen and sweet, but have not been reported as contributing to any flavors (*2*). Chloroanisoles are primarily produced by the fungal methylation of chlorophenols originating from use as fungicides but also may originate from the addition of chloride to phenol by chloroperoxidase action. Molds capable of methylating chlorophenols include many *Aspergillus* and *Penicillium* species (*3*). Chlorophenols can also cause taint, however the thresholds are much higher, thus a large direct contamination with a herbicide or fungicide would be necessary for detection (*4*).

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Sensory Studies

Research on the sensory properties of chloroanisoles has been conducted primarily by three groups: by the Agricultural Research Council's Food Research Institute in Norwich, England (*5-7*); CSIRO Division of Food Science and Technology, Sydney Laboratory, Australia (*8*); and TNO Division of Nutrition and Food Research in The Netherlands (*3, 9*). The group at the Food Research Institute studied the odor character of all the chloroanisoles and determined the aqueous thresholds for compounds that were most intense at 1 part per trillion. Whitfield and coworkers (*8*) determined thresholds for some of the chloroanisoles in various media. A study in the Netherlands looked at the character and threshold of some chloroanisoles incorporated in coagulated egg yolks. An additional study of musty flavored compounds by Chambers *et al.* gave more specific descriptors for some of the chloroanisoles (*2*).

The chloroanisoles are very potent. Thresholds for the more potent chloroanisoles range from 0.004 to 3×10^{-7} ppm in water and from 0.12 to 0.0024 ppm when incorporated in coagulated egg yolk (*5, 9*). Pentachloroanisole had high thresholds in all media and thus is not likely to contribute to taint odors at the low concentrations in which they have been found. Engel *et al.* fed chickens individual chloroanisoles, and found that the 2,4,6-trichloroanisole was more active than 2,3,4,6-tetrachloroanisole, while 2,4,5-trichloroanisole and pentachloroanisole were inactive. This inactivity corresponds to higher thresholds (*10*). Compounds with 2,6-chlorination were shown to have a strong fatiguing effect, combined with adaptation that lasted from 6 to 12 minutes (*6*). Griffiths and Fenwick studied the sensory effects of replacing chloro groups with methyl groups (*7*). They found that the most odorous chemicals have chlorine in the 2,6-position. With increasing chlorination the series became less solvent-like, antiseptic or sweet, and became more musty. This is in agreement with the work of Dyson in 1926, when he showed that the odor character of a series of isothiocyanates was far more dependent upon position than on structure of the substituent; a methyl group in any position can be replaced by a halogen group without changing the odor character (*11*).

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Chloroanisoles in Chicken Eggs and Broilers

Chloroanisoles have been found in musty flavored chicken broilers and eggs (*5, 9, 10, 12-14*). Gas chromatography olfactometry, GCO, on fat, skin and muscle from musty birds gave a strong musty odor detected consistently at the elution time of 2,3,4,6-tetrachloroanisole and at the elution time of pentachloroanisole when present at a high concentration (*12*). Sensory evaluation of chickens fed with chloroanisoles showed feeding either the tetrachloroanisole or 2,4,6-trichloroanisole gave musty tasting meat.

It is hypothesized that the primary source of this contamination is the fungal methylation of chlorophenols used as antifungal agents in the wood shavings used to cover the floor of the chicken coop (*5, 10, 12*). By inoculating pure wood shavings with 2,4,6-trichloroanisole, 2,3,4,6-tetrachlorophenol or pentachlorophenol, then combining them with chicken feces, there was nearly a quantitative conversion to the anisole form within 9 days for the tetrachlorophenol (*5*). Slower conversion was demonstrated for 2,4,6-tetrachlorophenol and pentachlorophenol. Many different microorganisms were found to be capable of this methylation, and so it cannot be attributed to a single organism (*12*). In surveying the chloroanisole levels in chickens within a coop, there was a correlation found between the high concentrations of tetrachloroanisole in the litter and the high concentrations of taint in the chickens. Chloroanisoles are highly volatile, thus contamination may occur simply through absorption through the lungs. Additionally, chloroanisoles and chlorophenols were found in some feed (*14*).

Cork Taint

Cork taint occurs in all grades of cork and can affect all bottled products that utilize cork stoppers such as wine and brandy. It is a major problem for the industry, as it affects 0.5-6% of all corked bottles (*15*). In 1981 Tanner *et al.* identified the microbial metabolite 2,4,6-trichloroanisole as the major cause of cork taint. At low concentrations this compound dulls the aroma, freshness and fruity characteristics of wine. At slightly higher concentrations, it imparts a musty, moldy, dank, or cellar-like aroma to the wine. With a threshold range of from 1 to 10 parts per trillion in wine, a small amount of this chemical has a very large consequence on the sensory characteristics of the contaminated substance (*16-19*). Corks are highly absorptive, and are easily tainted with volatile chemicals. Microbial growth can produce volatile metabolites in, on or nearby the cork that can leach into wine affecting flavor.

2,4,6-Trichloroanisol is not the only cause of the problem generally referred to as "cork taint." Other contributing compounds are also microbial metabolites, and include 1-octen-3-ol, guaiacol, 2-methylisoborneol, and geosmin. However, 2,4,6-trichloroanisol is the only halogen-containing compound, has the lowest threshold of any of these compounds, and is the primary cause of cork taint.

Corks are bleached with hypochlorite for cosmetic purposes as well as to sterilize the corks. In 1959 Burtschell proposed a chemical pathway of the formation of chlorophenols under slightly alkaline conditions with hypochlorite, as might happen under cork bleaching conditions (*20*). This could lead to the production of 2,4,6-trichloroanisole. Several routes are possible for the successive chlorination of the naturally occurring cork phenol, but in all cases, *ortho* and *para* positions to the hydroxyl group are preferred over *meta* positions. Other paths have also been proposed for the synthesis of 2,4,6-trichloroanisole (*21*). These can be spontaneous or assisted through enzymatic means. As well as the addition of chlorine atoms to phenol, 2,4,6-trichloroanisole can be formed through the de-chlorination of a highly chlorinated compound, such as pentachlorophenol or hexachlorobenzene. Reductive dehalogenation is one of the major pathways for enzymatic degradation of halogenated compounds in microorganisms.

Simply in the presence of chlorine or hypochlorite, some microorganisms can fully biosynthesize 2,4,6-trichloroanisole (*22*). In some *Penicillium* species phenols are synthesized via the shikimic acid pathway from a carbohydrate source, then are chlorinated and demethylated. Other microorganisms, such as *Basidimycetes*, a white wood-rot fungus, are capable of introducing a chlorine atom into a benzene ring by the action of a chloroperoxidase enzyme (*1*). Microorganisms, including molds and soil bacteria, can also dechlorinate pentachlorophenol to trichloroanisole, thereby detoxifying it (*23*). Large quantities of chlorophenols (mostly pentachlorophenol) have been used as general biocides, fungicides and wood preservers over decades. Most commercial preparations of pentachlorophenol are contaminated with tetra- and tri-chlorophenols, all of which can form chloroanisoles.

Chloroanisoles can form to a limited extent on the bark of trees, but the major point of formation is during cork processing. Traditionally, after bleaching, cork slabs were left to mature for several months in fairly humid circumstances; the perfect conditions for the microbial formation of cork taint. Often they were not considered 'done' until they were covered by mold. In the

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absence of microbial growth during subsequent processing after bleaching, corks had no greater levels of 2,4,6-trichloroanisole than in untreated corks (*19*). This is not the only place where chloroanisoles can form. Commercially available wood preservatives containing pentachlorophenol or 2,4,6-trichlorophenol as the active ingredients have been marketed and used for the treatment of shipping holds (*24*). Treatment of corks with paraffin and silicone oils to waterproof the cork increase the susceptibility of corks to certain airborne taints, such as the migration of chloroanisoles from packaging and shipping containers.

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Other Sources of Chloroanisoles and Chlorophenols

Since chlorophenols are commonly used as a fungicides, herbicides, and pesticides they can be found in many places, creating a potential for the formation of the chloroanisoles by mold methylation. Jute sacks can be treated with pentachlorophenol or bleached with chlorine, and thus are a common source of contamination with chloroanisoles to produce a musty contamination taint. Rice, coffee, potatoes, and flour have been found to have a musty off-flavor from chloroanisoles originating from the jute sacks they were transported in (*25-28*). Cardboard boxes are another potential source for chloroanisole contamination. Dried fruit with a musty chloroanisole taint were shipped in contaminated cardboard boxes (*29, 30*). Cocoa powder and biscuits were also contaminated by cardboard boxes (*31*). Canned beverages were contaminated by cardboard boxes which had been stored in the same area as the cans (*31*). Contamination of cheese rind was associated with chlorophenols used to preserve wood shelves on which the cheese were being stored (*3*). The contamination of wine, brandy, and gin, in addition to cork taint, has been attributed to the wood crates that the bottles were shipped in (*3*). In one instance, meat was contaminated by chlorophenols used as refrigeration material (*31*).

Chlorophenols can come by direct contamination with herbicides and fungicides, or result from in-plant chlorination of phenol. The major sources of chlorine for the formation of chlorophenol are treated water supplies and chlorine-based sterilizing agents (*4*). Phenols can be derived from plastic and resin fittings, paint, printing inks, packaging materials, boiler water additives and food itself. Chemical formation of chlorophenols in plant was the source of the musty taint in cheese, beer and milk (*32-34*). The source of chloroanisoles found in several essential oils is unknown (*35*). 2,6-dichlorophenol was found in carrots treated with sodium hypochlorite (*35*). Water treated with chlorine that comes in contact with phenol has been found to be tainted with chloroanisoles and chlorophenols (*36*). Canned vegetables were contaminated with condenser water containing chlorophenols (*31*).

Bromophenols

Sensory Studies

Bromophenols contribute to the aroma of many seafoods (*37-47*). Extensive sensory research on bromophenols was conducted by Boyle *et al.* at the University of Wisconsin-Madison to determine odor character and contribution of the bromophenols in seafood (*48*). Odor character was determined for the compounds in water, vegetable oil, shrimp meat, and fish meat. 2,6-Dibromophenol was by far the most potent, with a threshold in water (0.0005) three orders of magnitude lower than the next potent bromophenol. 3-Bromophenol, 4-bromophenol and 2,4-bromophenol had relatively high thresholds thus are presumed to seldomly contribute to aroma. At low concentrations, the bromophenols imparted flavor notes reminiscent of marine fish and seafood. In fish and shrimp muscle tissue and oil matrices 2,6-dibromophenol and 2,4,6-tribromophenol provided iodine-, shrimp-, crab-, and sea salt-like flavor attributes. When the two were combined in oil, briny, sea and fish qualities predominated. Monobromophenols enhanced sweet and overall seafood-like flavor characteristics in these matrices. 2,4-Dibromophenol always had a phenolic medicinal odor in all media and at all concentrations. The combination of bromophenols present in the fish produce the overall final flavor. At high concentrations bromophenols are associated with off-flavors in shrimp; however, at low concentration they enhance positive shrimp flavor. Thus, bromophenols might be used to improve the flavor of aquacultured species (*42*). Freshwater fish flavors lack the notes associated with marine flavor, while bromophenols appear to account for much of these flavors in marine fish and seafoods (*41, 49*).

Sources of Bromophenols

Bromophenol occurrence is more pronounced in the marine life which inhabits the sea bottom (*48*). Thus, organisms that feed on the sea bottom are likely to encounter bromophenol-containing species of prey, which include many species of marine crustacea, molluscs, worms and fish. Primary producers of bromophenols in the ocean include diatoms, cyanophyceae, rhodophyceae, and chlorophyceae. These species contain enzyme systems capable of producing bromophenols. Primary-producers are either consumed directly by predators, or degrade into debris which is filtered by larger organisms. These larger organisms are classified as secondary producers, and include crustaceans, protozoa, ostracods, as well as other herbivorous, carnivorous, and organic debris consuming animals. It has been shown to be likely that shrimp do not metabolize the bromophenols (*43, 48*). If the shrimp head is removed immediately after catching, the amount of bromophenol in the tail meat can be minimized. Secondary-producers become food for squid and fish, and these, in

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turn, are preyed upon by larger fish. It is likely that bromophenols are not metabolized by fish either (*43, 48*).

Formation Pathways in Algae

Algae contain many halo-metabolites, of which several bromophenols have been found to be odor-active and flavor-contributing. Red and green algae contain greater quantities of bromophenols than the brown algae (*50*). 2,4,6-tribromophenol is the predominant bromophenol found in algae, followed by 4-bromophenol, 2,4-dibromophenol, 2-bromophenol, and 2,6-dibromophenol (*51*). Bromophenol content is dependent upon the season, with bromophenol levels highest in summer (*51, 52*).

Bromine and other halides are incorporated into algal metabolites through the action of haloperoxidases, most notably bromoperoxidases. Bromoperoxidases are enzymes capable of brominating organic substances in the presence of bromide and hydrogen peroxide (*1, 53, 54*). Bromide is available to marine plants and algae via salt water. In marine algae, halometabolite formation is dependent on photosynthesis as a source of hydrogen peroxide (*55*). Hydrogen peroxide is produced during the Mehler reaction when oxygen is consumed in photosynthesis and in photorespiration during the conversion of glycolate to glyoxylate (*56, 57*). The substrates can be alkenes, alkanes, phenols, or terpenes (*1*).

Halogenation likely occurs during secondary metabolic processes, and the compounds produced are capable of acting as allelochemics, including defense chemicals, sex attractants, and pheromones in sea life (*48*). A pathway for the biosynthesis of 2,4,6-tribromophenol was proposed by Flodin and Whitfield, which was a continuation of the work from Landymore *et al.* (1978) in the algae *Ulva lactuca* (*52, 58*). Tyrosine is deaminated and successively oxidized and decarboxylated to yield 4-hydroxybenzaldehyde. This can be either oxidized to 4-hydroxybenzoic acid or reduced to 4-hydroxybenzyl alcohol. The addition of bromide to the *ortho* and *para* positions of the phenolic ring is preferred. Degradation of L-tyrosine to 4-hydroxybenzaldehyde takes place in a cell compartment separate from where the bromination reaction takes place. Halogenated compounds are localized in the chloroplasts, vesicles in cytosol, middle lamella, and outer cell walls (*59-62*).

There are several schools of thought about the reasons behind halometabolite production in algae. As hydrogen peroxide can be toxic at certain concentrations, halometabolites may be produced as a byproduct of hydrogen peroxide scavenging; or the halometabolites may function as chemical defenses of the algae. It has also been proposed that these halometabolites are biocides or fungicides, deterrents to grazers, or part of interalgal competition (*53, 63-66*). Recently, some of these functions have been disputed, as some fish feed on algae with high halometabolite content (*67*).

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Fresh Water Origins

Bromophenols can also be chemically formed through wastewater treatment processes. In the presence of bromide ions in water undergoing chlorination, bromide-containing oxidants may be formed (*47*). Fish are very susceptible to absorption of compounds in water since they are transmitted directly through the gills. Thus, any run off from chemical plants or farming that results in water contamination, will likely cause an off-flavor in fish found in the contaminated water (*68*). While water passes directly through the gills of freshwater fish, this does not occur in saltwater fish, so freshwater fish are more likely to absorb odorous pollutants than are saltwater fish.

Other Sources of Bromophenols

Trace levels of bromophenols have been identified in tainted fruit juices that were diluted from concentrate (*4*). The source of the bromophenols was presumed to be the use of solid bromine donors for cleansing the food processing line. When residual hypobromous acid reacts with phenol, bromophenols are subsequently formed.

Iodine and Fluorine Containing Compounds

While iodine is not as abundant as chlorine or bromine in the environment, iodometabolite formation is quite prevalent in marine creatures such as red algae, sponges, and coral (*1*). The relative instability of the carbon-iodine bond may explain why few iodometabolites have been discovered. Nucleophilic exchange with hydroxide ions occurs readily and results in a loss of iodine during sample preparation. The carbon-iodine bond is easily broken by thermal energy, for example in the injector port of a gas chromatograph. Thus, iodine can be lost from compounds during analysis with no indication of their presence.

Plants growing in areas containing either fluoride-rich bedrock or fluorspar mining waste have been found to contain high levels of fluoride. Some specialized sponges accumulate and metabolize large concentrations of fluorine in the oceans. Soya beans convert fluoroacetate to fluorocitrate. As with iodine, it is difficult to distinguish between C-H and C-F bonds in a basic analysis such as gas chromatography. Special methods must be used to look for the presence of fluorinated compounds (*1*). Since fluorinated compounds have bioactivity in several pheromones, it is likely that there are some fluorinated compounds with odor activity. As fluoride and iodide containing compounds are difficult to find, it is likely that they may have been overlooked in flavor analyses. It would not be surprising to learn in the future that fluorinated and iodinated compounds contribute to the aroma of some foods.

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Possible Aroma Contributors

There are additional studies that imply halogenated volatiles contribute to the aroma of other foods; however, no sensory studies were conducted to verify their contribution. 2-Acetyl-5-chloropyrrole, having an almond-like aroma, was reported in cocoa butter (*69*). Fourteen volatile halogenated compounds were found in baked potatoes (*70*). In a review of meat flavor volatiles, Shahidi *et al.* suggested that the volatile halogenated compounds present in various meats could potentially contribute to meat flavors (*71*).

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Conclusions

Halogenated compounds are often associated with unnatural contamination due to use in biocides and sanitizers; however, these compounds are also quite prevalent in nature. Halogenated compounds contribute to the aroma of foods and originate from both natural and pollutant sources. Chlorinated and brominated compounds are the most commonly reported halogenated aroma compounds, but this may simply be due to the difficulty associated with analyzing and identifying other halogenated compounds. The reported halogenated aroma compounds are very potent with very low thresholds, thus minor reactions and slight contaminations can contribute to an aroma. Since this class of compounds may impart an aroma even at low concentrations, halogenated aroma compounds may easily be overlooked when analyzing a sample.

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Chapter 18

Volatile Compounds Formed in a Glucose–Selenomethionine Model System

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Selenium (Se) is an essential component of enzyme glutathione peroxidase, which is important in the protection of red blood cell membranes and other tissues from damage by peroxides. Wheat and meat are the main source of Se in the diet, and selenomethionine is the primary form found in these foods. The Maillard reaction is a complex degradative reaction and occurrs extensively in processed foods as well as in vivo, where proteins, amino acids, nucleic acids, and amino phospholipids react nonenzymatically with reducing sugars. This study investigates the Maillard reaction of selenomethionine and glucose. The effects of pH, time, and added diallyl disulfide on the generation of volatile compounds were studied using a model system. The organoselenium and mixed organoselenium-sulfur compounds generated were identified by EI/MS and NH_3-CI/MS.

In Heteroatomic Aroma Compounds; Reineccius, G., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2002.

Intoduction

Chemical Properties of Selenium

Selenium, the 34th element on the periodic table, was first discovered by Berzelius in 1817. The name selenium is derived from the Greek word meaning "the moon". It be longs to Group VI A (oxygen, sulfur, selenium, tellurium, polonium) of the periodic system and possesses similar chemical properties with sulfur. Selenium is a semi-metal. Essentially, it is a nonmetal from a chemical viewpoint; however, it possesses some metallic characteristics.

Selenium has six stable isotopes: 74 (0.87%); 76 (9.02%); 77 (7.58%); 78 (23.52%); 80 (49.82%); 82 (9.19%), and is one of the few nonmetal elements which has variable valence within the redox range of biological systems. The four natural oxidation states are as follows: (0), elemental selenium, selenodiglutathione (dipeptide); (-2), sodium selenide (Na_2Se), hydrogen selenide (H_2Se); (+4), sodium selenite (Na_2SeO_3), selenium dioxide (SeO_2), selenious acid (H_2SeO_3); and (+6), sodium selenate (Na_2SeO_4), selenic acid (H_2SeO_4). Selenium (0) can be reduced to hydrogen selenide, oxidized to selenious acid or selenites, and further oxidized to selenic acid or selenates (*1*).

Selenium can easily replace sulfur to form a large number of organic selenium compounds (dimethyl selenide, trimethylselenium). Selenium occurs primarily as selenides on the side chains of selenocysteine at physiological pH values. Selenomethionine in nature occurs almost exclusively as the *l*-enantiomer. Inorganic selenate and selenite predominates in water. Organic selenium compounds (primarily selenomethionine and selenocysteine), which are incorporated into plant proteins, account for the major portion of selenium in vegetables and in cereals (*2*). Selenocystine is an oxidation product of selenocysteine, in which two –SeH groups become one Se-Se group. In the body, selenocystine is metabolized to selenocysteine. Selenocysteine is the form of selenium is present in all currently known enzymes (e.g., selenoprotein P, glutathione peroxidase), which contain selenium.

Biological Functions of Selenium

Selenium is an essential nutrient with a recommended dose of 50-200 µg/day considered being adequate and safe for adults (*3*). Observations in Japanese fishermen suggest that a selenium intake of 10 to 200 times above normal does not produce toxic effects. However, ingestion of 31 mg/day for 11 days produced toxicity, while individuals who ingested 312-617 mg/day

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chronically reported toxicity. More selenium is required if diets are also deficient in vitamin E. Specifically, selenium is an essential component of glutathione peroxidase, which destroys hydrogen peroxide and hydroperoxides; protecting cell membranes from oxidative damage. Vitamin E is also implicated in this system in which the role is to prevent the formation of lipid hydroperoxides (*4*).

Bioavailability

The bioavailability as well as the toxic potential for selenium and selenium compounds is related to chemical form, and most important, to solubility. Selenates are relatively soluble compounds, similar to sulfates, but selenites and elemental selenium are virtually insoluble. Elemental selenium is probably not absorbed from the gastrointestinal track. Absorption of selenite is from the duodenum. The bioavailability of selenium, including selenomethionine and selenocysteine, in plant-derived foods is high while the bioavailability of selenium from animal-derived food is low to moderate. Animal studies suggest that vitamins A and C promote absorption of selenite, although vitamin C could also be expected to reduce selenite to elemental Se, which is not absorbed (*5*).

Organoselenium Compounds in Garlic

Genus *Allium* plants, especially garlic (*Allium sativum*), contain more selenium than other vegetables. Garlic is claimed to contain selenoproteins, and ingestion of garlic is well known to cause bad breath. Now it is clear that garlic breath odor is from the lungs but not from particles of garlic retained in the mouth (*5*). Several compounds have been identified as responsible for the garlic flavor; they are allyl methyl sulfide, allyl methyl disulfide, diallyl sulfide, diallyl disulfide, hydrogen sulfide, 2-propenethiol, and (+)-limonene (*7*). Compared to organosulfur compounds, selenium is present in a very low amount in garlic. The ratio of S:Se in fresh garlic is 1.2 x 10^4:1. During analysis, selenium compounds usually coelute with other compounds. Because of the low concentrations present in foods, selenium analysis had not drawn much attention from flavor chemists before the 1990's.

In 1995 Cai et al. (*8*) reported the organoselenium compounds generated in human breath after ingestion of garlic. Several organoselenium and mixed organoselenium-sulfur compounds were identified. The major selenium-containing compounds found in garlic breath were dimethyl selenide (Me_2Se), allyl methyl selenide (MeSeAll), methanesulfenoselenoic acid methyl ester

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(MeSeSMe), 2-propenesulfenoselenoic acid methyl ester (MeSeSAll), and dimethyl diselenide (MeSeSeMe).

Maillard Reaction

The Maillard reaction is one of the most important reactions on flavor and color development in foods, especially during thermal processing of foods. It may create desirable aroma components like in baking and roasting processes. In some cases, Maillard reaction is undesirable such as in the formation of off-flavors or dark colors during storage or in dehydrated foods.

The Maillard reaction is a complex series of chemical reactions initiated by the interaction of a free amino group and a carbonyl group. The reaction is a degradative reaction and responsible for the generation of many volatiles. In 1998, Tsai et al. (*9*) studied the selenomethionine-glucose model system, and five organoselenium compounds were identified.

The main objectives of our study were to gain a better understanding of the formation of organoselenium in food systems. Our study investigated the effects of time of heating and pH on the volatile compounds formed in a selenomethionine-glucose model system. In addition, interactions between organoselenium and sulfur compounds formed by the Maillard reaction were monitored.

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Experimental

Thermal Reaction and Isolation of Volatiles

Selenomethionine (0.15 g) and glucose (0.2 g) were dissolved in either 25 mL of water (unbuffered solution) or 25 mL 0.05 M sodium phosphate buffer solution, and the pH of the solutions were adjusted to 3.0, 5.0, 7.0 and 9.0 using 85% phosphoric acid or 1 M NaOH solution. The solution was sealed in a 100 mL glass bottle. The solutions were heated at 160 °C for 40, 60 and 80 minutes.

After the reaction, the solution was adjusted to pH 7, and extracted with 50 mL of CH_2Cl_2. The organic phase was concentrated to 2 mL by a Kuderna-Danish concentrator, then to 1 mL under N_2.

Interactions between Organoselenium and Sulfur Compounds

Selenomethionine (0.15 g), glucose (0.2 g) and diallyl disulfide (10 µL) were dissolved in 25 mL 0.05 M sodium phosphate buffer solution, and the pH of the solutions were adjusted to 7.0. The solution was sealed in a 100 mL glass bottle. The reaction time was 60 minutes and the reaction temperature is 160°C.

After reaction, the solution was adjusted to pH 7, and extracted with 50 mL of CH_2Cl_2. The organic phase was concentrated to 2 mL by a Kuderna-Danish concentrator, then to 1 mL under N_2.

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Gas Chromatographic Analysis

The volatile compounds isolated from the thermal reaction systems were analyzed by a Siemens SiChromat 2-8 Gas Chromatograph with a AS-20 auto-injection system. The GC was equipped with a HP-5 fused silica capillary column (25 m x 0.32 mm i.d.; 0.52 µm film thickness) and a flame ionization detector. For each sample, 1 µL was injected with a split ratio of 15:1. The GC was run with injector and detector temperatures of 275°C. The column temperature was programmed from 40°C to 285°C at a rate of 5°C/min.

GC/Mass Spectrometry Analysis

GC/MS analysis was performed by an HP 5989A coupled with an HP 5890 II GC. Mass spectra were obtained by EI at 70 eV or NH_3-CI and a mass scan from 40-550 amu.

Results and Discussion

Volatile Compounds from Selenomethionine-Glucose Model System

The GC/MS profile and compounds identified are shown in Figure 1. The most abundant peak in this chromatogram is dimethyl diselenide, which possess strong garlic and somewhat metallic flavors. Selenium-containing compounds can easily be identified by EI/MS or NH_3-CI/MS because of the isotopic distribution. The EI/MS spectrum of dimethyl diselenide, with the monoisotopic molecular ion at *m/z* 190, is shown in Figure 2.

The heated model systems, the solvent extracts and the concentrates all possessed an aroma which can be described as roasted, nutty and garlic-like. It is believed that pyrazines are responsible for the roasted aroma and dimethyl diselenide is responsible for the garlic flavor. Due to the flavor properties of the extracts, this study focuses on the nitrogen-containing compounds, ethyl selenoacetate and dimethyl diselenide, although several selenium-containing and sugar degradation products were also identified.

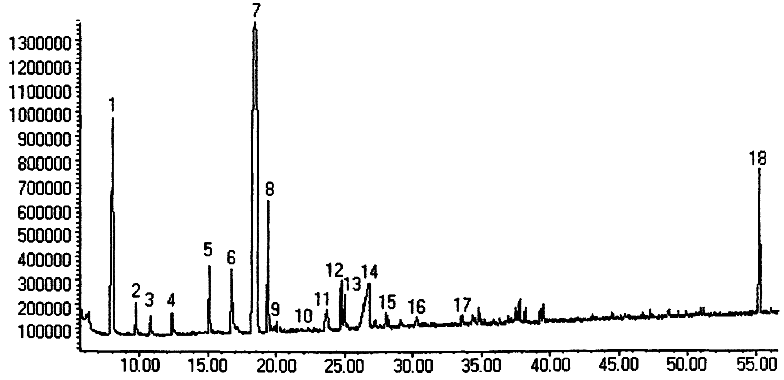

Figure1. GC/MS profile of volatile aroma compounds formed from glucose-selenomethionine model system: 1 = hydroxyacetone, 2 = 3-hydroxy-2-butanone, 3 = pyrazine, 4 = 1-hydroxy-2-butanone, 5 = methylpyrazine, 6 = 2-furanmethanol, 7 = dimethyl diselenide, 8 = 2,6-dimethylpyrazine, 9 = ethylpyrazine, 10 = ethyl selenoacetate, 11 = trimethylpyrazine, 12 = 4-hydroxy-5-methyl-3(2H)-furanone, 13 = 2-hydroxy-3-methyl-2-cyclopenten-1-one, 14 = 2,5-dimethyl-4-hydroxy-3(2H)-furanone, 15 = 3-ethyl-5-methyl-2(5H)-furanone, 16 = 5-methyl-1H-pyrrole-2-carboxaldehyde, 17 = selenotrithiolane, 18 = internal standard.

Effects of Reaction Time and pH

The pH is known to play an important role in the Maillard reaction. The formation of colored compounds in the Maillard reaction increases with increasing pH, especially at pH values above 7. Color formation is due to the formation of melanoidin pigments from reaction of amino groups with Maillard intermediates, which are inhibited at a lower pH as a result of the protonation of amino group. Higher pH conditions tend to favor the formation of nitrogen-

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containing volatiles, such as pyrazines, but some volatiles are favored under acid conditions (*10*), such as furanthiols and disulfides, which are believed to be important in meat aroma and favored by lower pHs (*11*).

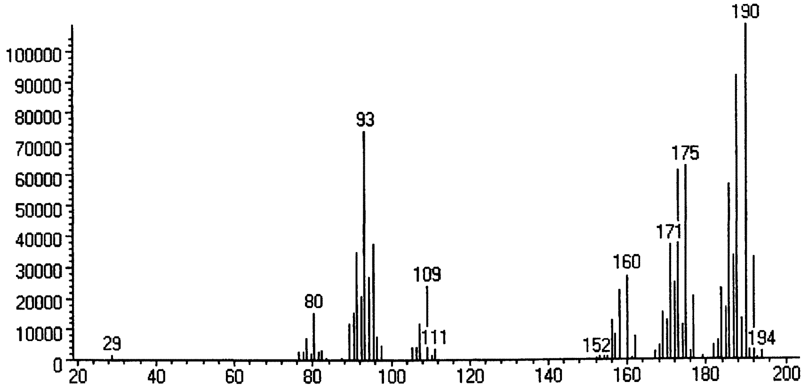

Figure 2. EI/MS spectrum of dimethyldiselenide.

In our model systems, after reaction all buffered solutions with pH3 were colorlessand those with pH7 and pH9 were brown. In terms of the effect of heating time, the longer the heating times the darker the resulting solutions. The yields of pyrazines, ethyl selenoacetate and dimethyldiselenide at different times and pH values are listed in Tables I-III. Unlike pyrazines and dimethyldiselenide, the formation of ethyl selenoacetate is favored in low pH, which has the maximum yield at pH3 and 80 min (Figure 3), and the yield decreases with increasing pH.

Table I. Yields of Major Volatile Compounds (mg/g of glucose) Generated in the Model System Heated for 40 Minutes (buffered solutions)

Compounds	*pH 3*	*pH 5*	*pH 7*	*pH 9*
Pyranize	ND	ND	0.021	0.041
Methylpyrazine	ND	ND	0.022	0.014
Dimethyl diselenide	0.061	0.029	0.743	0.938
2,6-Dimethylpyrazine	ND	ND	0.039	0.014
Ethylpyrazine	ND	ND	0.019	0.005
Ethyl selenoacetate	0.027	0.021	0.022	0.009
Trimethylpyrazine	ND	ND	0.006	0.003

ND: not identified

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Table II. Yields of Major Volatile Compounds (mg/g of glucose) Generated in the Model System Heated for 60 Minutes (buffered solutions)

Compounds	*pH 3*	*pH 5*	*pH 7*	*pH 9*
Pyranize	ND	ND	0.029	0.024
Methylpyrazine	ND	ND	0.033	0.164
Dimethyl diselenide	0.046	0.041	0.545	1.162
2,6-Dimethylpyrazine	ND	ND	0.067	0.144
Ethylpyrazine	ND	0.002	0.008	0.027
Ethyl selenoacetate	0.057	0.044	0.016	0.010
Trimethylpyrazine	ND	ND	0.011	0.043

ND: not identified

Table III. Yields of Major Volatile Compounds (mg/g of glucose) Generated in Model System Heated for 80 Minutes (buffered solutions)

Compounds	*pH 3*	*pH 5*	*pH 7*	*pH 9*
Pyranize	ND	0.014	0.252	0.260
Methylpyrazine	ND	0.009	0.202	0.169
Dimethyl diselenide	0.058	0.134	2.099	1.490
2,6-Dimethylpyrazine	ND	0.018	0.239	0.191
Ethylpyrazine	ND	0.008	0.034	0.027
Ethyl selenoacetate	0.542	0.098	0.077	0.007
Trimethylpyrazine	ND	0.008	0.098	0.052

ND: not identified

It is well known that pyrazines are only produced at pH values above 5.5, and the yield of pyrazines formed in the Maillard model system increased with pH values in the range of 5 to 9 (*12*). However, in our model system, 4 out of 5 pyrazines and dimethyl diselenide exhibited a maximum yield at pH7, 80 min. The yields of dimethyl diselenide at different times and pH values is shown in Figure 4. The possible mechanism for the formation of dimethyl diselenide from the Maillard reaction of selenomethionine is shown in Figure 5.

The products from the Maillard reaction are pH dependent due to the breakdown of the Amadori product during the intermediate stages. In many model studies on the effect of pH on the volatile products of the Maillard reported, large pH changes were usually used. However, it has also reported that small changes in pH over the range 4.5-6.5 may have significant effects on the types and concentrations of the volatile products in model systems such as those that contain cysteine and ribose (*13*).

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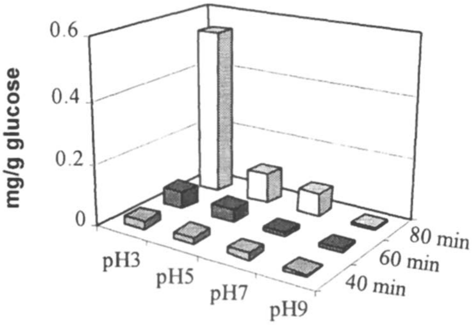

Figure 3. The yields of ethyl selenoacetate at 160 °C and different times and pHs.

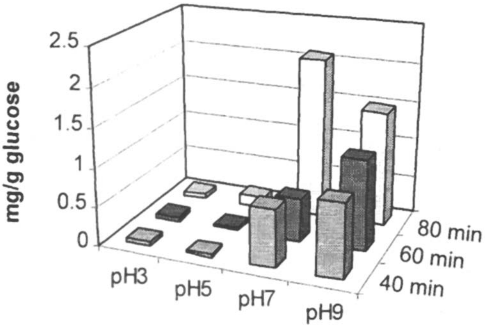

Figure 4. The yields of dimethyl diselenide at 160°C and different times and pHs.

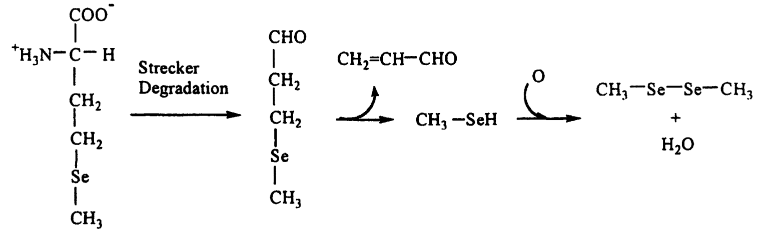

Figure 5. The formation of dimethyl diselenide.

In terms of pH change, in unbuffered model systems a pH change of 3 or more units is not unusual during heating, and this can affect both the rate and the pathway on the formation of volatile and colored products (*14*). Thus, it is very important to maintain a constant pH during heating when model systems are used to study the influence of pH on the Maillard reaction.

In our study, it is shown that the pH change of an unbuffered solution is significant after heating (Table IV), and this pH change dramatically affected the formation of pyrazines (Figure 6).

Table IV. pH Changes of Buffered and Unbuffered Systems

	Buffered Systems		*Unbuffered Systems*	
Initial pH	7.01	8.99	6.98	9.01
Final pH	6.87	8.92	4.78	5.12

Interactions Between Organoselenium and Sulfur Compounds

In order to investigate the potential interaction of organoselenium and organosulfur compounds, the model reaction of selenomethionine, glucose and diallyl disulfide was performed. The GC/MS profile of the reaction products is shown in Figure 7. Several organosulfur, organoselenium and mixed organoselenium-sulfur compounds were identified in this model system. They are allyl methyl selenide (MeSeAll), diallylsulfide, CH_3-Se-S-CH_3, CH_3-Se-S-CH_2-CH=CH_2 (Figure 8), CH_3-Se-Se-S-CH_3, CH_3-Se-S-S-CH_2-CH=CH_2, CH_3-Se-Se-S-CH_2-CH=CH_2, 2-acetyl-thiophene, and 5-methyl-2-thiophene-carboxaldehyde. As an example, Figure 8 shows the EI/MS spectrum of MeSeSAll. Some of them have previously been identified in garlic (*8*).

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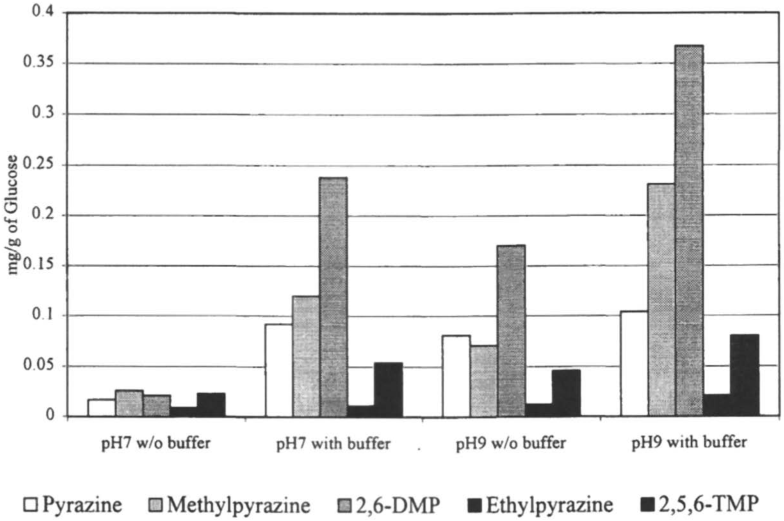

Figure 6. The yields of pyrazines in buffered and unbuffered solutions, (160°C, 80 min).

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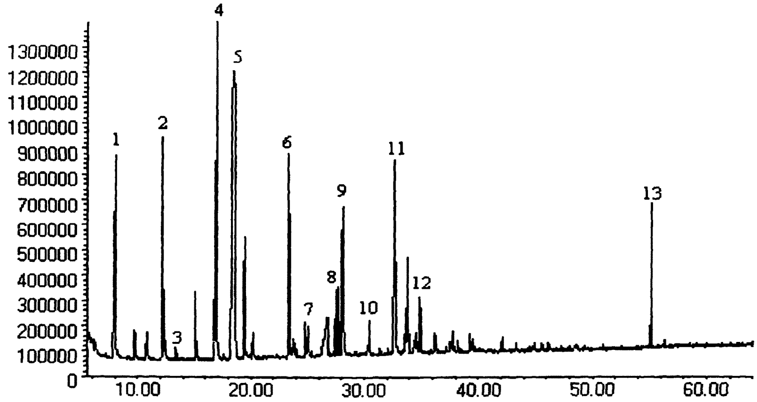

Figure 7. GC/MS profile of volatile compounds formed from glucose-selenomethionine model system with diallyl disulfide. 1 = hydroxyacetone; 2 = allyl methyl selenide; 3 = CH_3-Se-S-CH_3; 4 = diallylsulfide; 5 = dimethyl diselenide; 6 = CH_3-Se-S-CH_2-CH=CH_2,; 7 = CH_3-Se-S-S-CH_2-CH=CH_2; 8 = 5-methyl-2-thiophenecarboxaldehyde; 9 = 2-acetylthiophene; 10 = CH_3-Se-Se-S-CH_3, 11 = 4-hydroxy-5-oxohexanoic acid lactone, 12 = CH_3-Se-Se-S-CH_2-CH=CH_2; 13 = internal standard.

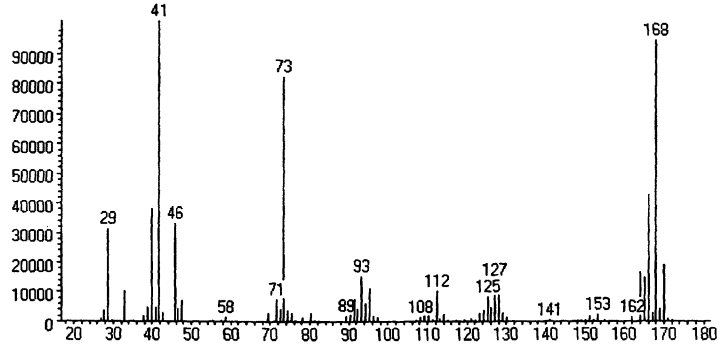

Figure 8. EI/MS spectrum of 2-propenesulfenoselenoic acid methyl ester (MeSeSAll).

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Chapter 19

Quantitation of the In-Mouth Release of Heteroatomic Odorants

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In order to determine the influence of the human physiology on the timing and extent of the release of odorants, the process of swallowing was observed by application of real-time magnetic resonance imaging (MRI). Using this technique the velum-pharyngeal performances were displayed during consumption of liquid and solid foods. Based on the physiological insights obtained, in-mouth investigations on heteroatomic odorants under defined conditions were performed. Using the recently developed SOOM (Spit-Off Odorant Measurement) technique, the in-mouth concentration changes of low concentrations of odorants were followed as a function of time and odorant structure. Thereby, the degree of adsorptive and resorptive effects on the odorants investigated as induced by the mouth mucosa was measured for the first time.

Flavor perception during eating directly depends on the transport of odorants released in mouth during mastication via the retronasal route to the nasal cavity and, thereby, to the olfactory epithelium. Although other factors also influence the amounts of odorants getting into the 23gas phase during mastication, such as shear forces, hydration or dilution of the food material due to salivation, enzymic processes and adsorption or resorption by the mouth mucosa, the transfer to the nasal cavity is the crucial point in flavor perception.

Up to now, the oral cavity was regarded as a kind of "bellows" with an open connection to the airways, "pumping periodically air in and out of the oral cavity" due to chewing movements (*1*). Gas flow as induced by breathing was, therefore,

In Heteroatomic Aroma Compounds; Reineccius, G., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2002.

regarded as the key factor in transporting odorants to the oral cavity and odorant transfer was directly related to the velocity of tidal breathing.

However, when looking at the swallowing process more closely, several physiological factors become evident, which may cause a major hindrance with respect to the free access of odorants into the nasal cavity. A schematic shows the anatomy of the naso-, oro- and hypopharynx in the sagittal plane (Figure 1).

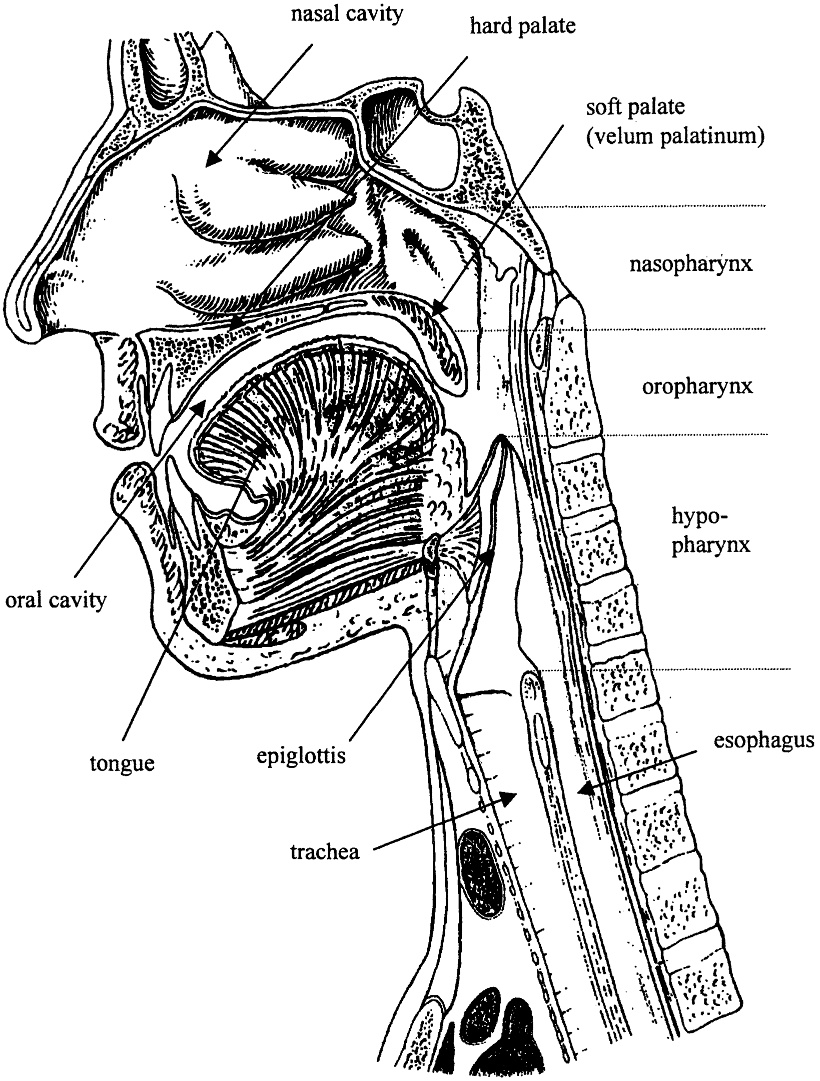
nasal cavity
hard palate
soft palate
(velum palatinum)
nasopharynx
oropharynx
hypo-
pharynx
oral cavity
esophagus
tongue
epiglottis
trachea

Figure 1. Schematic drawing of the anatomy of naso-, oro-, and hypopharynx in the sagittal plane.

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The oropharyngeal deglutition is a complex process, involving 26 muscle-groups acting within the very short period of about 0.7 s. A precise coordination is necessary to avoid aspiration or nasal penetration of the bolus. During the swallowing process, we have to be protected against three main events: prior to swallowing, portions of food material or saliva may not be transferred into the pharynx to protect us from the ingression of food material into the trachea. This is achieved by a seal formed by the soft palate and the pharyngeal base of the tongue retaining the food material in the oral cavity. Second, when the swallowing process is started, the food material and/or saliva has to pass the connection to the nasal cavity while being pressed into the pharynx in order to avoid the material being introduced into the nose. Again, the velum functions as a border, this time being tilted upward and backward to form a closure by approximation with the dorsal pharyngeal wall. Closing the epiglottis activates a third protective mechanism when the food material is actively transported into the hypopharynx. This alignment, again, prevents the food material from entering the trachea. The effectiveness of forming these closures against solid or liquid materials is well known in medicine and has been extensively investigated over the past decade (*2*). However, the effect of these physiological barriers, mainly of the two formed by the velum, on the timing and the extent of the odorant transfer to the nasal cavity has not yet been studied.

To observe the oral and pharyngeal performances, starting from the introduction of the food material into the mouth until the end of the actual swallowing process, we applied a novel technique, called real-time magnetic resonance imaging (MRI). Using MRI as a continuous, movie-like observation of the swallowing process, detailed information on both the drinking of liquid material as well as the eating of solid foods were obtained in this study.

The physiological insights allowed us to study the in-mouth behavior of highly odor-active, heteroatomic odorants (Table I), e.g., their retardation during mastication, as induced by their structural properties, without interference of an odorant transfer to the airways or the nasal cavity.

Table I. Some Important Heteroatomic Odorants in Foods

Odorant	*Odor Quality* [a]	*Odor Threshold in Water [µg/L]* [a]	*Concentration inFood [µg/kg]* [b]
3-isopropyl-2-methoxypyrazine	earthy	0.002	0.1-5 (bell pepper, coffee)
3-isobutyl-2-methoxypyrazine	earthy	0.005	10-100 (bell pepper, coffee)
3-sec-butyl-2-methoxypyrazine	earthy	0.002	0.1-10 (bell pepper, coffee)
Methional	potato	0.2	100-200 (coffee)
Methionol	potato	5	1000-2000 (beer)

a) Data from (*3*).
b) Data from (4,5,6,7).

Experimental Procedures

Materials

Ethylbutanoate 99 %, 3-isobutyl-2-methoxypyrazine, 3-isopropyl-2-methoxypyrazine, 3-sec-butyl-2-methoxypyrazine, methional and methionol were from Aldrich (Steinheim, Germany) and were purified by distillation prior to analysis. Helium-gas, purity 4.6, was from Messer-Griesheim (Krefeld, Germany).

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Real-Time Magnetic Resonance Imaging of Swallowing

Magnetic resonance imaging (MRI) was performed by means of Philips Gyroscan ACS NT 1.5 Tesla scanner, as described elsewhere (*8*). Temporal resolution was 6 images per second, spatial resolution was 1.2 x 2.4 mm with a slice thickness of 15 mm. A preparation of gadolinium-DTPA for oral use (magnevist-enteral®) was diluted with tap water in a ratio of 1 : 4 and served as fluid contrast medium. As the solid contrast medium, cookies coated with magnevist-enteral® were used. Images were acquired in the sagittal and coronal plane during deglutition of fluid and solid contrast medium, respectively.

Detection of Helium in Exhaled Air (*9*)

Helium (25 mL) was taken into the mouth and normal respiration was continued via the nose while keeping the lips closed. Experiment a): During three minutes, the air from the nose was exhaled into the glass device displayed in Figure 2. At the end of the device, the tip of a helium detector (GL 228, GL Sciences, detection limit: 0.01 mL helium/min) was positioned in the middle of the gas stream. Care was taken not to swallow. Experiment b): Same as a), but with swallowing actions. Experiments were carried out in duplicates with a panel of 12 persons (six males, six females).

Quantitation of Flavor Compounds

The following internal standards were synthesized according to the literature cited: [2,2,2-$^2H_3$]-ethyl butanoate (*10*), 3-isopropyl-2-[$^2H_3$]-methoxypyrazine (*11*), 3-isobutyl-2-[$^2H_3$]-methoxypyrazine (*12*), 3-sec-butyl-2-[$^2H_3$]-methoxypyrazine (*13*), 3-([$^2H_3$]-methylthio)-propanal (*14*), 3-([$^2H_3$]-methylthio)-propanol (*7*). The structures of the labeled standards are displayed in Figure 3. Concentrations of the labeled compounds were determined gas chromatographically using methyl octanoate as internal standard according to the method described previously (*15*). The calibration factors for the labeled compounds were obtained as reported in (*16*).

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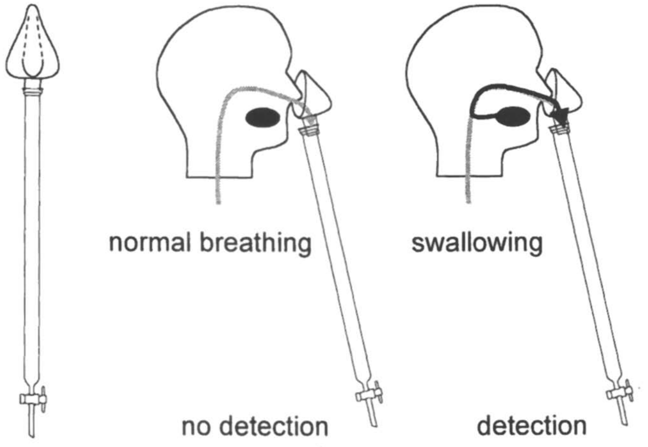

Figure 2. Device for the detection of helium in breath air. Left: breathing without swallowing; right: breathing with swallowing.

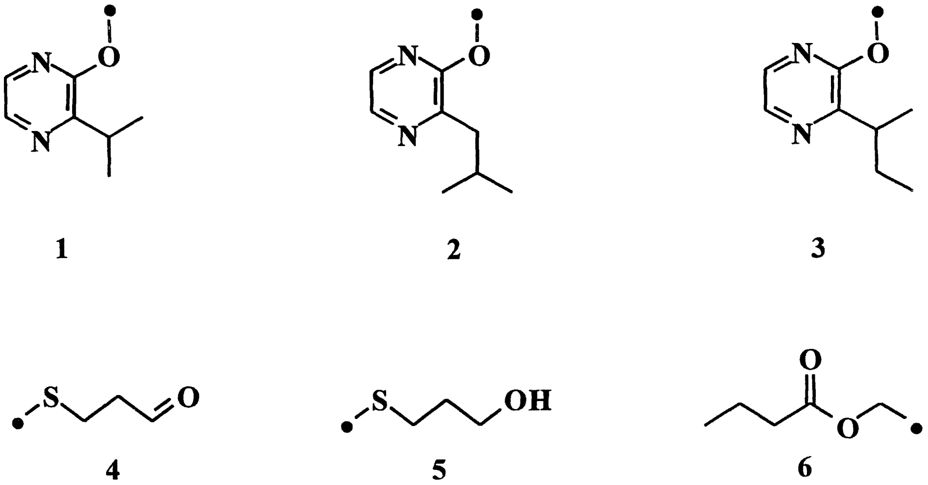

Figure 3. Labeled standards used in the stable isotope dilution assays: (1) 3-Isopropyl-2-[$^2H_3$]-methoxypyrazine, (2) 3-isobutyl-2-[$^2H_3$]-methoxypyrazine, (3) 3-sec-butyl-2-[$^2H_3$]-methoxypyrazine, (4) 3-([$^2H_3$]-methylthio)-propanal, (5) 3-([$^2H_3$]-methylthio)-propanol, (6) [2,2,2-$^2H_3$]-ethyl butanoate.

Determination of Exhaled Ethyl Butanoate by Application of the Exhaled Odorant Measurement- (EXOM) Technique

An aqueous solution (100 mg/L) of ethyl butanoate was freshly prepared using tap water. In each experiment 25 mL of this solution (containing 2.5-mg ethylbutanoate) was taken into the mouth and the sample was rinsed in the mouth during one minute with modest mouth and tongue movements.

In experiment I, the air being exhaled during one minute without swallowing was trapped using the device displayed in Figure 4. During sampling the device was connected to a rotary evaporator with a rubber tube. Application of a vacuum of 10 mbar to the system enabled the test person to exhale into the glass column normally and without any effort.

In experiment II, only the amount of odorants being exhaled during the short period of the swallowing breath were trapped (about 2 seconds, one exhalation). In experiment III the odorants, being exhaled during five minutes following the swallowing breath, were trapped (without trapping of the swallowing breath). Each experiment was performed five times and by four panelists. Quantitations were performed separately for each experiment as described elsewhere (*9*).

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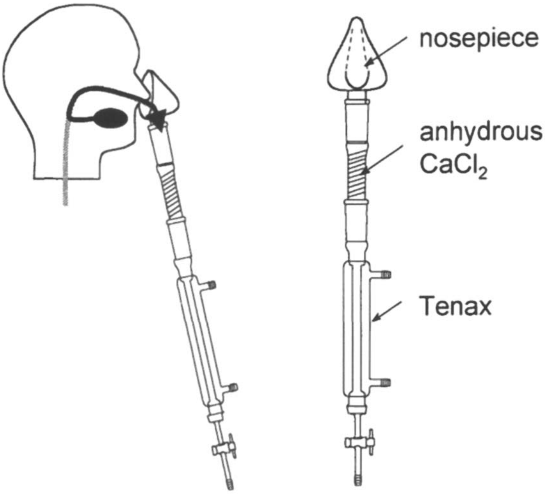

Figure 4. Device used for trapping of odorants from human breath.

Determination of Odorant Losses during Mastication by the Spit-Off Odorant Measurement - (SOOM) Technique

Aqueous solutions (100 µg/L) of each odorant (given in Table I) were freshly prepared. 25 mL of each solution, containing a total amount of 2.5 µg of an odorant, was rinsed in the mouth for a defined period of time (30 sec, 1 min, 2 min, 3 min, 4 min, 5 min, 7 min, and 10 min). After the specified times the solution was spit into a saturated $CaCl_2$-solution, in order to inhibit enzymic reactions, then spiked with the respective stable isotope labeled standard and stirred for equilibration (*17*).

The solution was extracted with diethylether (five times, total volume 150 mL) and the combined organic phases were dried over anhydrous Na_2SO_4. The obtained volatile fraction was subsequently isolated by high vacuum transfer and the aroma extract concentrated by careful distillation (*18*). Quantitation of the volatiles was done by multidimensional gas chromatography (MD-HRGC) with the MS-system ITD-800 running in the CI-mode with methanol as the reagent gas (*19*). At least four replicates were performed.

Results and Discussion

Real Time Magnetic Resonance Imaging (MRI) of the Swallowing Process

Liquids

In Figure 5, the important oral and pharyngeal stages during swallowing are shown in 6 pictures from a sagittal real time MRI series using fluid contrast medium. In Figure 5a the bolus is kept in the oral cavity between the tongue and the hard and soft palate (oropharynx). During rest, there is no connection to the dorsal oropharynx, the nasopharynx and the airways, preventing leaking and aspiration of food. At the beginning of swallowing, an elevation of the tip of the tongue against the hard palate and an adduction of the soft palate to the base of the tongue is observable (Figure 5b). The next step (Figure 5c) consists of an elevation and posterior movement of the soft palate in order to achieve complete velopharyngeal closure to prevent nasal penetration while the bolus is transported to the hypopharynx. Due to the anterior and superior movement of the larynx and closure of the epiglottis, the bolus cannot enter the airways and aspiration is prevented. At the same time (Figure 5d), the contraction of the dorsal wall of the pharynx starts at the height of the first cervical vertebra, resulting in a propulsion of the bolus in the direction of the esophagus. In Figure 5e, the peristaltic wave of the dorsal pharyngeal wall reaches the middle and lower parts of the pharynx and the bolus enters the esophagus with the upper esophageal sphincter still being open. Finally (Figure 5f), the bolus has left the pharynx, the

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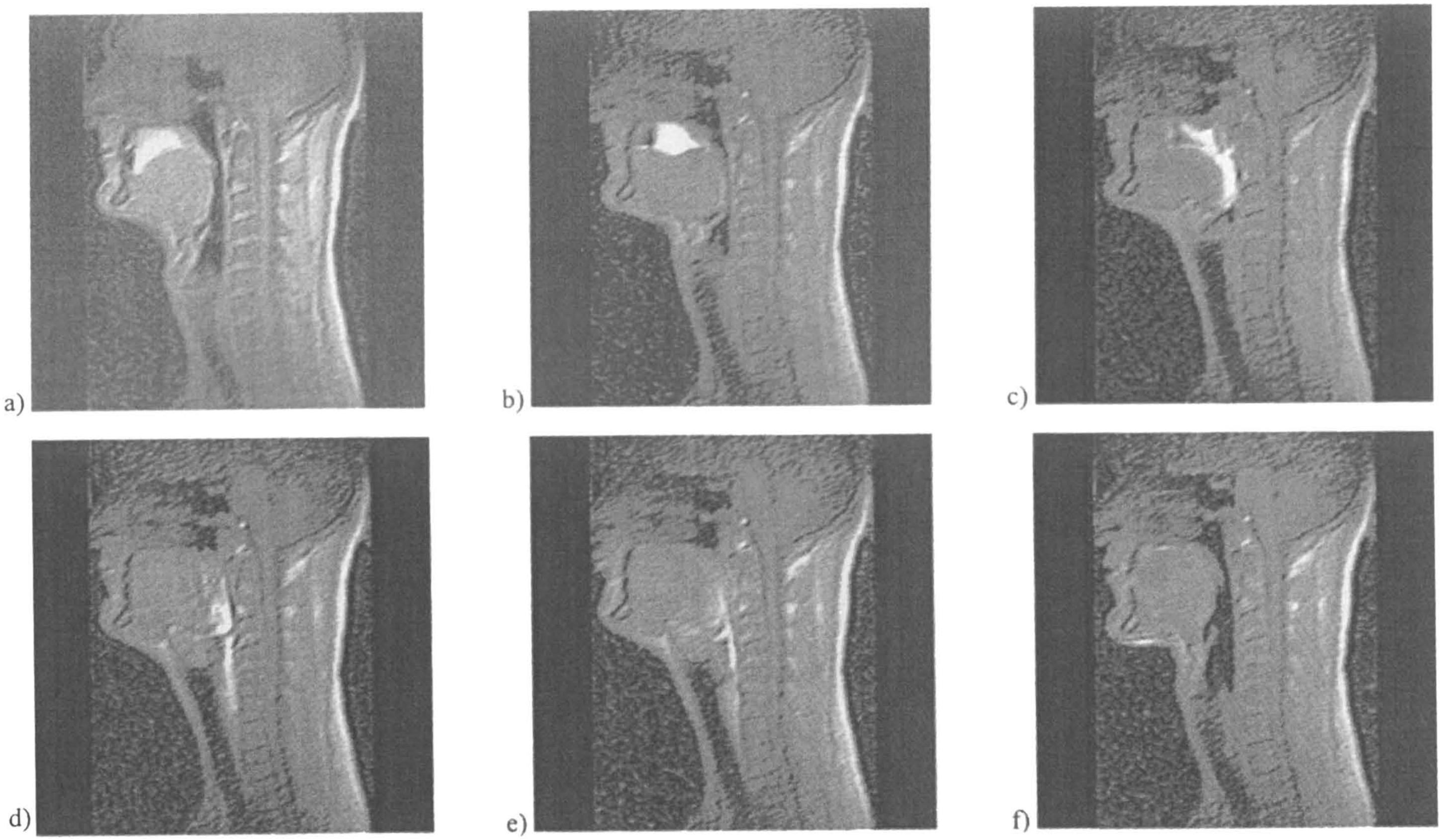

Figure 5. Stages of swallowing of a liquid bolus in a sagittal MRI series. For explanation see text.

upper esophageal sphincter is closed and larynx, epiglottis and velum return to their original position, followed by a short pulse of respiration, the so called "swallow breath" (*20*).

Solid Foods

During mastication, intermittent opening of the connection of the oral cavity to the naso- and dorsal oropharynx was observed, depending on the texture and the amount of the bolus (Figure 6a open oral cavity, Figure 6b closed oral cavity).

In general, the more fluid the texture of the bolus and the higher the amount of the bolus, the more efficient the closure of the oral cavity against the nasopharynx and dorsal oropharynx. Both events were observed alternately (the open and the closed state), even for very dry foods such as cookies.

This observation shows that even during mastication of dry foods, odorants are, at times, hindered to pass continuously into the oropharynx. As a consequence, transfer of the odorants is not only related to the tidal gas flow of breathing but also on the extent and timing of the opening of the velum-tongue border. Furthermore, even when the border is open, the oral cavity still behaves like a cave containing a certain air reservoir. This reservoir is more or less separated and undisturbed from the sinus-like breath-flow occurring in the airways, because the diameter of the velum/tongue passage is not as wide as the oral cavity itself.

Only pumping actions, induced by up- and down-movements of the tongue, could then, in a very varying pattern, lead to a propulsion of air portions into the oropharynx. This, again, directly depends on the effort the eating person spends on mastication and is, therefore, a highly variable process.

However, it can be concluded that the main impulse should still result from single swallowing events. It is very important to note, that human beings swallow considerably often during one eating sequence. In addition to the swallowing of the food material itself (which is usually swallowed in several little portions), there are many small intermittent swallowing events, simply to remove excess amounts of saliva produced during mastication to ease the further mastication and breakdown of the food material. Saliva, acting as a carrier of food odorants, then becomes an important factor in flavor perception.

The most striking example for this phenomenon is chewing bubble gum. When chewing, one will notice an aroma pulse each time when swallowing portions saliva. This effect is also perceivable, when someone is sucking a peppermint candy for curing a blocked nose. With each tiny amount of swallowed saliva one can feel a bit more of relief.

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Studies on the Effectiveness of Velum Closure using Helium

The MRI studies have shown that the velum-tongue border is highly capable of keeping foods more or less completely in the oral cavity until swallowing is initiated. However, the question still remained whether odorants are able to penetrate this closure.

One very simple and effective way to prove this is to use helium as a highly volatile, inert gas which can be detected on-line even in trace amounts. Two experiments were performed: one with just keeping a "bubble" of helium in the mouth (closed lips) and without swallowing, the second with performing swallowing actions.

During the first experiment, when keeping the mouth in the resting position after introduction of helium into the oral cavity, no helium could be detected in the air exhaled from the nose. However, when swallowing actions were performed (by e.g. swallowing the saliva present in the oral cavity), helium could immediately be detected in the exhaled air. Based on the fact that the oral cavity was filled with helium and that the helium detector would indicate helium in the exhaled gas stream even in trace amounts, this experiment clearly demonstrates the effectiveness of the velum-tongue border, to retain even a highly volatile gas such as helium.

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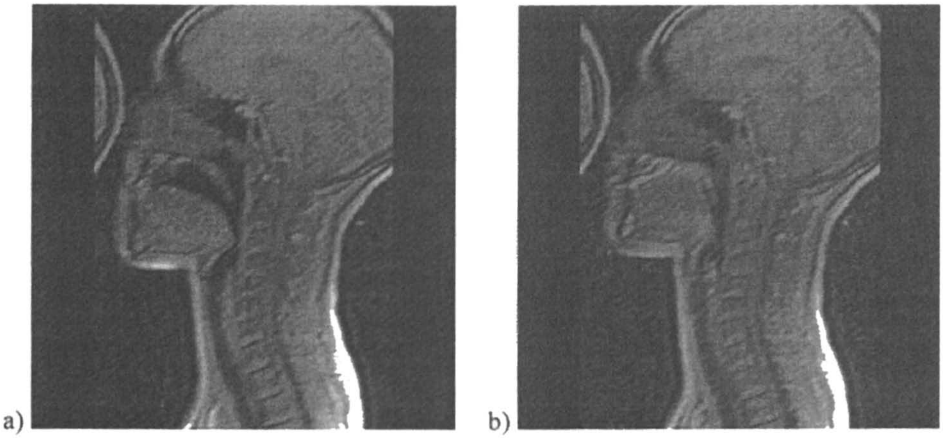

Figure 6. The process of mastication of a solid bolus in a sagittal MRI series. For explanation see text.

Determination of Exhaled Ethyl Butanoate during the Swallowing Process Using the EXOM-Technique

The determination of exhaled ethyl butanoate during different intervals when swallowing an aqueous solution (2.5 mg ethyl butanoate/25 mL water) clearly showed that the main aroma "pulse" is indeed related to the moment of swallowing. Before swallowing, only trace amounts of ethyl butanoate (in total 20 ng, Figure 7) were detected over a period of 5 min, when the model solution was kept in the mouth with closed lips and without swallowing (performing modest mouth and tongue movements).

Immediately after swallowing, when the so-called "swallow-breath" was trapped, by far the highest amounts of ethyl butanoate (5 µg, 92.3 % of total exhaled ethyl butanoate) were determined. During the next 4 min, following the "swallow breath", only 400 ng of ethyl butanoate were exhaled. This result confirmed that, indeed, the main aroma pulse was related to the "swallow breath".

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Determination of Losses of Heteroatomic Odorants in the Mouth by Application of the SOOM-Technique

3-Alkyl-2-methoxypyrazines

Our results allowed us to conclude that the oral cavity is an efficiently closed system for liquids, unless any swallowing or deliberate pumping actions of the oral cavity (as e.g. performed by winetasters when evaluating wine) takes place.

Taking advantage of this "static" state, the retardation of 3-alkyl-2-methoxypyrazines was studied. Eight experiments were performed to follow the effect of different time interval exposures on the odorants behavior, starting from 30 seconds up to ten minutes. Although the in-mouth dwell-time of foods, in general, is very short, we considered that adsorptive effects would become more evident over a longer period of time. These differences are possibly related to the persistency of food odorants in the oral cavity, e.g., in the so-called aftertaste.

The data show (Figure 8) that 3-isopropyl-2-methoxypyrazine, 3-isobutyl-2-methoxypyrazine and 3-sec-butyl-2-methoxypyrazine behave in a very similar pattern, all being continuously reduced in the spit-off solution down to about 65 to 70% after a period of 10 min (as related to the original concentration).

It is important to note that the lips were kept closed during each single experiment and swallowing was avoided. According to the experiments discussed above, losses of odorants, e.g., by transfer into the airways, can be excluded.

When considering the air-water partition coefficients of the three methoxypyrazines, it becomes evident that the observed losses can not only be

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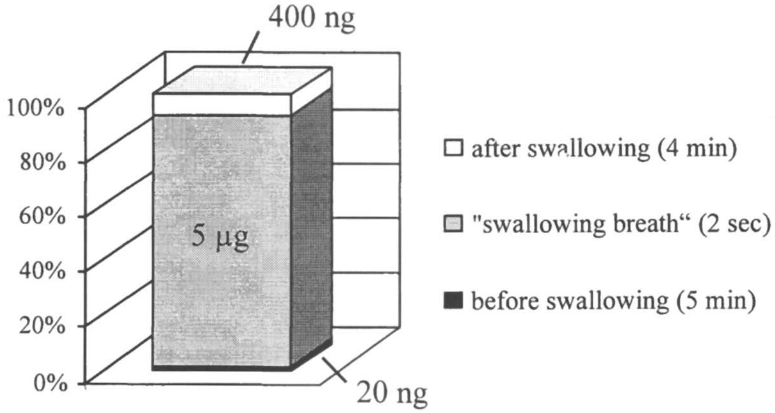

Figure 7. Amounts of ethyl butanoate exhaled via the nose before, during and after swallowing of an aqueous model solution containing 2.5 mg/25 mL water.

induced by the partitioning of the pyrazines into the air present in the oral cavity. For example, even when it is assumed that the same volume of air and water phase (25 mL each) would be present in the mouth (which is, in fact, an unrealistical high volume of air), one would only find a maximum reduction of approximately 0.2 % of the methoxypyrazines in the water phase (at equilibrium). This is based on the partition coefficient of 3-isobutyl-2-methoxypyrazine of 2x10^{-3} at 25°C (according to (*21*)). Therefore, the main portion of the odorants lost is undoubtedly caused by adsorptive or resorptive effects of the mouth mucosa.

When looking more closely at the decrease of the methoxypyrazines, one finds a slight differentiation of the two butyl methoxypyrazines in comparison to the shorter-chain and more polar isopropylmethoxypyrazine, which was a bit less reduced in the spit-off solution (by about 5 % after ten minutes as related to the initial concentration). The observation that within homologous series the in-mouth decrease of odorants is related to their polarity agrees with our previous findings on homologous aldehydes and esters (*17*). However, it was not yet proved whether additional factors, e.g. saliva, may have an influence.

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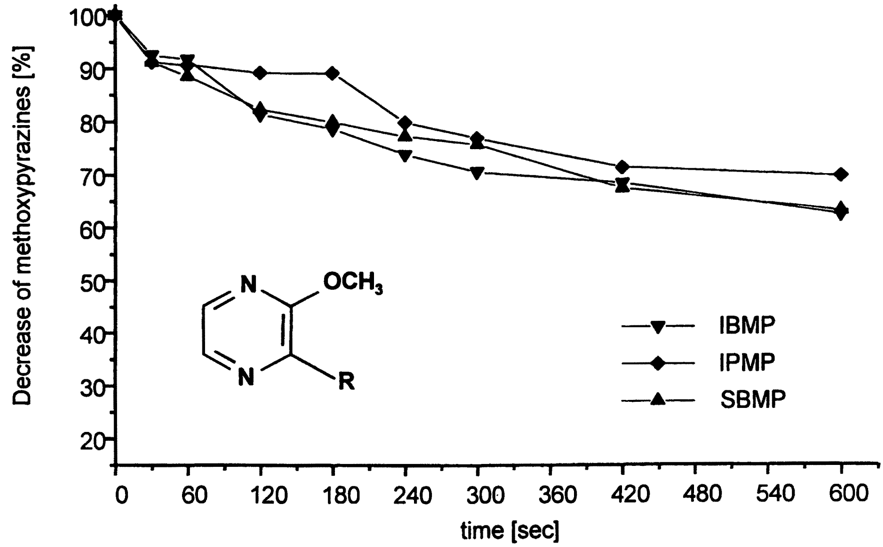

Figure 8. Losses of 3-isobutyl-2-methoxypyrazine (IBMP), 3-isopropyl-2-methoxypyrazine (IPMP) and 3-sec-butyl-2-methoxypyrazine (SBMP) in spit-off aqueous solutions after different times of rinsing in mouth. Values are the means of four replicates.

Ethyl butanoate, methional, methionol

In further experiments, the in-mouth losses of odorants belonging to different substance groups were measured. Methional and ethyl butanoate were significantly reduced during rinsing in the mouth. Only about 25 % of the original amounts were present in the spit-off solutions after 10 min (Figure 9), while methionol was recovered in considerably higher amounts.

Again, the decreases determined for ethyl butanoate, methionol and methional can not be solely explained by partitioning from the water phase into the gasphase. For example, for ethyl butanoate, an approximate maximum reduction of about 3 % at equilibrium would be calculated, assuming same volumes of air and water (partition coefficient of ethyl butanoate: 26.2×10^{-3} at 25°C (*22*)). This is, however, a considerably lower amount than we observed in our experiments.

However, it is also evident that these losses can not be due to differences in the polarity of an odorant. Decreases do not go in parallel with the reduction as we would expect due to their partition coefficients, e.g. we would expect ethyl butanoate (log P of ethyl butanoate: 1.77 ± 0.21 (calculated by means of ACD/logP DB, Science Serve, Pegnitz, Germany)) to be even less reduced than the 3-alkyl-2-methoxypyrazines (calculated log P of 3-isopropyl-2-methoxypyrazine: 2.09 ± 0.27; 3-isobutyl-2-methoxypyrazine: 2.62 ± 0.27; 3-sec-butyl-2-methoxypyrazine: 2.62 ± 0.27) or at least in a more similar pattern.

Therefore, other factors, which are not yet clarified, have to be regarded such as, enzymic processes or interactions with the constituents of the saliva, (e.g. the mucins). Besides adsorption at the mouth mucosa, the resorption of the odorants by the oral mucosa, as described in medicine for several physiological active substances (*23, 24*), can not be excluded. Further investigations have to clarify the influence of these factors.

Conclusions

Observation of eating and drinking processes by use of real-time MRI showed that the oral cavity is a temporarily closed system, with closure strength and frequency directly depending on the texture and amount of food material being masticated. This closure is even effective in retaining helium gas within the oral cavity. Swallowing of aqueous solutions of ethyl butanoate and trapping of the exhaled odorant amounts at distinct time intervals showed an aroma pulse to be directly related to the moment of swallowing. This static aspect of the oral cavity is a natural physical property that may be used to directly measure the influences of saliva, adsorption or even resorption of odorants by mouth mucosa, and flavor release at non-equilibrium conditions avoiding the need for the use of model mouth systems.

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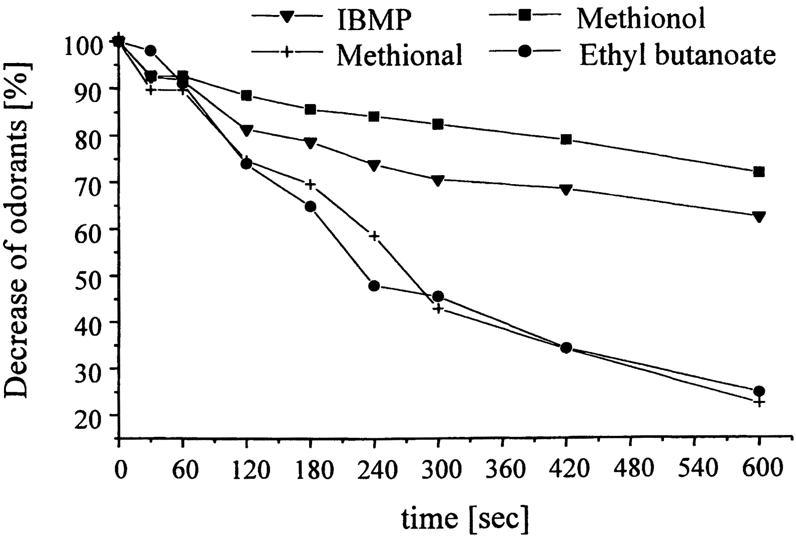

Figure 9. Losses of 3-isobutyl-2-methoxypyrazine (IBMP), methional, methionol and ethyl butanoate in spit-off aqueous solutions after different times of rinsing in mouth. Values are the means of four replicates.

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Chapter 20

Use of Omission Tests to Evaluate Taste-Active Compounds in Food: Application to Cheese and Tomato

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Use of omission tests have allowed us to overcome masking, additive, and synergistic effects that are problematic limitations in studing the effects of tast-active compounds in foods. From the results obtained from physico-chemical analyses of water-soluble food extracts matching synthetic taste mixtures were created. The taste equality of the synthetic mixtures to that of the food extracts were verified by sensory analysis. Omission of one or several components (e.g. sugars, salts, peptides, acids and minerals) in the synthetic mixtures determined the direct and indirect contributions of each compound to taste. Examples of omission tests with goat and camembert cheese, and tomato juice extracts are presented.

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Introduction

Taste is an important part of the organoleptic quality and acceptance of food. To date, the taste of a food has been studied by global description and monadic profiles of basic taste constituents. The classical method to evaluate taste-active compounds is to sub-fractionate the water-soluble fraction of a food by ultrafiltration and gel permeation chromatography, which are the only techniques that produce edible fractions. However, this method is limited because of the low efficiency to separate taste-active compounds. Also, the sensory evaluation of purified fractions or isolated compounds does not take into account interactions between components such as masking, additive, or synergistic effects.

The molecules responsible for taste, often recovered in the water-soluble fraction of cheeses *(1,2)* or in the juice of fruits and vegetables *(3)*, are mainly small molecules such as sugars, organic acids, mineral salts, amino acids, mononucleotides, peptides. The concentrations vary according to the nature and origin of a food. Even if the taste properties of single compounds are demonstrated, the individual effect remains unclear when incorporated in a complex mixture such as a food extract or a juice. It is known that masking and enhancing effects between taste-active compounds may occur even in some very simple synthetic mixtures *(4)*.

Most of the previous studies on crude extracts of food are based on the simplification of the taste fraction by separation techniques to evaluate the taste-active compounds. With such methods, the mixture context is not or only partially taken into account.

In the past twenty years, several studies were carried out on the taste-active components of cheese, with a special interest in small peptides *(1,5,6)*. Chromatographic methods and especially gel permeation were primarily used to separate the taste components *(2,7)*. In contrast with other methods, these procedures allow for edible eluents as pure water is used as the solvent. However, these studies have produced very limited results and have many disadvantages. The chromatographic resolution is generally very poor because water is not an efficient solvent to this kind of separation. Moreover, this technique does not permit easy preparation of large quantities of fractions which are necessary for sensory evaluation experiments.

The approach generally used to evaluate the taste-active compounds in a complex mixture is the comparison of the concentration of each component quantified in the crude extract or in the fractions with the threshold value measured in water or in another simple medium *(2,8,9)*. The main criticisms of this approach are that the evaluation of threshold values does not take into account the mixture context of complex solutions and since a total assessment of the evaluated extract or fraction is not made, the relation made between composition and sensory data is rather uncertain.

Another strategy consists in studying statistical correlations existing between physio-chemical and sensory data. However, the non causative nature of the mathematical linkage between both types of data diminishes the relationship between the conclusions to the original hypotheses.

Omission tests have already been used to characterize taste-active compounds in foods such as chicken meat extract *(10)*, stewed beef juice *(11)*, and goat cheese extract *(12)*. Omission tests consist in the taste evaluation of solutions where one or several components are omitted. In principle these omission tests seem to be a good way for studying the contribution of compounds placed in a complex mixture context. However, for these studies, no total assessment of the components was made and the selection of the quantified compounds was realized only on the basis of qualitative previous knowledge of the taste active molecules.

The objective of this paper was to evaluate taste active compounds of the WSE of food using omission tests. The relative taste impact of individual water-soluble components of goat cheese is qualitatively and quantitatively evaluated. A camembert cheese is also studied, mainly focusing on its intense bitterness to better understand the nature of molecules involved in this taste. The third application of omission tests concerns another model, tomato juice, which contains different taste-acive compounds and is characterized by other taste properties.

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Materials and Methods

Food Products

A semi-hard goat cheese called "Bouton de culotte" was bought at the “Lycée Agricole of Davayé” (Mâcon, France) after two and half weeks of ripening. Experimental French camembert cheeses were manufactured at the "Lycée Agricole de Saint Lô Thére" (Saint Lô, France) after 31 days of ripening. Details on the cheese-making process and preparation are given in references *13* and *14* for goat and camembert cheese, respectively.

The variety of tomato studied was selected among 22 other varieties on the basis of taste quality and intensity. The gustatory profile of the selected tomato juice presented high intensities of the main basic tastes: sourness, sweetness, saltiness and the presence of the secondary tastes : umami, bitterness and astringency (to be published).

Chemicals

The following food grade compounds were purchased from commercial suppliers: L-aminoacids (Rexim, Courbevoie, France); lactose, glucose, fructose, lactic acid, citric acid, malic acid, mineral salts (Merck, Darmstadt, Germany); potassium aluminum sulfate, adenosine monophosphate (AMP), uridine monophosphate (UMP) (Sigma, Saint Quentin Fallavier, France); and L-monosodium glutamate and capsaicin (Fluka, Saint Quentin Fallavier, France). Pure water was obtained from a milliQ system® (Millipore, Bedford, MA).

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Extraction Procedures

Goat and Camembert Cheeses

One part of cheese was dispersed in two parts of pure water and homogenized *(13,14)*. The suspension was centrifuged at 4000x *g* for 30 min at 4°C. The supernatant was collected and ultracentrifuged at 100,000x *g* for 30 min at 4°C. The obtained supernatant constituted the crude water-soluble extract (WSE). This crude extract was submitted to two successive tangential ultrafiltrations with a molecular weight cut-off of 10,000 Da and 1000 Da, respectively, and then to a tangential nanofiltration (500 Da). This was performed mainly to collect the different classes of peptides present in the cheeses. For camembert cheese, the ultracentrifugation was substituted by a tangential microfiltration step (0.05 µm cut-off membrane)

Tomatoes

The tomatoes were crushed, homogenized, immediately frozen in liquid nitrogen and the kept frozen until use. After thawing, the slurry was centrifuged at 20,000x *g* for 30 min at 4°C. The crude supernatant was filtered using cotton gauze to obtain a tomato juice. A part of the juice was ultrafiltered by frontal filtration on a membrane with a molecular weight cut-off of 1000 Da. The juice was stored at -20°C until use.

Physio-Chemical Analysis

All the measurements were made in triplicate. The pH values were measured using a pH meter equipped with a glass electrode. For the cheese studies, a drying oven (106°C) was used to determine the dry matter content. For tomato juice, the dry matter content was determined using a centrifugal concentrator (60°C, 53 Pa) The concentrations of the minerals: sodium, potassium, calcium and magnesium were determined by atomic absorption. Inorganic phosphorus and chloride ions were quantified using diagnostic kits (Sigma). Lactose, glucose and fructose were quantified using Boehringer-Mannheim kits (Meylan, France). Nucleotides, citric and malic acids were determined by HPLC methods *(15)*. The free and total amino acids were determined by amino acid analyses with ninhydrin as post column derivatization reagent, before and after acid hydrolysis (6 N HCl, 110°C, 24 h).

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HPLC-MS Analyses of Extracts

The HPLC peptide fractions were prepared from the retentate obtained from 1000 Da ultrafiltration (RUF1) and 500 Da nanofiltration (RNF) *(14)*. The conditions for HPLC-MS analysis were as follow. Twenty microliters were injected on an ODS nucleosil 50-5 RP18ec column (200 x 2.1 mm, 5 µm; Macherey Nagel, Hoerdt, France) kept at 40°C. The flow-rate was 0.25 ml/min. Separations were made using a binary gradient. The eluents were: A) 0.106% TFA in pure water, and B) 0.1 % TFA in 80 % acetonitrile. The elution was made with pure eluent A for 5 min then the gradient was formed by linearly increasing the concentration of eluent B to reach 44% B at 45 min, 88% B from 55 min to 57 min then 100% A from 59 min to 65 min. The first peak contained mainly mineral salts and was discarded. The remaining eluent was introduced in the mass spectrometer (Sciex API-III; Thornill, Canada) equipped with an electrospray ionization source at a flow-rate around 30 µl/min (split : 1/8).

Sensory Analyses

Panel Training

The panels were composed of 16, 17 and 10 panelists for the goat, camembert and tomato studies, respectively. They were trained to recognize and quantify each basic taste, astringency and sharpness in single, binary and ternary solutions, and were familiarized with the test procedures.

General Protocol

The evaluations were conducted under red light in an air-conditioned room (21°C). To suppress olfactive sensations, panelists' nostrils were pinched. At each session, products were presented in a monadic way according to an experimental design to balance reporting and positional effects.

For each sensation studied, an appropriate reference solution was chosen. The quantification of each attribute intensity was evaluated in comparison with the perceived intensity of the corresponding reference solution. For the goat cheese study, the reference solutions were: lactic acid 1.38 g/L (sour), L-leucine 8 g/L (bitter), sodium chloride 4.5 g/L (salty), L-monosodium glutamate 0.6 g/L (umami), D-lactose 23.75 g/L (sweet), potassium alum 0.33 g/L (astringent), capsaicin 0.15 mg/L (sharp). For the camembert cheese study, the reference solutions were the same as for goat cheese apart from L-leucine 10 g/L (bitter), sodium chloride 3 g/L (salty). For tomato study, the references solutions were : citric acid 1 g/l (sour), L-leucine 8 g/L (bitter), sodium chloride 2 g/L (salty), glucose 12 g/L + fructose 12 g/L (sweet) monosodium glutamate 0.6 g/L (umami), potassium alum 0.6 g/L (astringent), capsaisin 0.1 mg/L (sharp).

The panelists were asked to taste the reference solutions and to memorize the respective intensity corresponding to 50% of their respective assessment scale. Then, the panelists were instructed to taste each product and to score the intensity of each gustatory attribute on an unstructured scale anchored from "no sensation" to "strong". The middle of the scale corresponded to the intensity of the corresponding reference solution. During the tests, the assessors could taste some reference solutions to recall their gustatory sensation and intensity. Between samples, the panelists cleaned their pallet with bread and then rinsed their mouth with water.

Model Water-Soluble Extracts (MWSE)

The MWSE were created in physio-chemical accordance with the crude WSE, using commercial chemical compounds and ultrafiltration and nanofiltration retentates as the source of peptides.

Omission Testing

Sensory evaluation by omission tests were carried out to evaluate the modification of the intensity of each basic taste including additive or synergistic effects. The taste profile of a model mixture (MWSE) containing all the components identified and quantified in the WSE of the food was compared to

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the same model mixture (MWSE) where one or several components were omitted. This lead to the evaluation of the impact of the omitted component on each of the considered descriptors. The volume of solutions tasted was 4.5 ml for the cheese studies and 6 ml for tomato juice study. Each solution was presented twice in the same session.

Statistical Analysis

The data were processed with the SAS statistical package version 6.11, 4th edition (SAS Institute, Inc., Cary, NC). ANOVA analyses were performed at level $\alpha = 0.05$, according to the model attribute = product + subject + product x subject, with subject as the random effect. Means were compared with the Newman-Keuls multiple comparison test (Student *t*-test). To quantify the relative impact of each compound on each attribute, stepwise multiple linear regressions were performed with procedure REG with the stepwise option to select the variables.

Results and Discussion

Goat Cheese

The values of the components contained in the WSE are presented in Table I. This WSE contained mainly mineral salts (39.7%), lactose (30.6%), peptides (17.5%), lactic acid (8.5%) and a small quantity of free amino-acids and lipids. The total amount of the compounds identified and quantified in the WSE represented around 95% of the dry matter quantity. These results allowed for a model water soluble extract (MWSE) to be constructed. In this MWSE, all components were present at the same concentration as in the WSE.

From a sensory point of view, no significant difference was pointed out between the WSE and MWSE (Figure 1). Moreover, a triangular test made on the overall taste of these two fractions determined no difference between the two solutions *(13)*.

As this model solution appeared representative of the WSE, omission tests were performed to determine qualitatively and quantitatively the relative gustatory impact of each of the water soluble components in WSE of the cheese. As sourness, bitterness and saltiness were the three main tastes perceived in the WSE, the following results reported for the goat cheese study concerns only these three gustatory attributes.

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No significant difference was found between the MWSE with and without peptide *(16)*. This means that the total amount of the peptides present in the WSE did not have any significant direct impact on the taste profile. To control the possible indirect effects of peptides on other taste active compounds, omissions of MWSE components were made in the presence and in the absence of peptides. Only mineral salt omission had an impact on the taste profile of the extract but independently of the presence of peptides *(16)*. So, as the total amount of peptides did not have any direct or indirect effect on taste, peptides were always omitted in subsequent tests.

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Table I. Composition of Goat and Camembert WSE and Tomato Juice

	Goat Cheese WSE (g/kg Cheese)	*Camembert Cheese WSE (g/kg Cheese)*	*Tomato Juice (g/kg Tomato)*
Dry matter	45.59	37.84	49.77
Na	3.69	4.98	0.01
K	1.81	0.65	2.85
Ca	1.23	0.32	0.007
Mg	0.14	0.03	0.067
Cl	7.89	10.19	0.19
Phosphates	3.34	0.64	0.54
Total minerals	18.1	16.63	3.75
Lactic acid	3.86	3.50	nm
Citric acid	nd	nd	3.75
Malic acid	nd	nd	3.19
Galactose	nd	0.16	nm
Lactose	13.95	nd	nm
Glucose	nm	nm	15.81
Fructose	nm	nm	16.06
AMP	nd	nd	0.04
UMP	nd	nd	0.02
Amino acids	0.67	6.49	5.04
Peptides	8.03	14.17	0.03
Total lipids	0.77	nm	nm
Total assessment	45.38	41.50	47.60

The standard deviation for each measure is lower than 5 %. nm : not measured. nd : not detected.

Concerning the omission of the other compounds, the results are presented in Table II. No significant deviation from the model solution was observed for lactose and amino acids omissions. In contrast, the omissions of minerals and lactic acid led to a significant mean taste deviation. Mineral salts were responsible for the salty note and for a partial decrease of sourness. Lactic acid increased the intensity of sourness.

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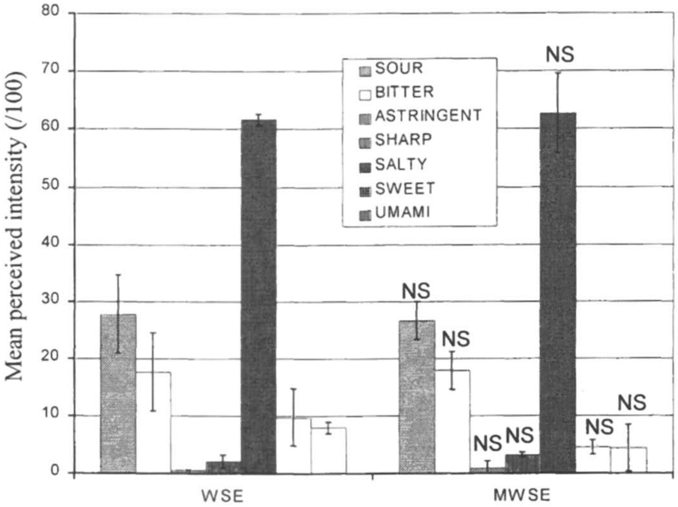

Figure 1. Gustatory profile of both WSE and MWSE. The bars represent the mean gustatory intensity of the considered attribute for three replications. Standard deviation is drawn at the top of each bar. NS indicates that there is no significant difference between WSE and MWSE for the considered attribute. (Reproduced from reference 13. Copyright 2000 American Chemical Society)

In order to specify the relative contribution of the different mineral salts: sodium, potassium, calcium and magnesium chloride and phosphate, individual omissions were performed (Table III). Concerning saltiness, all the minerals contributed positively to this note. Though sodium chloride appeared to have the biggest impact, the other salts were necessary to explain the whole effect of saltiness. Concerning sourness, phosphates had a decreasing effect whereas sodium chloride had an enhancing influence. The effect of phosphates can be related to the role on pH. For sodium chloride, its contribution to sourness was due to the enhancing effect on compounds acting on the pH level, phosphate and lactic acid.

Table II. Omissions of Lactose, Amino Acids, Lactic Acid and Mineral Salts Performed on the MWSE

Omitted	*Mean Taste Intensity Deviations from the MWSE (/100)*		
Components	*Saltiness*	*Bitterness*	*Sourness*
Minerals	-64.0*	-14.5	34.9*
Amino acids	-1.0	4.4	0.3
Lactose	1.9	-1.0	-3.9
Lactic acid	-6.5	-15.6	-16.7*

For each attribute, means affected with * are significantly different from MWSE at the level of 5% according to Student t-tests.

Table III. Individual Omissions of Mineral Salts Performed on the MWSE

Omitted	*Mean Taste Intensity Deviations from the MWSE (/100)*		
Components	*Saltiness*	*Bitterness*	*Sourness*
Minerals	-64.0*	-14.5*	34.9*
NaCl/PO_4	-49.0*	31.8	11.7*
NaCl	-41.0*	12.2	-11.0*
KCl	-12.5*	-3.6	-2.4
$CaCl_2$/$MgCl_2$	-11.7*	-20.9*	-7.8
PO_4	-13.1*	-1.4	26.3*

For each attribute, means affected with * are significantly different from MWSE at the level of 5 % according to Student tests.

The result of the simultaneous omission of phosphates and sodium chloride confirmed this hypothesis showing that the significant increase of sourness due to phosphate omission was partially compensated by the omission of sodium. For the bitter taste, the omission of both calcium and magnesium chlorides led to a significant decrease in bitterness. So, these two salts might be at least partially implicated in the bitterness of the WSE. The bitter taste of these compounds was previously mentioned by Tordoff *(17)*. However, the bitterness intensity of a simple solution of calcium chloride and magnesium chloride in the same concentrations as in the MWSE was around 80/100 while the level of bitterness was only 30/100 for the MWSE. This last result showed that some other constituents present in the MWSE might act as partially masking agents to the bitter taste. The simultaneous omission of phosphate and sodium chloride

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significantly increased the perceived bitterness whereas the phosphate omission alone had no effect. The quantity of phosphate being the same in the two cases, the increase of the perceived bitterness was probably due to the larger quantity of sodium omitted. This masking effect of sodium chloride on bitterness has already been mentioned by other authors *(18,19)*.

In order to quantify the contribution of each compound in the WSE, stepwise multiple linear regressions were processed on the omission tests data. Concerning saltiness, 97.4% of this taste was explained. Sodium, calcium, magnesium, potassium chlorides and phosphates appeared as being responsible for 75, 13.8, 5.8 and 4.4% of the salty taste, respectively. The impact of these components were positively correlated with the intensity of saltiness, confirming the additive contributions described above.

Concerning bitterness, it was mainly due to the opposing effects between calcium and magnesium chloride which contributed positively for 41.3% and both sodium chloride and phosphate chloride which contributed negatively, for 29.2 % and 9.4 %, respectively. As for saltiness the impact of sodium phosphates might mainly be due to sodium ions. It is noticeable that mineral compounds explained 84.3% of the total bitterness of the WSE of goat cheese.

In the case of goat cheese, mineral salts and lactic acid were the main taste-active compounds, whereas lipids, the volatile fraction, lactose, amino acids and peptides did not have any significant impact. Among these taste-active compounds, the following complex interactions were observed: additive effects of salts on saltiness, an enhancing effect of sodium chloride on sourness due to the balance between phosphate and lactate species, and the masking effect of sodium chloride on bitterness mainly due to calcium and magnesium.

Camembert Cheese

Since the bitter taste of cheeses is often related to proteolysis and, more importantly, to the presence of bitter peptides, we decided to focus our study on the taste-active compounds of a typically bitter cheese.

The typically bitter camembert cheese was treated as described previously for the goat cheese. The total assessment of the components contained in the WSE accounted for about 95% compared to the dry matter quantity of the extract *(14)*. The WSE contained a high ratio of peptides (about 30%). A model solution (MWSE) was elaborated in physio-chemical accordance with this total assessment and the gustatory properties were compared to the WSE. In the same way as for the goat cheese study, no significant difference between the gustatory

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profile of the WSE and MWSE was observed, thus confirming the chemical and sensory validity of the MWSE.

This latter study was focused on the three main and most intense tastes of the WSE: saltiness, bitterness and sourness. The omission tests were performed on the main families of components (Table IV). The omission of amino acids, potassium, magnesium and calcium chlorides did not show any effect on the MWSE gustatory profile. In contrast, a significant and drastic decrease of the salty and sour taste intensities was observed for the omission of sodium chloride. The omission of peptides led to a significant decrease in bitterness, showing the contribution of these compounds to this taste.

Table IV. Omissions of Peptides, Amino Acids, Peptides/Amino Acids, NaCl, KCl, $CaCl_2$ / $MgCl_2$ from the MWSE of Camembert Cheese

Omissions	*Mean Taste Intensity (/100)*		
	Sourness	*Bitterness*	*Saltiness*
Model WSE	17.79^a	36.5^a	79.25^a
- Peptides	15.14^a	15.46^b	78.54^a
- Amino acids	14.39^a	40^a	72.36^a
- Peptides / amino acids	18.81^a	13.92^b	69.77^a
- NaCl	3.71^b	34.61^a	7.68^b
- KCl	16.68^a	33.68^a	74.57^a
- $CaCl_2$ / $MgCl_2$	11.89^a	31.21^a	73.39^a

For each attribute, the means with the same letter (a-b) are not significantly different at the level of 5 % according to Newman-Keuls tests.

To further ascertain the role of peptides on the bitterness of the WSE, omission tests were performed with each peptide fraction obtained by the successive filtrations (Table V). As it was previously shown for the global peptide fraction, we did not notice any significant effect for each of the three fractions on the sourness and saltiness of the extract. For bitterness, we observed that the omission of peptides contained in the retentate obtained by 1000 Da ultrafiltration and 500 Da nanofiltration led to a significant decrease in bitterness while no effect was observed for the retentate obtained by ultrafiltration cutoff 10,000 Da. This finding suggests that the bitter peptides had molecular weights less than 10,000 Da and were contained in the 1000 Da ultrafiltration and 500 Da nanofiltration retentates.

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The analysis of these two bitter peptide fractions by HPLC-MS showed that a high proportion of these peptides had molecular weights lower than 1000 Da, and none exceeding 3000 Da *(14)*. So, the bitterness of this Camembert cheese extract seemed mainly due to small peptides.

Table V. Omissions of Peptide Fractions from the MWSE of Camembert Cheese

Omissions	*Mean Taste Intensity (/100)*		
	Sourness	*Bitterness*	*Saltiness*
Model WSE	16.68	35.04^{a}	76.11
-Total peptides	15.54	13.32^{c}	72.25
- RNF	15.36	18.18^{c}	72.36
- RUF1	14.54	23.29^{bc}	68.5
- RUF10	15.61	30.93^{ab}	74.29

For each attribute, the means with the same letter (a-c) are not significantly different at the level of 5 % according to Newman-Keuls tests. RNF, RUF1 and RUF10 are the retentate obtained respectively by ultrafiltration of the WSE on 10 000 Da, 1000 Da cut-off membranes and nanofiltration on 500 Da cut-off membrane.

In the case of the ripened camembert cheese, mineral salts, lactic acid and peptides were the main taste-active compounds. As for goat cheese, complex interactions were observed between them. However, in contrast with goat cheese, which was less ripened than camembert, bitterness was mainly due to small and medium size peptides resulting from the proteolysis, without any significant contribution of divalent cations.

Tomato Juice

Tomato juice constituted another model with a different system of taste components that we studied in the same way as cheeses. A total assessment of the crude tomato juice was made by physicochemical methods. Most of the components present in the dry matter (97%) of the juice were identified, and a model juice was then created from this assessment, with chemical compounds. The taste profiles of the model and of the tomato juice permeate obtained from ultrafiltration on a 1000 Da cutoff membrane were not significantly different (5 % level) for saltiness, sweetness, sourness, bitterness, umami and astringency. For sharpness, a significant difference was observed between the two solutions

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but the intensities were low (< 20/100). These results indicate that compounds responsible for sharpness in the crude juice fraction were not identified.

Omission tests were made from the model mixture, with omissions of amino acids, nucleotides, glucose, fructose, citric and malic acids, and minerals. The results obtained from the omission tests performed on sugars and acids are presented in Table VI for the three main taste characteristics: sourness, saltiness and sweetness. The omission of all the sugars led to an almost total disappearance of sweetness and a significant increase of sourness and saltiness which were partly masked by the sugars. The omission of the acids led to a total disappearance of sourness and saltiness. Considering the pH of the tomato and the pKa of the organic acids, the acid form of organic acids was probably responsible for the total sourness and the dissociated form was probably mainly implied in saltiness. The single omission of one acid or one sugar demonstrated the same trend, only less so, as was just discussed for all of the acids and sugars..

Omissions tests were made also with amino acids, nucleotides and mineral salts. No omission test was able to show a particular effect of mineral salts, amino acids or nucleotides on saltiness, sourness or sweetness. But, the omission of mineral salts did not include potassium, which is mainly associated in malic and citric salts and likely contributed to the salty taste of the model mixture.

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Table VI. Omissions of Organic Acids, Sugars, Amino Acids, Mononucleotides and Mineral Salts from the Model Tomato Juice

Omissions	*Mean Taste Intensity (/100)*		
	Sourness	*Saltiness*	*Sweetness*
Model juice	45.8^{a}	25.3^{a}	42.6^{ab}
- Acids	1^{b}	3.2^{b}	64.5^{a}
- Sugars	66.2^{c}	52.8^{c}	8.3^{c}
- Amino acids	34.7^{a}	17.5^{ab}	43.5^{b}
- AMP/UMP	46.7^{a}	29.7^{a}	34.7^{b}
- Minerals	55.9^{ac}	30.4^{a}	42.2^{b}

Acids: citric + malic acids ; Sugars: glucose + fructose ; AMP/UMP: adenosine- and uridine monophosphate ; Minerals: $CaCl_2$, $MgCl_2$, Na_2HPO_4, K_2HPO_4. For each attribute, the means with the same letter (a-c) are not significantly different at the 5% level according to Newman-Keuls tests.

The evaluation of the relative contributions of each main component of the model solution by stepwise multiple linear regressions confirmed the results obtained by omission tests. An important part of sourness (95%), saltiness

(89.9%) and sweetness (85.8%) were explained. For sourness, we observed an important positive contribution of citric acid (64.4%) and a lower contribution of malic acid (19.1%). A large part of the saltiness may be explained by the presence of potassium citrate and malate. We also observed a negative contribution of fructose (27%) which seemed to have a masking effect on saltiness. Concerning sweetness, this taste was mainly explained by glucose and fructose. We pointed out the higher contribution of fructose compared to glucose. As these compounds were present approximately in the same ratio, this result is explained by the higher sweetener property of fructose (20).

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Conclusion

To conclude, our approach with omission tests determined the molecules responsible for the taste characteristics of goat and camembert cheeses and tomato juice. In most cases, it was impossible to attribute one taste to one particular molecule because several taste-active compounds had a masking or enhancing effect and the global taste of the food results from a balance between these different effects.

Future Work

Our continued research efforts, in the case of camembert cheese, will focus on the identification of the bitter peptides contained in the retentates of ultrafiltration and nanofiltration by HPLC-MS-MS. The evolution of the identified peptides during the ripening will then be studied to try to correlate the presence of some particular peptides with bitterness. In addition, the quantity of taste and aroma compounds released during mastication of a food or model mixture incorporated in a matrix such as cheese model may be evaluated using an artificial mouth system.

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Chapter 21

Development of Selective Chemosensors for the On-Line Monitoring of Coffee Roasting by SOMMSA and COTA Technology

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Gas chromatography combined with a sniffing device (GC/O) or a chemosensor array (GC/SOMMSA) was used to develop a fast and objective method for monitoring flavor generation during roasting of coffee beans. The responses of the human nose and those of the chemosensors to single roast coffee volatiles were compared. Human odor perception and artificial volatile detection revealed 2-furfurylalcohol as an indicator volatile in the coffee headspace. This compound was selectively detected with high sensitivity by the chemosensors and was found to quantitatively correlate with 2-furfurylthiol, a character impact odorant of coffee. The chemosensors used in these experiments were then used to monitor the coffee roasting process on-line. To overcome problems occurring with the on-line use of chemosensors, such as contamination and overloading of the sensing metal oxides, we developed the Cyclic Operating-Temperature Alteration (COTA)-technology. This new technique, which involves switching each chemosensor between a measuring and a desorption mode, proved to be a powerful tool for monitoring flavor formation during coffee roasting on-line.

In Heteroatomic Aroma Compounds; Reineccius, G., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2002.

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Besides color and taste, aroma in particular is one of the most important characteristics determining consumer acceptance of processed foods. For many foods such as coffee, bread, and meat, thermal processing of the raw material is necessary to generate the desired flavors from non-odorous precursor compounds.

For economic reasons, modern food manufacturing often requires shortening of manufacturing time and variations in raw materials compared to traditional methods. Very often changes in the overall aroma of the products are observed. The noted capability of a consumer to discern food flavors as different, good, or bad, induces the food industry to follow and control flavor development during food processing in order to maintain or optimize the quality of the final product.

Flavor analysis is traditionally performed either by using trained sensory panels, instrumental analysis, or by techniques combining the human nose with instrumental analysis, e.g. gas chromatography/ olfactometry GC/O (*1*). Both sensory methods are very costly time-consuming and furthermore, these methods do not allow the on-line measurement of flavor generation during food processing. A need exists in the food industry for fast, objective, and non-destructive techniques to monitor on-line flavor development during food production. Such a system would permit manufacturers to better control flavor development and to assess the reproducibility of flavor formation, e.g. during the coffee roasting process.

In an attempt to meet this demand, arrays of chemical sensing elements (also termed "electronic noses") aimed at mimicking human olfaction electronically have been developed. Although the concept of these electronic noses was inspired by the attempt to imitate the signal processing in the human nose, in practice, the background of molecular recognition and signal processing significantly differs from that believed to be operative in the biological nose. In contrast to human olfactory receptors, most of the artificial sensing systems are based on the phenomenon that the reaction of hot metal oxides, such as SnO_2 or ZnO with organic compounds induces changes in conductivity (*2*).

Measurement by chemical sensors involves the contact of hundreds of volatiles present in the headspace above a food (e.g. more than 830 volatiles are known to exist in the vapor of roasted coffee (*3*)), with the chemically sensitive coatings of the sensor array; transducers then enable signal conversion. A computer records the signals of each sensor, the data are then processed by pattern recognition software and a resulting finger print profile of the volatile food fraction is displayed (*4*). Various techniques, such as, metal oxide sensors (*5*, *6*), bulk acoustic wave (BAW)-devices (*7*), and polypyrrols (*8*) have been used as artificial sensors. However, the specificity of electronic sensors currently differs greatly from the biological counterparts. In particular, this non-specificity of chemosensing elements is responsible for the discrepancies between "...the proposed ideal machine and what is available..." (*9*). Unfortunately chemosensors respond to all the predominant chemicals present in the headspace, whereas the human olfactory receptors can selectively perceive intense, trace (sub. ppt) odor-active compounds that are typically present in a majority of odorless volatiles (*10*).

To bridge the gap between the technical and the biological world of odor detection, and to improve the reliability of chemosensor measurements, more specific sensing materials have to be developed, and the data obtained from artificial molecular recognition must be correlated with those obtained by human perception (*9*). To achieve this we developed the GC/SOMMSA technique, which combines a chemosensor array with high-resolution gas chromatography. This method is designed to systematically check the responses of chemosensors to food volatiles. In addition, GC/SOMMSA permits one to tune the selectivity and sensitivity of sensor surfaces by optimizing the chemical composition, doping, and operating temperature of the chemically sensitive coating (*10*, *12-14*).

The purpose of the present investigation was to develop a chemosensor array- based methodology for on-line monitoring of flavor formation during thermal food processing using the roasting of coffee beans as an example.

Experimental

Green and roasted coffee beans (*Coffea arabica*) were from Colombia (var. tipica). Three different roasting degrees were obtained from the coffee industry: light: CTn value 110, roasting loss 14.5 %; medium: CTn value 85, roasting loss 16.2; dark: CTn value 60, roasting loss 18.9.

Identification of Key Coffee Odorants by HS-GC/O

To develop tailor-made chemosensors for measuring the flavor generation during roasting of coffee beans, it was a prerequisite to know those volatiles in the coffee vapor which are selectively and sensitively detected by the human nose. Odorants contributing to a food flavor can be sorted out from the bulk of non-odorous compounds by using approaches combining the olfactory receptors of the human nose, as very selective and sensitive biosensors, with analytical techniques, such as GCO (*1*). In order to elucidate the key compounds determining the flavor quality of roasted coffee, first the headspace of a medium roasted coffee was analyzed by HS-GC/O.

For HS-GC/O or HS-GC/MS, ground coffee (100 mg) was equilibrated for 30 min at 35°C in a septum-sealed vessel (250-mL). Headspace volumes (0.3-25-mL) were then withdrawn using a gas-tight syringe and injected into the purge-and-trap system of the GC as detailed recently (*15*).

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Evaluation of Chemosensing Elements

To compare biological odor recognition to artificial volatile detection, headspace samples of roasted ground coffee were then analyzed by means of GC/selective odorant measurement by multisensor array (GC/SOMMSA) technology (*10, 12-14*).

The principle of this method is as follows: The volatiles in a headspace sample are separated by GC and the effluent is split 1:1:1 into an flame ionization detector (FID), a sniffing port, and an array of 6 chemosensors arranged in a temperature controlled brass box (1.8-mL internal volume). Using a constant voltage circuit, 0.5 V is applied to the interdigitated electrodes of the metal oxide sensor layer. The d.c. current is measured by an operational amplifier as a voltage and is digitized with an digital analog converter. The semi-conducting metal oxide sensors used were either supplied by UST (Umweltsensortechnik, Geschwenda, Germany), or self-prepared by applying suspensions of metal oxides (ZnO, SnO_2, ZnO/SnO_2, WO_3) on ceramic substrates followed by sintering for 1 week at 900 K as recently reported (*12*).

On-line Measurements

For on-line measurements, the experimental set-up consisted of various hardware modules that measured the responses of the chemosensors regulated by the Cyclic Operating Temperature Alteration (COTA) technology (*16*): (the humidity (capacitive polymer sensor, MiniCap2, Panametrics GMBH, Hofheim, Germany); temperature (Pt100 sensor) of the roast vapor; and control of the relays for switching valves. The data of the modules were received by interface hardware, processed, and then saved by using self-programmed software.

Results and Discussion

Identification of Key Odorants in the Headspace of Roasted Coffee Powder by HS-GC/O

As shown in Figure 1A, in a 10-mL sample of headspace above ground coffee powder, 14 odorants were detected at the sniffing port. In order to rank these aroma compounds based on odor-activity, the gas volumes analyzed were successively reduced by half until the respective compound could just be detected at the sniffing port.

This aroma dilution analysis (ADA) revealed that the coffee-like smelling 2-furfurylthiol and the buttery smelling butan-2,3-dione were still detectable in

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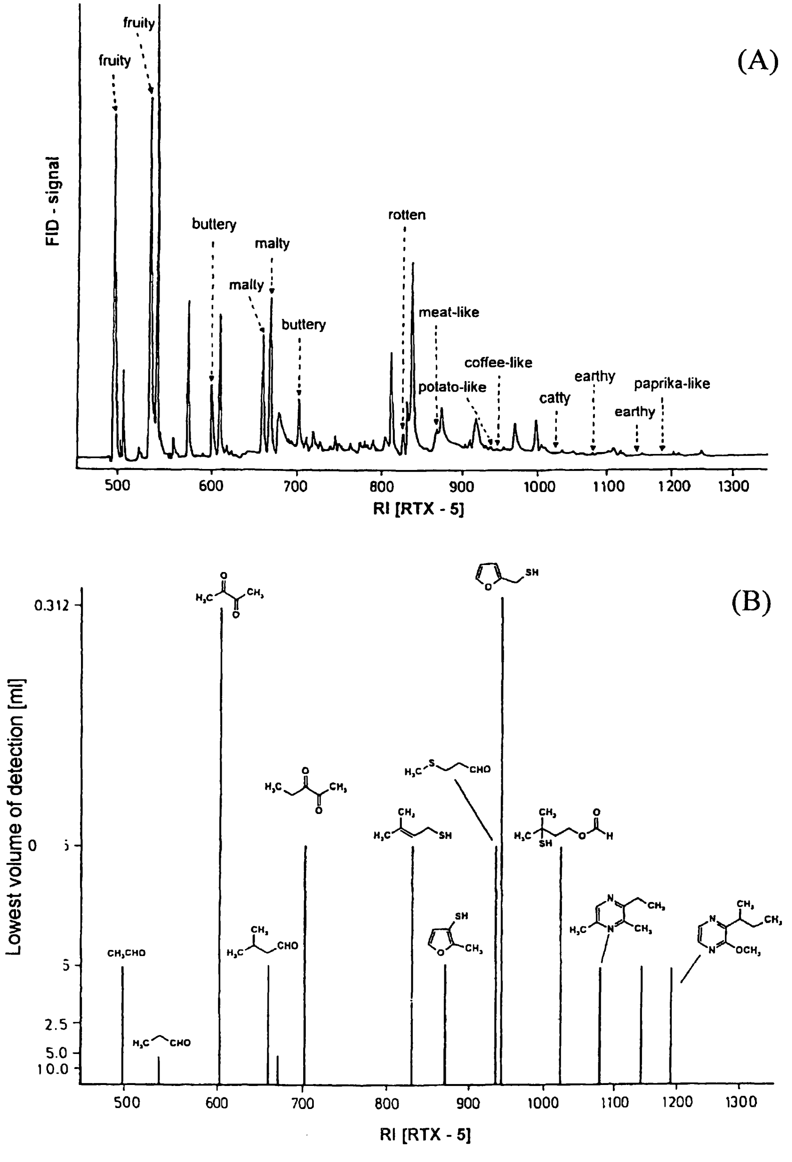

Figure 1. (A) Headspace-chromatogram (FID) and (B) flavor dilution chromatogram of a medium roasted arabica coffee.

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the lowest headspace volume of 0.3 mL and, therefore, were selected as compounds that likely contribute most to the overall odor of roasted coffee powder (Figure 1B). In addition, the buttery smelling pentan-2,3-dione, the cooked potato-like smelling methional, the rotten, skunky smelling 3-methyl-2-buten-1-thiol and the catty-like smelling 4-mercapto-4-methylbutylformate were detected in a slightly higher HS volume of 0.625 mL and were also assigned as odor contributors, but at somewhat lower odor impacts (Figure 1B). These data confirmed earlier results reported in the literature (*15*, *17*, *18*) and established, in particular, 2-furfurylthiol as one of the most important key contributors to the typical aroma of roast coffee powder.

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Development of Chemosensors by Means of SOMMSA Technology

The FID chromatogram of a coffee sample and the response patterns obtained from three sensor elements are displayed in Figure 2. A commercially available UST 5000 sensor, containing a Pt-doped SnO_2 material, sensitively detected an abundance of volatiles in the coffee vapor (Figure 2A); however, the sensor was not very selective. On the other hand, a self-prepared ZnO sensor showed relatively weak sensitivity, but a significantly higher selectivity than the UST 5000. This sensor detected only three higher-boiling volatiles, all which eluted at the end of the GC run program. Substitution of ZnO by WO_3 led to a significant change in the response pattern (Figure 2C). Only two volatiles were detected with high selectivity and sensitivity, thereby demonstrating that the type of transition metal used significantly influences the selectivity and the sensitivity in artificial molecular recognition.

Comparing these sensor results to those volatiles detected by the human nose at the sniffing port (Figure 1) demonstrates that the coffee-like smelling 2-furfurylthiol, eluting after 1220 sec, was not clearly detected by the chemosensors. In contrast, both volatiles to which the WO_3 sensor responded were not detected at the sniffing port.

These data demonstrate that in thermally processed foods, such as roasted coffee, the artificial detection of odor-active trace compounds, such as, 2-furfurylthiol, might be obscured by a high sensor response to odorless volatiles present at much higher concentrations. However, if such an odorless volatile is selectively detected by the chemosensors, and is quantitatively correlated with a key odorant, then selective detection of the odorless compound an as indicator compound for the trace odor-active compound can be proposed to monitor flavor development in the roasting process (*10*). To achieve this, first the indicator volatile has to be identified and confirmed that it is in fact quantitatively correlated with the odor significant compound (e.g. 2-furfurylthiol in coffee).

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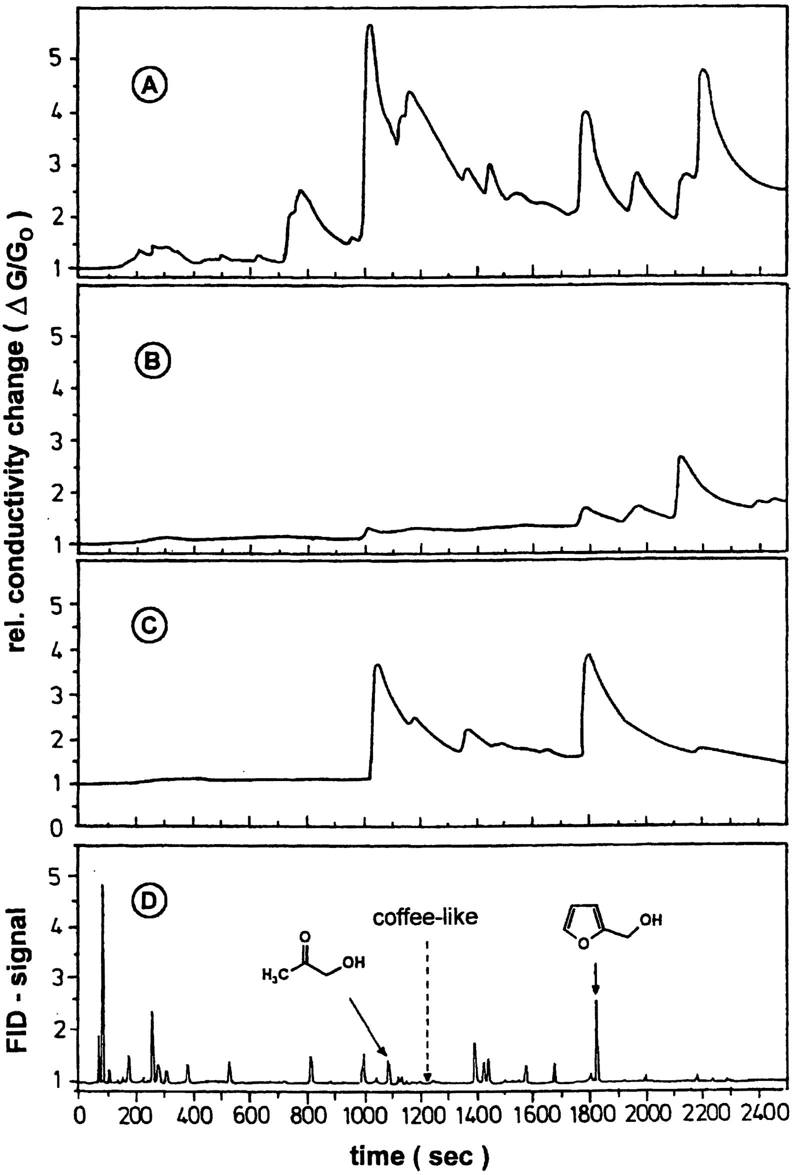

Figure 2. Response of three chemosensors, UST5000 (A), ZnO (B), WO_3 (C), and FID (D) to the volatiles present in the headspace above roasted ground coffee.

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By comparison of the mass spectra, retention times, and chemosensor responses with the data obtained from synthetic reference compounds, the volatiles detected by the WO_3 sensor were unequivocally identified as hydroxy-2-propanone (R_t = 1092 sec; Figure 2) and 2-furfurylalcohol (R_t = 1820 sec; Figure 2).

Because 2-furfurylalcohol has been proposed as an important precursor of 2-furfurylthiol by reacting with H_2S (which is liberated from cysteine during coffee roasting (e.g. *22*)), 2-furfurylalcohol was discerned as a likely sensor-active indicator volatile. To confirm this assumption, the amounts of 2-furfurylalcohol and 2-furfurylthiol were quantified in three arabica coffees that varied in the degree of roast. Independent of roasting degree, 2-furfurylalcohol was formed in amounts ca. 200-fold higher than that of the odor-active 2-furfurylthiol (Figure 3). However, the amounts of both volatiles were significantly influenced by the roasting degree and these concentrations increased greatly during roasting from light to medium. After reaching a maximum concentration at the medium roast degree, the amounts of these compounds decreased slightly during roasting to dark (Figure 3). Because the concentrations of 2-furfurylalcohol formed corresponded to the amounts of 2-furylfurylthiol formed, respectively, 2-furfurylalcohol was concluded to be a suitable indicator volatile to monitor on-line the formation of the key odorant 2-furfurylthiol during coffee roasting (*16*).

To further improve the sensitivity of 2-furfurylalcohol detection, additional SOMMSA experiments were performed using sensing elements varying either in chemical composition or in operating temperature (Figure 4). Taking the responses of all chemosensors into account, 16 volatiles were detected by the sensors in the headspace of the coffee powder. The UST 5000 sensor, when operated at 190°C, responded to eight compounds, amongst which compounds no. 8 and 16 were identified as hydroxy-2-propanone and 2-furfurylalcohol, respectively (Figure 4A). Increasing the temperature to 310°C led to significant changes in the response patterns as well as to an increase in sensitivity, while the selectivity decreased.

A sensing element consisting of a 1:1 mixture of ZnO and SnO_2 and operating at 190°C (B; Figure 4) selectively detected hydroxy-2-propanone (no. 8) and an unknown compound, but showed low sensitivity (Figure 4B). An increase in the operating temperature neither improved the sensitivity, nor the selectivity. In contrast, a UST 7000 sensor when operated at 190°C (C; Figure 4), allowed for the specific detection of hydroxy-2-propanone, but showed significantly lower selectivity with increasing operating temperature. The self-prepared WO_3 sensor at 190°C selectively recognized hydroxy-2-propanone (no. 8) and 2-furfurylalcohol (no. 16, Figure 4D). Increasing the operating temperature resulted in an increase in hydroxy-2-propanone detection but a slight decrease in the response for 2-furfurylalcohol.

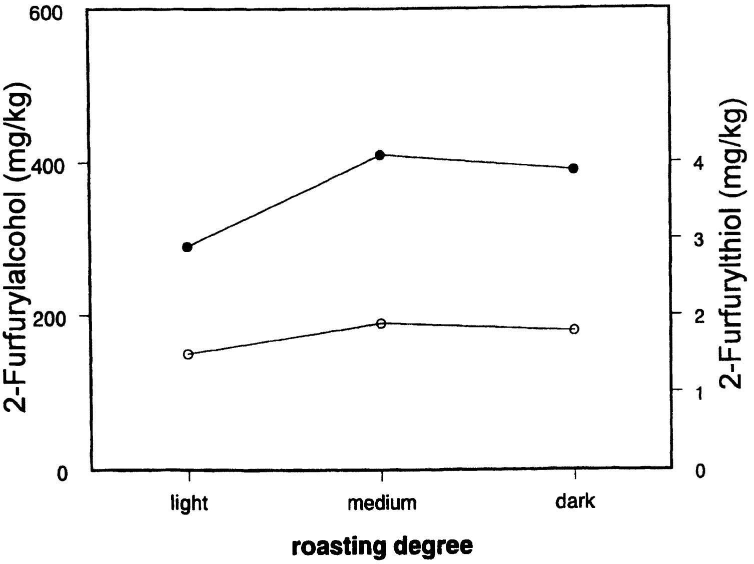

Figure 3. Influence of roasting degree on the concentrations of 2-furfurylalcohol (●) and 2-furfurylthiol (O) in roasted arabica coffees.

On the basis of these data, WO_3, operated at a lower temperature (e.g. 170°C), was selected as the chemically sensing material for the detection of hydroxy-2-propanone and of the coffee indicator volatile 2-furfurylalcohol (*16*). In addition, the UST 7000 was chosen for the selective detection of hydroxy-2-propanone. The WO_3 sensor was found to give exactly twice the response for the detection of hydroxy-2-propanone as the UST 7000 sensor (data not shown). Because the UST 7000 gave no response to 2-furfurylalcohol, the amounts of 2-furfurylalcohol can be calculated from the differences in both sensor signals (*16*).

In order to check the suitability of the two sensing elements for the measurement of 2-furfurylalcohol, a coffee headspace sample was analyzed by the sensors without pre-separation by GC. As displayed in Figure 5, the signal of the WO_3 sensor to coffee vapor directly corresponded to the roasting degree of the coffee beans. The sum signal of this sensor (☐ in Figure 5), responding exclusively to hydroxy-2-popanone and furfurylalcohol, increased from the light to the medium roasted sample and decreased during roasting to dark. In comparison, the UST 7000, provided information on the amounts of hydroxy-2-propanone in the coffee vapor. Detection by this sensor clearly demonstrated that hydroxy-2-propanone decreased during roasting from light over medium to

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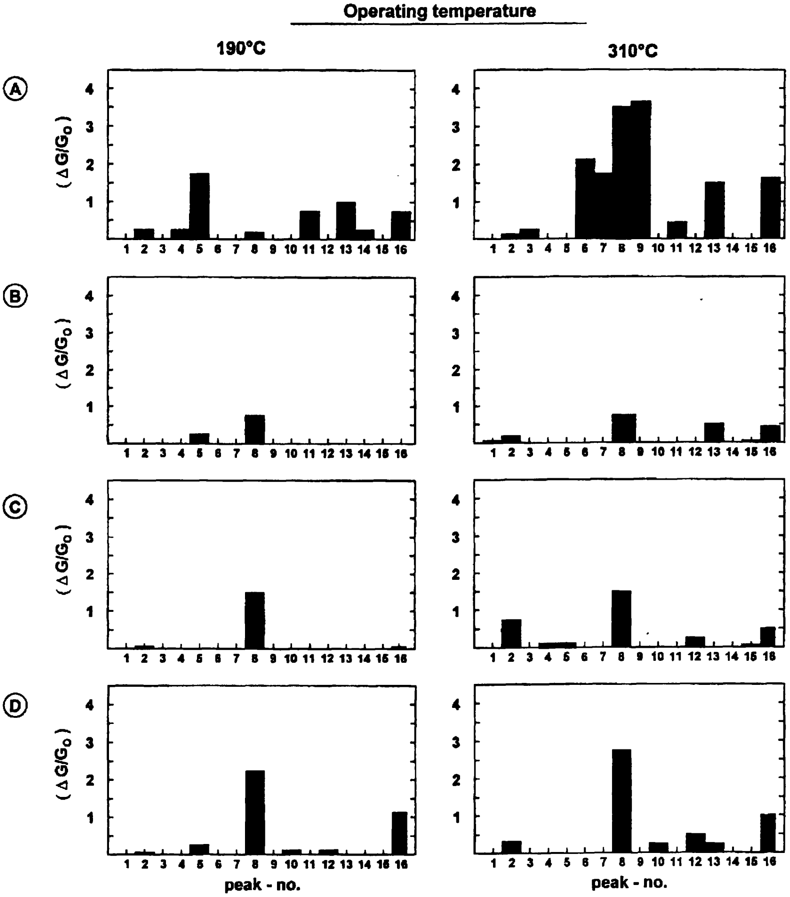

Figure 4. Influence of operating temperature of sensing elements on the response pattern to coffee headspace. (A) UST5000; (B) ZnO/SnO$_2$; (C) UST7000; (D) WO$_3$.

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dark (■ in Figure 5). This time course of hydroxy-2-propanone degradation was confirmed by independent quantitative experiments (data not shown).

Taking into account that the WO_3 sensor showed twice the sensitivity for hydroxy-2-propanone as the UST 7000, the time course of 2-furfurylalcohol formation was calculated from the differences in the sensor signals (+ in Figure 5). The influence of the roasting degree on the sensor response to 2-furfurylalcohol showed a reasonable fit with the formation of 2-furfurylalcohol and 2-furfurylthiol, respectively, as determined by quantitative analysis using GC/MS (Figure 3).

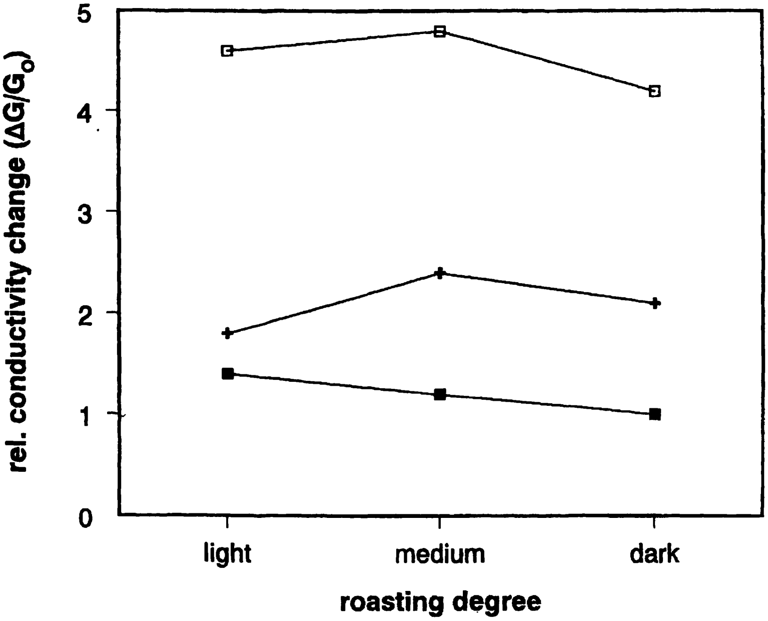

Figure 5. Influence of the roasting degree of coffee beans on the relative conductivity change of selected chemosensors: □: $\Sigma(WO_3)$; ■: $\Sigma(UST7000)$; +: $\Sigma(WO_3)-2\Sigma(UST7000)$.

These data demonstrate that the HS-GC/SOMMSA technique is a very useful tool to develop tailor-made chemosensors for the selective detection of indicator volatiles such as 2-furfurylalcohol, which have to be quantitatively correlated with key aroma compounds. Once specific chemically sensing elements are selected by SOMMSA experiments, these can be used to monitor the flavor development on-line during the coffee roasting process.

Model Roasting Experiments and On-line Measurements by COTA-Technology

To simulate the coffee roasting process on a laboratory scale, green coffee beans were roasted at 230°C in a self-constructed heating device shown in Figure 6 (*16*). To prevent local overheating of the beans and to establish a homogeneous heat-transfer, the beans were swirled around by a stream of nitrogen. At the end of the roasting process, the beans were ground in a mortar, evaluated by a sensory panel, and analyzed by HS-GC. The ground coffee material gave similar sensory and analytical data as that of a commercially available roasted coffee.

At the top of the roasting device, a temperature sensor monitored the temperature inside the roasting chamber (Figure 6). Because metal oxide sensors respond to the water that is released upon roasting, the water content in the stream of the roast exhaust was continuously measured by a non-destructive humidity sensor. Based on the data obtained, the responses of the WO_3- and the UST 7000 sensors to the volatiles in the coffee vapor were corrected to those responses obtained from a calibration experiment (*16*).

Because constant dosages of the chemosensors with the roast coffee vapors results in a rapid overload and contamination of the sensing layers, volatiles were introduced to the sensors semi-continuously by using two coupled 3-way valves and a 1:1 split of the nitrogen stream. In the first position (*Path 1* in Figure 6), an aliquot of the nitrogen was directly transferred to the sensor array without aroma enrichment. The other aliquot was led through the roasting chamber to take up the volatiles and, after humidity measurement, was flushed into a sniffing port where a person evaluated the quality of the aroma on-line during roasting. Upon switching the valves in the second position (*Path 2* in Figure 6), the gas stream, now enriched with coffee vapor, was directed into the sensor array, while the pure nitrogen stream was vented to the environment.

An additional requirement of this semi-continuous sampling technique is that the sensing layers must be cleaned between the single measurements in order to avoid contamination or signal drift of the chemosensor during the roasting process. To achieve this, we developed Cyclic Operating Temperature Alteration (COTA) technology (*16*). This technology is based on a cyclic variation of the sensor temperature between an upper and a lower level (Figure 7). The cycle has a period of 40 sec. As shown in Figure 7 (upper curve), during the first 10 seconds of a cycle, the heater voltage is increased from 2.2V to 9.5V and is then adjusted to 5.5V. This V corresponds to a sensor temperature of about 420°C. During this "desorption mode" the sensing surface is cleaned by thermal desorption of the volatiles. In the following 30 sec, the sensor is operated in the "measuring mode" at 2.2V, which corresponds to a temperature of about 170°C. This temperature was determined by SOMMSA experiments as the optimum temperature for the detection of 2-furfurylalcohol.

Figure 7 (-Δ-) displays the time course of the signal from the WO_3 sensor measured in a control experiment without coffee beans. The sharp increase in the operating temperature in the "desorption mode" is well reflected by a

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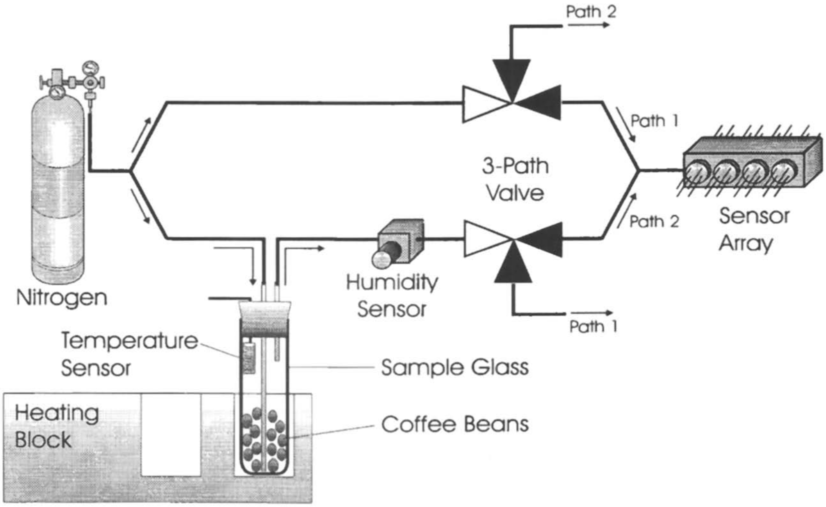
Path 2
Path 1
3-Path Valve
Path 2
Sensor Array
Nitrogen
Humidity Sensor
Path 1
Temperature Sensor
Sample Glass
Heating Block
Coffee Beans

Figure 6. Experimental set-up for on-line chemosensor measurement of flavor development during roasting of coffee beans.

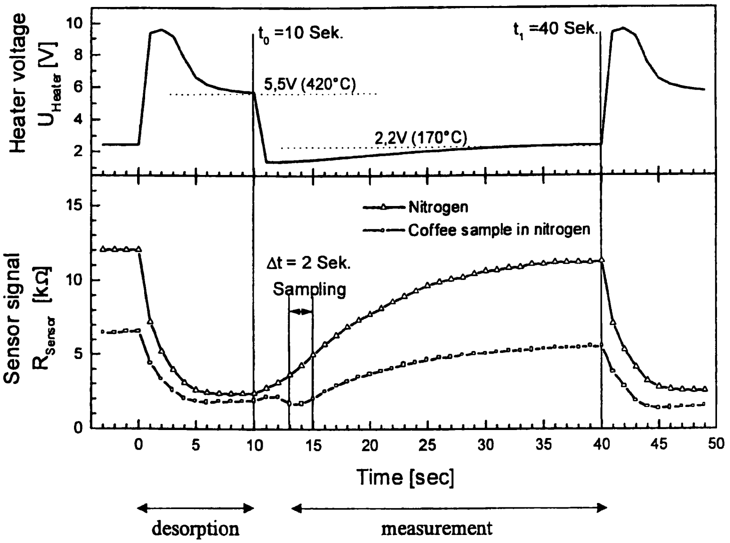

Figure 7. Principle of the cyclic operating temperature alteration (COTA) technology (sensor: WO_3).

corresponding decrease in sensor resistance to an approximate minimum value of 2.2 kΩ (Figure 7; - -). Lowering the temperature to 170°C in the "measuring mode" then resulted in an increase in the resistance approximating the base line after 40 sec.

When the response of the sensor to roast coffee vapor was measured, sampling was performed for exactly 2 sec after switching to the lower operating temperature. At this time, the roast volatiles are directed into the sensor array, where they are adsorbed at the sensing surface. The molecules adsorbed react with the metal oxides; this absorption is reflected in a partial break in the time course of the sensor resistance (Figure 7; - -). After two seconds of sampling, the sensors (still operating at 170°C) are flushed again with pure nitrogen. Now, no additional volatiles are adsorbed to the sensing layer, and the volatiles already accumulated react with the sensor surface and are partially desorbed again. This event is clearly reflected in the time course of the sensor signals showing that the resistance increases much more slowly as compared to the control experiment without coffee volatiles. After the "measuring mode", the next period starts again with the "desorption mode" to clean the sensor surface completely.

Using COTA technology, a roasting process was followed over 15 min encompassing 22 cycles of temperature change between 420°C and 170°C. The measured changes in resistance of the WO_3 sensor are shown in Figure 8. The relationship between the sensor signals and the roasting degree is more obvious, when calculating the difference between the sensor resistance at low and high temperature after 40 sec (c-f. Figure 7). These data show (Figure 8; lower) that the signal difference decreased until a minimum difference was reached at about 9 min. The resistance difference then increased again with extended roasting time.

Based on the results of the SOMMSA experiments, the amount of 2-furfurylalcohol was accurately determined from the signal difference between the WO_3 total response for hydroxy-2-propanone and 2-furfurylalcohol, and the hydroxy-2-propanone-specific UST 7000 sensor, (Figure 9). The time course of 2-furfurylalcohol formation determined by a chemosensor array showing a minimum at 9 min (upper curve) is well in line with the concentrations and the maximum amount of 2-furfurylalcohol at 9 min determined by independent quantitative analysis (lower curve). This correlation, in combination with the results of sensory evaluation of the aroma quality of the roast stream (sniffing at *path 1* in Figure 6, which had revealed an optimum aroma of a medium roasted coffee between 8 and 10 min), clearly demonstrated that the roasting process could successfully be monitored by the chemosensors selected by means of SOMMSA experiments. Because 2-furfurylalcohol is quantitatively related to the coffee-like smelling character impact compound 2-furfurylthiol, a powerful sensor system, which can be calibrated with a defined standard solution of 2-furfurylalcohol, is now available to monitor coffee roasting processes semi-continuously.

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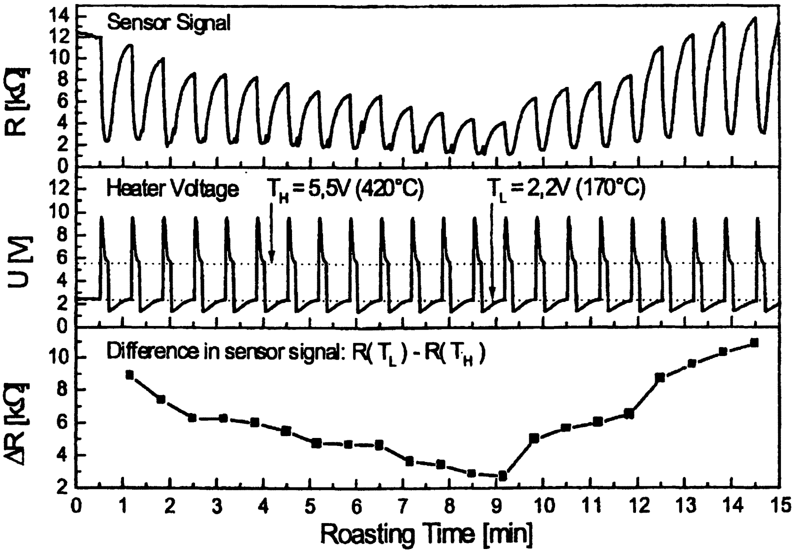

Figure 8. Signals of a WO_3-sensor operated by COTA technology during coffee roasting.

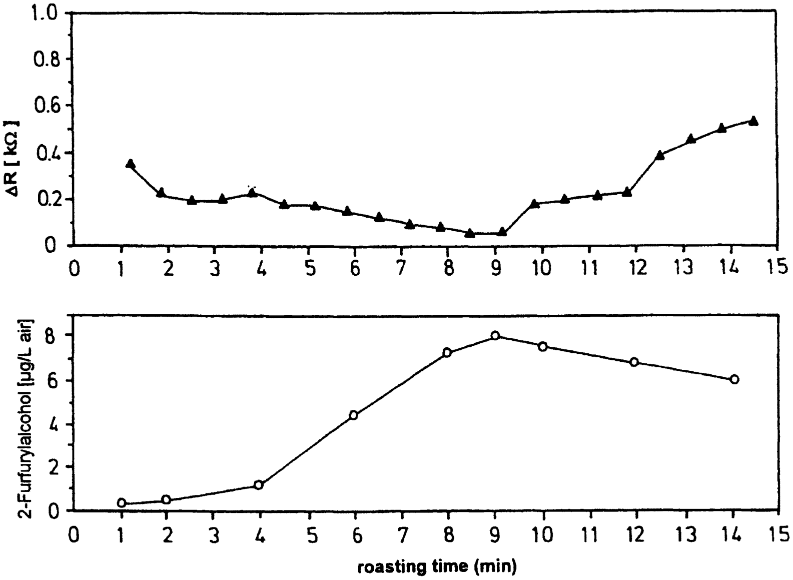

Figure 9. (Upper) Chemosensor recognition of 2-furfurylalcohol, as determined from the sensors WO_3 and UST7000, and (lower) headspace concentrations of 2-furfuryl-alcohol determined by independent quantitative analysis.

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Publication Date: August 7, 2002 | doi: 10.1021/bk-2002-0826.ch021

Author Index

Beer, A., 296
Bel Rhlid, R., 179
Blank, Imre, 25, 179
Bock, J., 328
Bonnarme, P., 54
Budin, John T., 192
Buettner, A., 296
De Kimpe, Norbert, 158
Deibler, Kathryn D., 269
Demyttenaere, Jan, 158
Devaud, S., 179
Elmore, J. Stephen, 93
Engel, E., 312
Fay, L. B., 179
Fleury, Y., 179
Fournier, Nicole, 166
Hannig, C., 296
Heinert, L., 328
Henry, Robert, 166
Ho, Chi-Tang, 281
Hofmann, Thomas, 207, 328
Jella, P., 102
Juillerat, M. A., 179
Kim, Young-Suk, 258
Kittel, Katherine M., 269
Kohl, C.-D., 328
Langlois, Dominique, 166
Le Quéré, Jean-Luc, 166, 312
Leconte, N., 312
Lin, J., 102
Martin, N., 54
Mottram, Donald S., 73, 93
Mottram, Hazel R., 73
Nicklaus, S., 312
Pandya, Rajesh N., 114
Peppard, Terry L., 114
Peterson, Devin, 227
Qian, Michael, 243
Reineccius, Gary A., 227, 243, 258
Reineccius, Terry A., xi
Rizzi, George P., 132
Rouseff, Russell L., 2, 102
Salles, C., 312
Schieberle, Peter, 207, 296, 328
Septier, C., 312
Settles, M., 296
Spinnler, H. E., 54
Tehrani, Kourosch Abbaspour, 158
Wei, Guor-Jien, 281

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Subject Index

A

Acetic acid, odor activity value, 252*t*
5-Acetyl-2,3-dihydro-1,4-thiazine, synthesis, 156
Acetylformoin (AF), 4-hydroxy-2,5-dimethyl-3(*2H*)-furanone (4-HDF) formation, 219*t*
2-Acetyl-1-pyrroline
  analogues, 154*f*
  first reported synthesis, 155*f*
  flavor compound of bread and cooked rice, 151
  identification, 153
  methods for synthesis, 155*f*
  oxidation of 2-acetylpyrrolidine, 155*f*
  production by bacteria, 160–162
  proposed reaction mechanism for formation from 1-pyrroline and 1,2-propanedione, 156*f*
  synthesis, 154
  See also Maillard reaction
6-Acetyl-1,2,3,4-tetrahydropyridine
  addition of Grignard reagents to 6-cyano-2,3,4,5-tetrahydropyridine for bread-like flavor compounds, 159*f*
  alkylation at 5-position, 157
  flavor compound of bread and cooked rice, 151
  Hodge mechanism, 158
  Maillard compound, 157–159
  proposed condensation of 1-pyrroline and hydroxyacetone, 158, 159*f*
  synthesis, 158–159
  synthetic pathways, 160*f*
  tautomeric equilibrium, 157*f*
  See also Maillard reaction
2-Acetyl-2-thiazoline (2-AT)
  detection by GC/olfactometry, 185*t*
  gas chromatograms showing amounts by bioconversion and bio/thermal processing, 188*f*
  generation via fermentation using baker's yeast, 184
  hypothetical formation, 189*f*
  roasted aroma character, 179–180
  structure, 180*f*
  See also Roasted notes
Acrolein, derivatization method, 197
Adenosine phosphosulfate (APS), sulfate incorporation, 55
Affinity, selective, column materials for thiols, 11
Aldehydes, thiazolidine derivatization, 198
Algae, bromophenol formation, 275
Alkylpyrazines. *See* Pyrazines
Alkylthiophenes, selected foods, 77*t*
Allium
  character-impact compounds, 35
  gas chromatography (GC), 11, 13*f*
  high performance liquid chromatography (HPLC), 12, 14
  organoselenium compounds, 283–284
  sulfur compounds, 74
Amines
  arylamines, 136
  biosynthesis, 134–138
Amino acids
  conversion into aroma compounds of banana, 231*f*
  cysteine, 57–58
  formation, 133–134
  glutathione, 68–69

  L-methionine, 58, 62–67
  normal metabolism, 135
  precursors to oxygenated aroma compounds, 230
  Strecker degradation, 81, 82*f*
  See also Biogeneration of sulfur compounds; Oxygenated aroma compounds
Ammonia, formation, 133–134
Amyl mercaptan, production from amyl alcohol, 58, 59*f*
Analytical methods
  artifact formation, 15
  atomic emission detection (AED), 19
  beef sulfur volatiles at two purging temperatures, 9*f*
  chemiluminescence, 16–17
  comparing sulfur volatiles in garlic headspace and garlic breath, 20*f*
  comparison of detection limits for sulfur specific GC detectors, 20*t*
  comparison of detection techniques, 19, 21
  comparison of recoveries from standard mixture of four sulfur compounds for Carboxen–PDMS fibers, 7*f*
  detection and identification for halogenated aroma compounds, 264–266
  detection and quantitation, 15–21
  direct injection, 259
  distillation, 4, 117–118, 262
  dynamic headspace concentration, 260–261
  dynamic headspace-purge and trap, 6–8, 122–123
  effect of temperature gradient on volatile decomposition, 13*f*
  electron capture detector, 264
  flame photometry, 15–16
  gas chromatography (GC), 11–12, 263–264
  Hall (electrolytic conductivity) detector, 264–265
  high performance liquid chromatography (HPLC), 12, 14
  HPLC of thiosulfinates in white onion, 14*f*
  influence of physicochemical properties of nitrogen heterocycles, 115–117
  influence of sample temperature during sparging, 8*f*
  ion exchange, 122
  isolation for halogenated aroma compounds, 259–262
  isotope dilution analysis, 21, 22*f*
  liquid–liquid extraction, 4–5, 261–262
  low temperature vacuum distillation, 11
  mass spectrometer (MS) detector, 265
  precipitation, 10
  pulsed flame photometry detection (PFPD), 17–19
  sample enrichment techniques, 10
  separation techniques, 11–14
  simultaneous distillation–extraction (SDE), 4, 119, 122
  solid phase micro extraction (SPME), 5–6, 123–124, 263
  solvent extraction, 118–119
  static headspace extraction, 260
  sublimation, 5
  sulfur volatiles from coffee powder using cryogenic trap, 10*f*
  supercritical fluid extraction (SFE), 262–263
  See also Nitrogen heterocycles; Oxygen-containing aroma compounds
Aroma extract dilution analysis (AEDA)
  ranking odorants, 214
  screening potent odorants, 28
  screening technique, 212
  sniffing technique, 201

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Publication Date: August 7, 2002 | doi: 10.1021/bk-2002-0826.ix002

See also Parmesan cheese
Aroma impact compounds
  pasteurized not-from-concentrate grapefruit juice, 107*t*
  See also Grapefruit juice
Aroma in heated foods
  breakdown pathways for thermal degradation of thiamin, 80*f*
  classes of volatile sulfur compounds, 74–77
  degradation products of thiamin, 80*f*
  dithiazines, 87, 89*t*
  flavor formation in Maillard reaction, 79, 81
  furanthiols and sulfides, 83–84
  number of sulfur- and nitrogen-containing compounds in foods, 76*t*
  occurrence and formation, 81, 83–87
  odor characteristics of thiazoles and thiazolines, 85*t*
  odor characteristics of thiols, sulfides, and disulfides, 84*t*
  odor threshold values of some sulfur compounds, 78*t*
  reactions between hydrogen sulfide and intermediates, 82*f*
  routes to formation of furan sulfides and disulfides, 86*f*
  route to alkylthiazoles, 85, 86*f*, 87
  Strecker degradation of amino acids, 81, 82*f*
  thialdine formation, 88*f*
  thiamin content of some foods, 79*t*
  thiamin-derived aroma compounds, 78–79
  thiazoles and thiazolines, 84–85, 87
  See also Oxygen heterocycles
Aromas, sulfur compounds, 3
Artifact formation, sulfur compounds, 15
Arylamines, aroma compounds, 136
Aspergillus oryzae, pyrazine production, 161
Atomic emission detection (AED)
  characteristics of detector, 125, 127*t*
  comparing garlic headspace and garlic breath, 20*f*
  compound independent calibration (CIC), 199
  oxygen-containing aroma compounds, 199
  sulfur, 19
  See also Detection and quantitation

B

Bacillus cereus
  2-acetyl-1-pyrroline production, 162
  pyrazine production, 161
Bacillus magnetarium, pyrazine production, 161–162
Bacillus subtilis, pyrazine production, 160–162
Basil leaves, concentrations and odor activity values of odorants, 33*t*
Beef
  character-impact sulfur compounds, 34*t*
  concentrations of cyclic oxygen-containing carbohydrate degradation products, 217*t*
  dithiazines, 87, 89*t*
  key odorants in stewed meat juices, 41*t*
  quantities of alkylthiophenes and alkylthiapyrans for polyunsaturated fatty acid (PUFA) rich diet, 99*t*
  relative flavor dilution (Rfd) factors for cyclic oxygen-containing carbohydrate degradation products, 216*t*
  sulfur volatiles at two purging temperatures, 7, 9*f*
  thiamin content, 79*t*
  thiazoles and thiazolines, 95–96

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Publication Date: August 7, 2002 | doi: 10.1021/bk-2002-0826.ix002

thiophenes and thiapyrans, 96–97
See also Cooked meat
Beer
chemiluminescence, 16–17
dimethyl sulfide concentration, 67
fermentation, 55, 57
mass spectrometer as specific detector for nitrogen heterocycles, 127, 128*f*
methional, 58
methylthioacetate (MTA) concentration, 65
off-flavor generation, 49*f*
sulfates and sulfites, 55–57
See also Biogeneration of sulfur compounds
Beverages
nitrogen heterocycles, 115
physicochemical data for nitrogen heterocycles, 116*t*
See also Grapefruit juice
Biogeneration. *See* Oxygenated aroma compounds; Roasted notes
Biogeneration of sulfur compounds
amyl mercaptan from amyl alcohol, 59*f*
cheese ripening process, 63, 65
conversion of methionine and acyl CoA to thioesters, 69*f*
cysteine, 57–58
development of cheese flavor, 62
dimethyl sulfide (DMS) in lager beer, 67
efficiency of diverse ripening bacteria, 68*f*
Ehrlich–Neubauer pathway for L-methionine degradation, 58, 60*f*
flavor producing capabilities in *Geotrichum candidum*, 65, 67
major amino transferases, 62–63
L-methionine, 58, 62–67
L-methionine degradation by amino transferases, 64*f*
methylthioacetate (MTA) concentration in lager beer, 65
possibilities for S-terminal degradation, 61*f*
production of MTA, 63, 66*f*
soft cheese technology, 63
sulfate assimilation, 56*f*
sulfates and sulfites, 55–57
sulfur compounds in *G. candidum*, 65, 66*f*
synthesis of MTA and cell free extracts (CFE) of *G. candidum*, 67
thioacetal production, 67
transamination of L-methionine for α-keto-γ-methylthiobutyric acid (KMBA), 62
Biosynthesis of nitrogen aroma compounds
alkylpyrazines, 143–144, 146*f*
amines and imines, 134–138
arylamines, 136
Bacillus cereus, 136, 138
Chichibabin reaction for pyridine formation, 142*f*
Cruciferae family, 138
dihydropyrazines reacting with aldehydes, 146*f*
formation of ammonia and amino acids, 133–134
formation of isothiocyanates, thiocyanates, and nitriles from glucosinolates, 140–141
glucosinolates, 139*f*
glucosinolates, oximes, and nitroalkanes, 138, 140
glucosinolates and decomposition products, 138–141
glucosinolates undergoing enzymatic and non-enzymatic reactions, 142*f*
imine linkage formation with pyridoxal phosphate (POP), 135, 137*f*
kynurenine degradation, 139*f*

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Publication Date: August 7, 2002 | doi: 10.1021/bk-2002-0826.ix002

  mechanism for 2-methoxy-3-isopropylpyrazine (MIP) formation, 145, 146*f*
  methoxypyrazines, 144–145, 147
  occurrence of 2-methoxy-3,5-dialkylpyrazines, 147, 148*f*
  pyrazines, 143
  pyridines, 141, 143
  reductive amination of carbonyls via putative biochemical route, 137*f*
  trimethylamine and dimethylamine from trimethylamine oxide, 136
  volatile *N*-heteroaromatic compounds, 141–147
Blue cheeses. *See* French blue cheeses
Boiling point, isolation of aroma compounds, 193
Bordeaux red wine
  aromas, 40
  See also Wine
Brandy, cork taint, 271–273
Brassica, sulfur compounds, 74
Bread
  concentrations of cyclic oxygen-containing carbohydrate degradation products, 217*t*
  important flavor compounds, 150–151
  relative flavor dilution (Rfd) factors for cyclic oxygen-containing carbohydrate degradation products, 216*t*
  thiamin content, 79*t*
  See also Maillard reaction
Broilers, chloroanisoles, 271
Bromophenols
  formation pathways in algae, 275
  fresh water origins, 276
  sensory studies, 274
  sources, 274–275
  tainted fruit juices, 276
  See also Halogenated aroma compounds
2,3-Butandione, odor threshold, 211*f*, 212
Butanoic acid
  OAV (odor activity value), 252*t*
  OAVs of brands of Parmesan cheese, 253*t*
  See also Parmesan cheese

C

Camembert cheese
  composition, 319*t*
  extraction procedure, 315
  future work, 326
  omissions of peptide fractions, 324*t*
  omissions of peptides, amino acids, peptides/amino acids, NaCl, KCl, $CaCl_2/MgCl_2$, 323*t*
  omission testing, 322–324
  omission testing method, 317–318
  sample, 314
  See also Cheese; Omission tests
Carbohydrates
  flavor contribution of, derived oxygen-containing compounds, 212–214
  structures of odorants from, 213*f*
  See also Oxygen heterocycles
Carbon, trapping material for sulfur compounds, 7
Carbonyl compounds, derivatization with 2,4-dinitrophenyl hydrazine, 197
β-Carotene, biogeneration of β-ionone, 236*f*, 237
Character-impact compounds, foods, 34–35
CHARM analysis
  screening potent odorants, 28
  screening technique, 212
  sniffing technique, 201
Cheese
  ammonia elimination, 62
  flavor development, 62

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Publication Date: August 7, 2002 | doi: 10.1021/bk-2002-0826.ix002

  flavor producing capabilities of *Geotrichum candidum*, 65, 67
  G. candidum in ripening process, 63, 65
  production of methylthioacetate (MTA), 66*f*
  sulfur compounds in *G. candidum*, 65, 66*f*
  thioacetal production, 67
  yeasts of soft technology, 63
  See also Biogeneration of sulfur compounds; Camembert cheese; French blue cheeses; Goat cheese; Omission tests; Parmesan cheese
Chemiluminescence
  detection of sulfur compounds, 16–17
  nitrogen-selective detectors, 125, 127*t*
  odor-active sulfur compounds by sulfur chemiluminescence detection (SCD), 110–112
  SCD method, 105
  See also Grapefruit juice
Chemosensors
  measurement by, 329–330
  See also Selective chemosensors development
Chichibabin reaction, pyridines, 141, 142*f*
Chicken
  dithiazines, 87, 89*t*
  relative flavor dilution (Rfd) factors for cyclic oxygen-containing carbohydrate degradation products, 216*t*
Chicken eggs, chloroanisoles, 271
Chlorinated aroma compounds
  chloroanisoles in chicken eggs and broilers, 271
  cork taint, 271–273
  sensory studies, 270–271
  sources of chloroanisoles and chlorophenols, 273
  See also Halogenated aroma compounds
Chloroanisoles
  chicken eggs and broilers, 271
  cork taint, 271–273
  sources, 273
Chlorophenols, sources, 273
Coffee
  aroma, 45–46
  aroma of roasted model, 46*t*
  character-impact sulfur compounds, 34*t*
  concentrations of cyclic oxygen-containing carbohydrate degradation products, 217*t*
  experimental set-up for on-line chemosensor measurement of flavor development during roasting, 340*f*
  furanthiols and sulfides, 83–84
  2-furfurylalcohol detection, 335–336
  hydroxy-2-propanone detection, 335–336
  identification of key odorants by headspace–gas chromatography/olfactometry (HS–GC/O), 330, 331–333
  influence of operating temperature of sensing elements on response pattern to headspace, 337*f*
  influence of roasting degree of beans on relative conductivity change of chemosensors, 338*f*
  isotope dilution analysis, 21, 22*f*
  model roasting experiments, 339–341
  omission tests, 45–46
  relative flavor dilution (Rfd) factors for cyclic oxygen-containing carbohydrate degradation products, 216*t*
  response of three chemosensors to volatiles, 334*f*

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Publication Date: August 7, 2002 | doi: 10.1021/bk-2002-0826.ix002

sulfur volatiles from powder after boiling using cryogenic trap, 8, 10*f*
thiamin content, 79*t*
See also Selective chemosensors development
Compound independent calibration (CIC), atomic emission detection, 199
Cooked meat
aldehydes from autooxidation of linolenic and linoleic acids, 98*f*
alkylthiophenes and alkyl-(*2H*)-thiapyrans in model systems containing linoleic and linolenic acids, 97–99
aroma recombination studies on stewed beef meat juice, 44*t*
aromas, 40–44
concentrations and odor activity values (OAV) of potent odorants of boiled beef, 45*t*
effect of fatty acid composition on meat volatiles, 94–99
effect of odorants on aroma profile of model mixtures, 43*f*
2-ethyl-(*2H*)-thiapyran formation, 98–99
key odorants in stewed meat juices, 41*t*
Maillard reaction, 94
modeling aromas, 40, 42*f*
off-flavor generation, 49*f*
omission experiments, 43
polyunsaturated fatty acids (PUFA), 94
quantities of alkylthiophenes and alkylthiapyrans in beef and lamb on PUFA rich diets, 99*t*
reactions occurring during cooking, 93
results of omission experiments, 45*t*
studying interaction of odorants, 43
sulfur heterocycles, 94
thiazoles and thiazolines in beef and lamb, 95–96
thiophenes and thiapyrans, 96–97
Cooked rice. *See* Rice
Cork taint, chlorinated aroma compounds, 271–273
Cruciferae family, glucosinolate biosynthesis, 138
Cryogenic traps, purge and trap technique, 8, 10*f*
Cucumbers, biogeneration of (*E,Z*)-2,6-nonadienal, 240*f*
Cyclic operating temperature alteration (COTA) technology
model roasting experiments and on-line measurements, 339–341
on-line measurements, 331
principle, 340*f*
signals of WO_3-sensor during coffee roasting, 342*f*
See also Selective chemosensors development
Cysteine
amyl mercaptan from amyl alcohol, 58, 59*f*
degradation pathways, 57
precursors of volatile compounds, 57–58

D

β-Damascenone
biogeneration from allenic acid and acetylenic carotenoids, 238*f*
biosynthesis, 237–238
odor threshold, 211*f*, 212
See also Oxygenated aroma compounds
Decanoic acid
OAV (odor activity value), 252*t*
OAVs of brands of Parmesan cheese, 253*t*
See also Parmesan cheese
Derivatization

Downloaded by 217.66.152.21 on September 14, 2012 | http://pubs.acs.org
Publication Date: August 7, 2002 | doi: 10.1021/bk-2002-0826.ix002

  analysis of formaldehyde, acrolein, and malonaldehyde, 197
  carbonyls with 2,4-dinitrophenyl hydrazine, 197
  combination with solid phase microextraction (SPME), 197–198
  halogenated aroma compounds, 265–266
  oxygen-containing aroma compounds, 196–198
Detection and quantitation
  artifact formation, 15
  atomic emission detection (AED), 19
  chemiluminescence, 16–17
  comparing detection limits for three sulfur specific GC detectors, 20*t*
  comparison of detection techniques, 19, 21
  flame photometry, 15–16
  isotope dilution analysis, 21, 22*f*
  nitrogen heterocycles, 124–129
  pulsed flame photometry detection (PFPD), 17–19
  See also Analytical methods
Detectors
  halogenated aroma compounds, 264–266
  oxygen-containing aroma compounds, 198–201
3,5-Dihydroxy-2-methyl-5,6-dihydropyran-4-one, formation from 1-desoxyosone of maltose, 223*f*
Dimethyl diselenide
  effects of reaction time and pH, 286–290
  EI/MS (electron ionization/mass spectrometry) spectrum, 287*f*
  EI/MS spectrum of 2-propenesulfenoselenoic acid methyl ester, 292*f*
  EI/MS spectrum of dimethyl diselenide, 287*f*
  formation, 290*f*
  formation of dimethyl diselenide, 290*f*
  gas chromatography (GC) analysis, 285
  GC/MS analysis, 285
  GC/MS profile of volatile aroma compounds, 286*f*
  GC/MS profile of volatiles from, with diallyl disulfide, 292*f*
  interactions between organoselenium and sulfur compounds, 285, 290, 292*f*
  pH changes of buffered and unbuffered systems, 290*t*
  thermal reaction and isolation of volatiles, 284
  volatile compounds from, 285–286
  yields after 40, 60, and 80 minutes heating, 287*t*, 288*t*
  yields at 160°C, 289*f*
  yields of dimethyl diselenide at 160°C, 289*f*
  yields of ethyl selenoacetate at 160°C, 289*f*
  yields of major volatile compounds after 40, 60, and 80 minutes heating, 287*t*, 288*t*
  yields of pyrazines in buffered and unbuffered solutions, 291*f*
  See also Selenomethionine-glucose model system
Dimethyl disulfide, frequently listed volatile, 25–26
Direct injection, halogenated aroma compounds, 259
Distillation
  halogenated aroma compounds, 262
  method for free fatty acids in Parmesan cheese, 245
  nitrogen heterocycles, 117–118
  sulfur compounds, 4
  See also Analytical methods
Disulfides, odor characteristics, 84*t*

Dithiazines
  occurrence and formation, 87, 89*t*
  thialdine formation, 88*f*
  See also Aroma in heated foods
Dynamic headspace (DHS)
  combination with liquid–liquid solvent extraction, 194
  halogenated aroma compounds, 260–261
  oxygen-containing aroma compounds, 193–194

E

Eating
  flavor perception, 296
  swallowing process, 297–298
  See also In-mouth release of heteroatomic odorants
Eggs, chicken, chloroanisoles, 271
Ehrlich–Neubauer pathway
  degradation of L-methionine, 58, 60*f*
  methional, 58
Electrolytic conductivity detector (ELCD)
  characteristics, 125, 127*t*
  halogenated aroma compounds, 264–265
Electron capture detector, halogenated aroma compounds, 264
Electronic noses. *See* Selective chemosensors development
Enrichment, sample, techniques, 10
Enzymes
  oxygen insertion into biomolecules, 208
  See also Oxygen heterocycles
Ethyl butanoate
  determination using exhaled odorant measurement (EXOM) technique, 301
  exhalation during swallowing process using EXOM technique, 306, 307*f*
  losses by spit-off odorant measurement (SOOM) technique, 309, 310*f*
  See also In-mouth release of heteroatomic odorants
Ethyl selenoacetate
  yields after 40, 60, and 80 minutes heating, 287*t*, 288*t*
  yields at 160°C, 289*f*
  See also Selenomethionine-glucose model system
Exhaled odorant measurement (EXOM)
  determination of exhaled ethyl butanoate, 301, 306, 307*f*
  See also In-mouth release of heteroatomic odorants

F

Fatty acids
  biosynthesis in plants, 230*f*
  branched in cheese, 249–250
  lipid oxidation mechanisms, 228–229
  normal metabolism, 230
  oxidative degradation of linoleic and linolenic acids, 229*f*
  precursors to oxygenated aroma compounds, 228–230
  See also Oxygenated aroma compounds; Parmesan cheese
Fermentation
  nitrogen depletion in wine, 57
  See also Beer
Fish, bromophenols, 274–275
Flame ionization detector (FID)
  halogenated aroma compounds, 265
  oxygen sensitive, 200
Flame photometry
  low concentration sulfur compounds, 15–16

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Publication Date: August 7, 2002 | doi: 10.1021/bk-2002-0826.ix002

  thioester detection in yellow passion fruit, 17*f*
  See also Detection and quantitation
Flavor dilution (FD), acidic fraction of Parmesan cheese, 249*f*
Flavor formation, Maillard reaction, 79, 81
Fluorine containing compounds, sources, 276
Food flavors
  chemical classes of sulfur compounds, 3
  nitrogen-containing volatiles, 133
  volatile organic sulfur compounds, 25
  See also Oxygen heterocycles
Food processing
  oxygen-containing aroma compounds, 209
  reactions leading to loss of oxygen from biomolecules, 210*f*
Foods
  character-impact sulfur compounds, 34*t*, 35*f*
  important heteroatomic odorants, 298*t*
  nitrogen heterocycles, 115
  number of sulfur- and nitrogen-containing compounds, 76*t*
  physicochemical data for nitrogen heterocycles, 116*t*
  See also Aroma in heated foods; Nitrogen heterocycles; Omission tests; Sensory relevance of sulfur compounds in food
Formaldehyde, derivatization method, 197
Free fatty acid (FFA)
  branched in cheese, 249–250
  quantification method, 247–248
  See also Parmesan cheese
French blue cheeses
  characteristics of main unknowns, 170*t*
  chiral analyses by multi-dimensional gas chromatography (MDGC) method, 168
  chirality and enantiomeric ratios, 175, 178
  EI mass spectra of 3-oxazolines, 176*f*, 177*f*
  EI mass spectrum of unknown, 170*f*
  extraction and fractionation, 167
  gas chromatography (GC) method, 167
  GC–Fourier transform infrared spectrometry (GC–FTIR) method, 168
  GC–FTIR spectra of major compounds, 171
  GC–mass spectrometry (GC–MS) method, 168
  ^{1}H nuclear magnetic resonance (NMR) data of unknowns, 171, 172*t*
  NMR method, 169
  odor and chiral properties of main 3-oxazolines, 174, 175*t*
  3-oxazolines, 173*f*
  preparative GC method, 168
  reaction scheme between ammonia, acetoin and methyl ketones to form 2,5-dihydro-oxazoles, 173*f*
  series of 2-alkyl-2,4,5-trimethyl-3-oxazolines, 171
  syntheses of 3-oxazolines, 169
  syntheses of major compounds, 174
  thermoionic (NPD) detector, 167
  vacuum cheese distillates, 169–170
French fries
  concentrations of cyclic oxygen-containing carbohydrate degradation products, 217*t*
  relative flavor dilution (Rfd) factors for cyclic oxygen-containing carbohydrate degradation products, 216*t*
Fresh water, bromophenols, 276

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Publication Date: August 7, 2002 | doi: 10.1021/bk-2002-0826.ix002

Fruits
  pyridines, 141, 143
  sulfur-containing aroma compounds, 74
Furan disulfides, formation routes, 84, 86*f*
2(*5H*)-Furanones
  formation, 221, 222*f*
  See also Oxygen heterocycles
3(*2H*)-Furanones
  formation, 218–221
  See also Oxygen heterocycles
Furan sulfides, formation routes, 84, 86*f*
Furanthiols
  occurrence and formation, 83–84
  See also Aroma in heated foods
2-Furfurylalcohol. *See* Selective chemosensors development
2-Furfurylthiol
  aroma recombination studies on stewed beef meat juice, 44*t*
  stewed meat juices, 40, 41*t*

G

Garlic
  atomic emission detection (AED), 19, 20*f*
  character-impact sulfur compounds, 34*t*
  organoselenium compounds, 283–284
Gas chromatography (GC)
  capillary, 12
  effect of temperature gradient on volatile decomposition, 13*f*
  multidimensional capillary, 12
  separation for halogenated aroma compounds, 263–264
  separation technique, 11–12
Gas chromatography–mass spectrometry (GC–MS), acetal formation, 200
Gas chromatography–olfactometry (GC–O)
  method, 104–105
  odor-active sulfur compounds, 110–112
  odor-active volatiles in hand-squeezed grapefruit juice, 108
  screening potent odorants, 28, 29*f*
  See also Grapefruit juice
Glucose. *See* Oxygen heterocycles; Selenomethionine-glucose model system
Glucosinolates
  biosynthesis, 138, 140
  enzymatic and non-enzymatic reactions, 142*f*
  formation of isothiocyanates, thiocyanates, and nitriles, 140–141
  intermediates, 139*f*
Goat cheese
  composition, 319*t*
  extraction procedure, 315
  gustatory profile of water-soluble extract (WSE) and model WSE (MWSE), 320*f*
  individual omissions of mineral salts, 321*t*
  omissions of lactose, amino acids, lactic acid, and mineral salts, 321*t*
  omission testing, 318–322
  omission testing method, 317–318
  sample, 314
  See also Omission tests
Grapefruit juice
  aroma impact compounds in pentane-ethyl acetate aroma extract, 107*t*
  aroma impact compounds of pasteurized not-from-concentrate (NFC), 105–109
  chemical structure of sulfur-containing odorants, 109*f*
  effect of pasteurization on sulfur compounds in early season, 110*f*

effect of thermal processing on sulfur compounds, 109–110
experimental, 103–105
FID-chromatogram and time-intensity aromagram, 106*f*
gas chromatography–olfactometry (GC–O) method, 104–105
NFC sample, 103
odor-active sulfur compounds by GC–O and sulfur chemiluminescence detection (SCD), 110–112
odor-active volatiles in hand-squeezed, 108
sample preparation, 104
SCD, 105
sulfur compounds in aroma, 103
time-intensity aromagram and GC–SCD chromatogram of pasteurized NFC, 111*f*

H

Hall detector, halogenated aroma compounds, 264–265
Halogenated aroma compounds
- bromophenols, 274–276
- chlorinated compounds, 270–273
- derivatization, 265–266
- direct injection, 259
- distillation, 262
- dynamic headspace concentration, 260–261
- electron capture detector, 264
- flame ionization detector (FID), 265
- Hall (electrolytic conductivity) detector, 264–265
- infrared detector, 265
- iodine and fluorine containing, 276
- liquid–liquid extraction, 261–262
- mass spectrometer (MS) detector, 265
- naturally occurring, 269
- possible aroma contributors, 277
- sniffing, 265
- solid phase microextraction (SPME), 263
- static headspace extraction, 260
- supercritical fluid extraction (SFE), 262–263
- *See also* Chlorinated aroma compounds

Headspace analysis
- dynamic purge and trap, 6–8, 122–123
- halogenated aroma compounds, 260–261
- oxygen-containing aroma compounds, 193–195
- static solid phase micro extraction (SPME), 5–6
- *See also* Analytical methods

Heated foods. *See* Aroma in heated foods; Oxygen heterocycles
Helium
- device for detection in breath air, 300*f*
- effectiveness of velum closure using, 305
- method for detection in exhaled air, 299
- *See also* In-mouth release of heteroatomic odorants

Herbs, pyridines, 141, 143
Heterocycles, N/S, selected foods, 77*t*
Hexanoic acid
- OAV (odor activity value), 252*t*
- OAVs of brands of Parmesan cheese, 253*t*
- *See also* Parmesan cheese

High performance liquid chromatography (HPLC), separation technique, 12, 14
Human nose, gas chromatograph coupling, 200–201
Hydrogen sulfide
- cysteine degradation byproduct, 57

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Publication Date: August 7, 2002 | doi: 10.1021/bk-2002-0826.ix002

reaction with Maillard reaction intermediates, 81, 82*f*
3-Hydroxy-4,5-dimethyl-2(*5H*)-furanone (3-HDF)
  concentration in foods, 217*t*
  formation from 2,3-butandione and hydroxyacetaldehyde, 222*f*
  relative flavor dilution (Rfd) factors in foods, 216*t*
  See also Oxygen heterocycles; Sotolon
4-Hydroxy-2,5-dimethyl-3(*2H*)-furanone (4-HDF)
  amounts from acetylformoin (AF), 219*t*
  amounts from different carbohydrates, 219*t*
  aroma compound, 213
  concentration in various foods, 217*t*
  formation by Aldol reaction, 220*f*
  formation from rhamnose, 218*f*
  influence of pH on formation, 220*t*
  relative flavor dilution (Rfd) factor, 216*t*
  See also Oxygen heterocycles
2-(1-Hydroxyethyl)-4,5-dihydrothiazole (HDT)
  application in pizza, 182–183
  biogeneration, 183*f*
  generation using baker's yeast, 184
  identification by GC/olfactometry, 185*t*
  impact as aroma precursor, 186, 190*t*
  structure, 180*f*
  See also Roasted notes
3-Hydroxy-5-ethyl-4-methyl-2(*5H*)-furanone, odor threshold, 211*f*, 212
4-Hydroxy-2-ethyl-3-methyl-3(*2H*)-furanone
  amounts from different carbohydrates, 219*t*
  concentration in various foods, 217*t*
  flavor of roasted coffee, 213
  relative flavor dilution (Rfd) factor, 216*t*
  See also Oxygen heterocycles
p-Hydroxymercuric acid (pHMB), trapping thiols, 26–27
4-Hydroxy-5-methyl-3(*2H*)-furanone
  amounts from different carbohydrates, 219*t*
  formation from ribose, 218*f*
  See also Oxygen heterocycles
3-Hydroxy-2-methylpyran-4-one
  concentrations of cyclic oxygen-containing carbohydrate degradation products, 217*t*
  formation from 1-desoxyosone of glucose, 223*f*
  formation from different carbohydrates, 222*t*
  relative flavor dilution (Rfd) factors in foods, 216*t*
  See also Oxygen heterocycles
Hydroxy-2-propanone. *See* Selective chemosensors development

I

Imines
  biosynthesis, 134–138
  linkages with pyridoxal phosphate (POP), 135, 137*f*
Infrared detectors
  halogenated aroma compounds, 265
  oxygen-containing aroma compounds, 199
In-mouth release of heteroatomic odorants
  detection of helium in exhaled air, 299, 300*f*
  determination of exhaled ethyl butanoate by applying exhaled odorant measurement (EXOM) technique, 301

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Publication Date: August 7, 2002 | doi: 10.1021/bk-2002-0826.ix002

determination of exhaled ethyl butanoate during swallowing using EXOM technique, 306
determination of odorant losses by spit-off odorant measurement (SOOM) technique, 306, 308–309
determination of odorant losses during mastication by SOOM technique, 302
important heteroatomic odorants in foods, 298*t*
losses of 3-alkyl-2-methoxypyrazines, 306, 308
losses of ethyl butanoate, methional, methionol, 309
losses of ethyl butanoate by exhalation before, during and after swallowing, 307*f*
losses of methoxypyrazine, methional, methionol, and ethyl butanoate after different times of rinsing in mouth, 310*f*
losses of methoxypyrazines, 308*f*
oropharyngeal deglutition, 298
process of mastication of solid bolus in sagittal magnetic resonance imaging (MRI) series, 305*f*
quantitation of flavor compounds, 299, 300*f*
real-time MRI, 298
real-time MRI of liquids, 302–304
real-time MRI of solid foods, 304, 305*f*
real-time MRI of swallowing, 299
real-time MRI of swallowing process, 302–304
schematic of anatomy of naso-, oro-, and hypopharynx of sagittal plane, 297*f*
stages of swallowing of liquid bolus in sagittal MRI series, 303*f*
studies of effectiveness of velum closure using helium, 305
swallowing process, 298

Iodine containing compounds, sources, 276
Ion exchange, nitrogen heterocycles, 122
β-Ionone
  biogeneration during black tea manufacture, 236*f*
  biogeneration from β-carotene, 236*f*
  biosynthesis, 235–237
  biosynthetic pathway from β-carotene, 237*f*
  See also Oxygenated aroma compounds
Isothiocyanates, formation from glucosinolates, 140–141
Isotope
  mass spectrometer (MS) detection, 200
  selenium, 282
  stable ^{18}O for oxygen, 201–202
Isotope dilution analysis
  quantitation of potent odorants, 28–29, 30*f*
  roast and ground coffee aroma, 22*f*
  sulfur compounds, 21
  See also Detection and quantitation

J

Juice
  bromophenols in tainted fruit, 276
  See also Grapefruit juice

K

Karamalt
  concentrations of cyclic oxygen-containing carbohydrate degradation products, 217*t*
  relative flavor dilution (Rfd) factors for cyclic oxygen-containing

  carbohydrate degradation products, 216*t*
Krill, dithiazines, 87, 89*t*
Kynurenine, degradation products, 136, 139*f*

L

Labrusca grapes, arylamines, 136
Lager beer. *See* Beer
Lamb
  quantities of alkylthiophenes and alkylthiapyrans for polyunsaturated fatty acid rich diet, 99*t*
  thiamin content, 79*t*
  thiazoles and thiazolines, 95–96
  thiophenes and thiapyrans, 96–97
  See also Cooked meat
Lignin, precursors to oxygenated aroma compounds, 232
Linoleic acid
  alkylthiophene and alkyl-(2*H*)-thiapyran formation, 97–99
  aroma compounds from oxidative degradation, 229*f*
  biogeneration of 1-octen-3-one from, in mushrooms, 239*f*
Linolenic acid
  alkylthiophene and alkyl-(2*H*)-thiapyran formation, 97–99
  aroma compounds from oxidative degradation, 229*f*
  biogeneration of (*E,Z*)-2,6-nonadienal from, in cucumbers, 240*f*
Lipid–Maillard interactions. *See* Cooked meat
Liquid–liquid extraction
  halogenated aroma compounds, 261–262
  method for free fatty acids in Parmesan cheese, 245
  sulfur compounds, 4–5
  See also Analytical methods

M

Magnetic resonance imaging (MRI), real-time
  studying swallowing, 299
  See also In-mouth release of heteroatomic odorants
Maillard reaction
  5-acetyl-2,3-dihydro-1,4-thiazine, 156
  2-acetyl-1-pyrroline, 153–154
  6-acetyl-1,2,3,4-tetrahydropyridine, 157–159
  flavor formation, 79, 81
  formation of organoselenium in food systems, 284
  interaction of lipid, 94
  nitrogen-containing compounds, 115
  nitrogen-containing volatiles, 133
  production of pyrazines and 2-acetyl-1-pyrroline by bacteria, 160–162
  proline-specific compounds, 152*f*
  proteins or amino acids with reducing sugars, 151
  reactions between hydrogen sulfide and intermediates, 82*f*
  safety debate, 151
  Strecker degradation of amino acids, 81, 82*f*
  structural requirements for roasty, biscuit or cracker-like odor, 152, 153*f*
  See also 2-Acetyl-1-pyrroline; 6-Acetyl-1,2,3,4-tetrahydropyridine; Selenomethionine-glucose model system
Maltol
  formation, 221–222, 223*f*
  See also Oxygen heterocycles
Malonaldehyde, derivatization method, 197

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Publication Date: August 7, 2002 | doi: 10.1021/bk-2002-0826.ix002

Marine life, bromophenols, 274–275
Mass spectrometer (MS)
  aroma active compounds using isotopes, 200
  nitrogen heterocycle detector, 127, 128*f*
  oxygenated aroma compounds, 198–199
Meat
  furanthiols and sulfides, 83–84
  polyunsaturated fatty acids (PUFA), 94–95
  See also Cooked meat
Methanethiol
  aroma recombination studies on stewed beef meat juice, 44*t*
  stewed meat juices, 40, 41*t*
Methional
  aroma recombination studies on stewed beef meat juice, 44*t*
  beer, 58
  Ehrlich–Neubauer pathway, 58
  loss after different times of rinsing in mouth, 310*f*
  losses by spit-off odorant measurement (SOOM) technique, 309
  odorant in foods, 298*t*
  reduction to methanethiol, 58
  stewed meat juices, 40, 41*t*
L-Methionine
  ammonia elimination, 62
  breakdown products, 58
  cheese flavor development, 62
  cheese ripening process, 63, 65
  cheese technology, 63
  conversion of, and acyl CoA to thioesters, 69*f*
  degradation by amino transferases, 63, 64*f*
  degradation via Ehrlich–Neubauer pathway, 58, 60*f*
  DMS in lager beer, 67
  flavor producing capabilities in *Geotrichum candidum*, 65, 67
  major amino transferases, 62–63
  methional to methanethiol, 58
  methylthioacetate (MTA) production, 63, 66*f*
  MTA concentration in lager beer, 65
  possibilities for S-terminal degradation, 58, 61*f*, 62
  second step of degradation by lyase, 63
  sulfur compounds in *G. candidum*, 65, 66*f*
  synthesis of MTA and cell free extracts (CFE) of *G. candidum*, 67
  thioacetal production, 67
  transamination producing α-keto-γ-methylthiobutyric acid (KMBA), 62
Methionol
  loss after different times of rinsing in mouth, 310*f*
  losses by spit-off odorant measurement (SOOM) technique, 309
  odorant in foods, 298*t*
Methoxypyrazines
  important heteroatomic odorants in foods, 298*t*
  labeled standards in stable isotope dilution assays, 300*f*
  loss after different times of rinsing in mouth, 310*f*
  losses by spit-off odorant measurement (SOOM) technique, 306, 308
  quantitation method, 299
  See also Pyrazines
Microorganisms
  diversification of products, 54–55
  See also Biogeneration of sulfur compounds
Modeling
  alkylthiophene and alkylthiapyran formation in fatty acid containing system, 97–99

cooked meat aromas, 40, 42*f*
  olive oil, 36–37
  See also Selenomethionine-glucose model system
Mouth. *See* In-mouth release of heteroatomic odorants
Mushrooms, biogeneration of 1-octen-3-one, 239*f*
Mutton, dithiazines, 87, 89*t*

N

Nitriles, formation from glucosinolates, 140–141
Nitroalkanes, biosynthesis, 138, 140
Nitrogen chemiluminescence detector (CLND), characteristics, 125, 127*t*
Nitrogen-containing compounds
  foods, 76*t*
  See also French blue cheeses; Nitrogen heterocycles
Nitrogen heterocycles
  acid/base fractionation simplifying chromatogram, 120*f*, 121*f*
  application of nitrogen-selective detection, 126*f*
  characteristics of nitrogen-specific detectors, 127*t*
  distillation, 117–118
  dynamic headspace (purge and trap) sampling, 122–123
  influence of physicochemical properties on analytical, 115–117
  ion exchange, 122
  isolation and concentration, 117–124
  mass spectrometer as specific detector for, 127, 128*f*
  nitrogen-selective detectors, 125
  physicochemical data, 116*t*
  separation and detection, 124–129
  simultaneous steam distillation/extraction (SDE), 119, 122
  solid phase micro-extraction (SPME), 123–124
  solvent extraction, 118–119
  supercritical carbon dioxide extraction, 119
Nitrogen–phosphorus detector (NPD), characteristics, 125, 127*t*
(*E,Z*)-2,6-Nonadienal
  biogeneration from linolenic acid in cucumbers, 240*f*
  biosynthesis, 239–240
  See also Oxygenated aroma compounds
Nose, gas chromatograph coupling, 200–201

O

Octanoic acid
  OAV (odor activity value), 252*t*
  OAVs of brands of Parmesan cheese, 253*t*
  See also Parmesan cheese
1-Octen-3-one
  biogeneration from linoleic acid in mushrooms, 239*f*
  biosynthesis, 238–239
  See also Oxygenated aroma compounds
Odor activity values (OAVs)
  definition, 32, 214
  odorants of fresh basil leaves, 33*t*
  potent odorants of boiled beef, 45*t*
  wine, 39
  See also Parmesan cheese
Odorants. *See* In-mouth release of heteroatomic odorants
Odor thresholds
  oxygen-containing sugar degradation products, 215*f*
  perception, of wine constituents, 39*t*
  sulfur compounds in relation to structure, 48*t*
  sulfur compounds in water, 78*t*
  sulfur-containing compounds, 30–31

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Publication Date: August 7, 2002 | doi: 10.1021/bk-2002-0826.ix002

Off-flavors
  processing or storage, 48–49
  sulfur-containing odorants, 48–50
Olive oil
  character-impact sulfur compounds, 34*t*
  food aromas, 36–37
  key odorants, 36*t*
  model mixture, 36, 37*f*
  sensory profiling of model, 37*f*
Omission tests
  basil leaves, 33
  basil model mixture, 33*t*
  boiled beef, 45*t*
  camembert cheese, 322–324
  characterizing test-active compounds, 314
  coffee, 45–46
  extraction of goat and camembert cheeses, 315
  extraction of tomatoes, 315
  extraction procedures, 315
  food products, 314
  future work for camembert cheese, 326
  goat cheese, 318–322
  gustatory profile of water-soluble extracts (WSE) and model WSE (MWSE), 320*f*
  high performance liquid chromatography–mass spectrometry (HPLC–MS) analyses of extracts, 316
  individual omissions of mineral salts on MWSE, 321*t*
  MWSE, 317
  omissions of lactose, amino acids, lactic acid, and mineral salts on MWSE, 321*t*
  omissions of organic acids, sugars, amino acids, mononucleotides, and mineral salts from model tomato juice, 325*t*
  omissions of peptide fractions from MWSE of camembert cheese, 324*t*
  omissions of peptides, amino acids, peptides/amino acids, NaCl, KCl, $CaCl_2/MgCl_2$ from MWSE of camembert cheese, 323*t*
  panel training, 316
  physico–chemical analysis, 315–316
  protocol for sensory analyses, 317
  sensory analyses methods, 316–318
  sensory evaluation by, 317–318
  statistical analysis, 318
  tomato juice, 324–326
Onion
  character-impact sulfur compounds, 34*t*
  effect of temperature gradient on volatile decomposition, 13*f*
  high performance liquid chromatography (HPLC), 14*f*
On-line monitoring. *See* Selective chemosensors development
Orange oil, 2-pentylpyridine, 141, 143
Organoselenium compounds
  garlic, 283–284
  interactions between, and sulfur compounds, 285, 290, 292*f*
  Maillard reaction, 284
  See also Selenomethionine-glucose model system
3-Oxazolines
  chirality and enantiomeric ratios, 175, 178
  compounds in blue cheeses, 173*f*, 174
  EI mass spectra, 176*f*, 177*f*
  identification, 171, 172*t*
  odor and chiral properties, 174, 175*t*
  syntheses, 169
  See also French blue cheeses
Oximes, biosynthesis, 138, 140

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Publication Date: August 7, 2002 | doi: 10.1021/bk-2002-0826.ix002

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Publication Date: August 7, 2002 | doi: 10.1021/bk-2002-0826.ix002

Oxygenated aroma compounds
  abundance, 228–232
  amino acid precursors, 230
  biogeneration of β-damascenone from allenic and acetylenic carotenoids, 238*f*
  biogeneration of β-ionone during black tea manufacture, 236*f*
  biogeneration of β-ionone from β-carotene, 236*f*
  biogeneration of (*E,Z*)-2,6-nonadienal from linolenic acid in cucumbers, 240*f*
  biogeneration of 1-octen-3-one from linoleic acid in mushrooms, 239*f*
  biosynthesis, 232–240
  biosynthetic pathway for formation of β-ionone from β-carotene, 237*f*
  biotechnological process for sotolon production, 235
  conversion of amino acids into aroma of banana, 231*f*
  β-damascenone, 237–238
  fatty acid biosynthesis in plants, 230*f*
  fatty acid precursors, 228–230
  flavor research, 228
  formation of sotolon as function of temperature and alcohol content, 233*f*
  3-hydroxy-4,5-dimethyl-2(*5H*)-furanone (sotolon), 232, 235
  β-ionone, 235–237
  kinetics of formation of sotolon, α-ketobutyric acid, and acetaldehyde, 234*f*
  lignin precursors, 232
  lipid oxidation mechanisms, 228–229
  (*E,Z*)-2,6-nonadienal, 239–240
  normal fatty acid metabolism, 230
  1-octen-3-one, 238–239
  oxidative degradation of linoleic and linolenic acids, 229*f*
  prevalence in food, 227–228
  proposed biogeneration of sotolon in French wine, 233*f*
  proposed formation of sotolon in fenugreek, 232, 234*f*
Oxygen-containing aroma compounds
  atomic emission detector (AED), 199
  boiling temperature and solubility, 193*t*
  derivatization, 196–198
  detectors, 198–201
  dynamic headspace, 193–194
  dynamic headspace with liquid–liquid solvent extraction, 194
  gas chromatography–mass spectrometry (GC–MS), 200
  headspace, 193–195
  human nose, 200–201
  infrared detectors, 199
  instrumentation, 198–202
  isolation techniques, 193–198
  isotopic ^{18}O techniques, 201–202
  mass spectrometer (MS), 198–199
  MS using isotopes, 200
  oxygen sensitive flame ionization detector (FID), 200
  sniffing techniques, 201
  solid phase microextraction (SPME), 194–195
  solvent extraction, 195–196
  SPME combination with derivatization, 197–198
  stable isotope of oxygen (^{18}O), 201–202
  volatile carbonyl compounds reacting with 2,4-dinitrophenyl hydrazine, 197
  See also Oxygen heterocycles
Oxygen heterocycles
  amounts of 3(*2H*)-furanones from different carbohydrates, 219*t*
  2,3-butandione, 211*f*, 212
  classification, 209, 212

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Publication Date: August 7, 2002 | doi: 10.1021/bk-2002-0826.ix002

concentration of carbohydrate degradation products, 217*t*
enzymes catalyzing insertion of oxygen into biomolecules, 208*f*
examples of potent food odorants, 211*f*
flavor contribution of carbohydrate derived, 212–214
formation of 4-hydroxy-2,5-dimethyl-3(*2H*)-furanone (4-HDF) by Aldol reaction, 220*f*
formation, 218–222
formation of 3-hydroxy-2-methyl-pyran-4-one and 3,5-dihydroxy-2-methyl-5,6-dihydropyran-4-one from 1-desoxyosone of glucose or maltose, 223*f*
formation of 3-hydroxy-2-methyl-pyran-4-one from different carbohydrates, 222*t*
formation of 3-hydroxy-4,5-dimethyl-2(*5H*)-furanone from 2,3-butandione and hydroxyacetaldehyde, 222*f*
formation of 4-hydroxy-5-methyl- or 4-HDF from ribose or rhamnose, 218*f*
2(*5H*)-furanones, 221, 221*f*
3(*2H*)-furanones, 218–221
4-HDF, 213
4-HDF from acetylformoin (AF), 219*t*
4-hydroxy-2-ethyl-3-methyl-3(*2H*)-furanone (4-HEMF), 213
influence of introduction of oxygen function into n-hexane on boiling points of volatiles, 211*f*
influence of pH on 4-HDF formation from 2-oxopropanal and 4-oxopropanol, 220*t*
influence of pH on 4-HDF formation from ribose, glucose, and rhamnose, 220*t*
maltol formation, 221–222, 223*f*
odor activity value (OAV) concept, 214
odor thresholds of oxygen-containing sugar degradation products, 215*f*
ranking odorants by aroma extract dilution analysis (AEDA), 214
reactions during food processing, 209
reactions leading to loss of oxygen from biomolecules during food processing, 210*f*
relative flavor dilution (Rfd) factors for carbohydrate degradation products, 216*t*
screening techniques, 212
sources in biosyntheses, 208–209
structures of carbohydrate derived odorants, 213*f*

P

Parmesan cheese
  analysis by gas chromatography/olfactometry–mass spectrometry (GC/O–MS), 246–247
  aroma extraction dilution analysis (AEDA), 248–250
  branched chain fatty acids, 249–250
  distribution of free fatty acid (FFA), 244
  experimental samples, 244–245
  extraction method, 245
  FFA description, 248
  FFA profiles of various brands, 253*t*
  FFA quantification method, 247–248
  FFAs in various brands, 252*t*
  flavor dilution (FD) values of acidic fraction, 249*f*
  fractionation method, 246
  high vacuum distillation method, 245
  OAV (odor activity value) method, 248
  OAV results, 250–252

- OAVs of selected aroma compounds, 252*t*
- OAVs of various brands, 253*t*
- sensory thresholds, 251
- straight short chain FFAs, 248
- use, 244
- *See also* Cheese

Pasteurization
- effect on sulfur compounds in grapefruit juice, 109–110
- *See also* Grapefruit juice

Peanuts
- dithiazines, 87, 89*t*
- thiamin content, 79*t*

Penicillium caseicolum, pyrazine production, 160

Peptides. *See* Biogeneration of sulfur compounds

Phosphoadenosyl phosphosulfate (PAPS), beer fermentation, 55, 57

Pizza
- applications of 2-(1-hydroxyethyl)-4,5-dihydrothiazole (HDT), 182–183
- impact of HDT as aroma precursor, 186, 190*t*

Polysulfides, selected foods, 77*t*

Polyunsaturated fatty acids (PUFA)
- alkylthiophenes and alkyl-(2*H*)-thiapyran formation, 97–99
- effect on meat volatiles, 94–95
- thiazoles and thiazolines, 95–96
- thiophenes and thiapyrans, 96–97
- *See also* Cooked meat

Pork
- dithiazines, 87, 89*t*
- key odorants in stewed meat juices, 41*t*
- thiamin content, 79*t*
- *See also* Cooked meat

Potent odorants
- quantitation, 28–29
- screening, 28

Precipitation, concentrating thiols, 10

Processing
- effect on sulfur compounds, 109–110
- off-flavor generation, 48–49
- *See also* Oxygen heterocycles

Pseudomonas perolens, pyrazine production, 160–161

Pulsed flame photometry detection (PFPD)
- characteristics of detector, 125, 127*t*
- organic compounds, 17–19
- use of gated amplifiers, 18
- *See also* Detection and quantitation

Purge and trap
- beef sulfur volatiles at two purging temperatures, 9*f*
- capillary cryogenic trap, 8, 10*f*
- carbon as trapping material for sulfur compounds, 7
- dynamic headspace, 6–8
- influence of sample temperature during sparging, 8*f*
- nitrogen heterocycles, 122–123
- *See also* Analytical methods

Pyrazines
- alkylpyrazines, 143–144, 146*f*
- biosynthesis, 143
- contribution to flavor of foods, 115
- dihydropyrazines reacting with aldehydes, 146*f*
- eliciting roasted note, 179
- formation of 3-alkyl-2-methoxypyrazine, 161*f*
- 2-methoxy-3-isopropylpyrazine (MIP) production, 144–145
- methoxypyrazines, 144–145, 147
- MIP formation mechanism, 145, 146*f*
- occurrence of 2-methoxy-3,5-dialkylpyrazines, 147, 148*f*
- production by bacteria, 160–162
- structural diversity of alkylpyrazines, 144
- vacuum distillation and solvent extraction, 118

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Publication Date: August 7, 2002 | doi: 10.1021/bk-2002-0826.ix002

- yields after 40, 60, and 80 minutes heating, 287*t*, 288*t*
- yields in buffered and unbuffered solutions of selenomethionine-glucose model, 290, 291*f*
- *See also* Methoxypyrazines; Selenomethionine-glucose model system

Pyridines
- biosynthesis, 141, 143
- Chichibabin reaction, 142*f*
- eliciting roasted note, 179
- 2-pentylpyridine in orange oil, 141, 143

Pyrrolines, eliciting roasted note, 179

Q

Quantitation
- potent odorants, 28–29, 30*f*
- *See also* Detection and quantitation

R

Real-time magnetic resonance imaging (MRI)
- method for studying swallowing, 299
- *See also* In-mouth release of heteroatomic odorants

Rhamnose. *See* Oxygen heterocycles

Ribose. *See* Oxygen heterocycles

Rice
- important flavor compounds, 150–151
- thiamin content, 79*t*
- *See also* Maillard reaction

Roasted notes
- analyses by gas chromatography (GC) and mass spectrometry (MS), 182
- applications of 2-(1-hydroxyethyl)-4,5-dihydrothiazole (HDT) in pizza, 182–183
- bioconversion, 180, 181*f*
- biogeneration of flavoring preparations, 183–186
- biogeneration of HDT, 183*f*
- compounds eliciting, 179
- experimental, 180–183
- GC showing amounts of 2-acetyl-2-thiazoline (2-AT) by bioconversion and bio/thermal processing, 188*f*
- HDT and 2-AT via fermentation using baker's yeast, 184
- hypothetical formation of 2-AT, 189*f*
- hypothetical formation of 2-methylthiazoline from *N*-acetyl cysteamine, 184*f*
- impact of HDT as aroma precursor, 186, 190*t*
- odorants by GC/olfactometry (GC/O), 185*t*, 187*t*
- preparation of flavoring samples, 182
- procedure for preparation of flavor samples, 181*f*
- sensory evaluation method, 182
- sensory evaluation of spiked samples, 190*t*
- thiazolines, 179–180

Roasting of coffee beans. *See* Selective chemosensors development

S

Safety, debate on Maillard reaction, 151

Sample enrichment, techniques, 10

Sauvignon blanc wine. *See* Wine

Scheurebe wine
- *S*-containing odorants, 40
- sensory relevance, 41*f*
- *See also* Wine

Seafood
- bromophenols, 274–275
- dithiazines, 87, 89*t*

Selective affinity, column materials for thiols, 11
Selective chemosensors development
  chemosensor recognition of 2-furfurylalcohol, 342*f*
  detection of hydroxy-2-propanone, 335–336
  development by means of selective odorant measurement by multisensor array (SOMMSA) technology, 333–338
  evaluation of chemosensing elements, 331
  experimental, 330–331
  experimental set-up for on-line chemosensor measurement of flavor development during coffee bean roasting, 340*f*
  2-furfurylalcohol as likely sensor-active indicator, 335
  GC/SOMMSA technology, 331
  Headspace (HS)-chromatogram and flavor dilution chromatogram of coffee, 332*f*
  identification of key coffee odorants by HS–GC/olfactometry (HS–GC/O), 330
  identification of key odorants in HS of roasted coffee powder by HS–GC/O, 331–333
  influence of operating temperature on sensing elements on response pattern to coffee headspace, 337*f*
  influence of roasting degree of coffee beans on relative conductivity change of selected chemosensors, 338*f*
  influence of roasting degree on 2-furfurylalcohol and 2-furfurylthiol concentrations in coffees, 336*f*
  model roasting experiments and on-line measurements by cyclic operating temperature alteration (COTA) technology, 339–341
  on-line measurements, 331
  principle of COTA technology, 340*f*
  response of three chemosensors to volatiles in headspace above coffee, 334*f*
  sensitivity of 2-furfurylalcohol detection, 335
  signals of WO_3-sensor by COTA technology during coffee roasting, 342*f*
  suitability for sensing elements for 2-furfurylalcohol, 336, 338
Selective odorant measurement by multisensor array (SOMMSA) technology
  chemosensor responses to volatiles in headspace above coffee, 334*f*
  development of chemosensors, 333–338
  evaluation of chemosensing elements, 331
  See also Selective chemosensors development
Selenium
  bioavailability, 283
  biological functions, 282–283
  chemical properties, 282
  Maillard reaction, 284
  organoselenium compounds in garlic, 283–284
  See also Selenomethionine-glucose model system
Sensory relevance of sulfur compounds in food
  aroma of roasted coffee model, 46*t*
  aroma recombination studies on stewed beef meat juice, 44*t*
  character-impact sulfur compounds in foods, 34–35
  chromatogram of Sauvignon blanc wine extract, 27*f*
  coffee, 45–46
  concentrations and OAVs (odor activity values) of odorants of fresh basil leaves, 33*t*

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- concentrations and OAVs of potent odorants of boiled beef, 45*t*
- cooked meat, 40–44
- effect of odorants on aroma profile of model mixtures, 37*f*
- experimental procedures, 26–33
- isolation of thiols, 26–27
- key odorants in Spanish olive oil, 36*t*
- key odorants in stewed meat juices, 41*t*
- key volatile constituents of fresh tomato aroma, 32*t*
- OAV concept, 32
- odor profile of stewed beef juice and model aroma, 42*f*
- odor thresholds, 30–31
- off-flavors, 48–50
- olive oil, 36–37
- omission experiments in stewed beef model aroma, 43
- omission tests with basil model mixture, 33*t*
- perception odor thresholds of wine constituents, 39*t*
- quantitation by isotope dilution assays, 30*f*
- quantitation of potent odorants, 28–29
- results of beef omission experiments, 45*t*
- Scheurebe wine, 40, 41*f*
- screening of potent odorants, 28, 29*f*
- sensory profiling of model aroma vs. Spanish olive oil, 37*f*
- sensory studies, 32–33
- structure-odor activity relationships, 46–48
- studying interaction of odorants, 43
- thiols eliciting common odor-activity relationships, 47*f*
- trapping thiols with *p*-hydroxymercuric acid (pHMB), 27*f*
- wine, 38–40

Separation techniques
- gas chromatography (GC), 11–12
- high performance liquid chromatography (HPLC), 12, 14
- nitrogen heterocycles, 124–129
- *See also* Analytical methods

Shrimp, dithiazines, 87, 89*t*

Simultaneous distillation–extraction (SDE)
- nitrogen heterocycles, 119, 122
- sulfur volatiles, 4

Solid phase microextraction (SPME)
- combination with derivatization, 197–198
- comparison to dynamic headspace, 195
- fiber-to-fiber sampling efficiency variation, 7*f*
- halogenated aroma compounds, 263
- isolation for oxygen-containing aroma compounds, 194–195
- nitrogen heterocycles, 123–124
- static headspace sampling for sulfur compounds, 5–6
- *See also* Analytical methods

Solubility, isolation of aroma compounds, 193

Solvent extraction
- acid/base fractionation, 120*f*, 121*f*
- nitrogen heterocycles, 118–119
- oxygen-containing aroma compounds, 195–196

Sotolon
- biosynthesis, 232, 235
- biotechnological process for production, 235
- formation as function of temperature and alcohol content, 233*f*
- kinetics of formation, 234*f*
- proposed biogeneration in French wine, 233*f*
- proposed formation in fenugreek, 232, 234*f*

  See also 3-Hydroxy-4,5-dimethyl-2(*5H*)-furanone (3-HDF); Oxygenated aroma compounds
Spanish olive oil. *See* Olive oil
Spinach, off-flavor generation, 49–50
Spit-off odorant measurement (SOOM)
  determining odorant losses during mastication, 302
  losses of 3-alkyl-2-methoxypyrazines, 306, 308
  losses of ethyl butanoate, methional, methionol, 309, 310*f*
  losses of heteroatomic odorants in mouth, 306, 308–309
  See also In-mouth release of heteroatomic odorants
Storage, off-flavor generation, 48–50
Structure-odor activity relationships
  sulfur compounds, 46–48
  thiols, 47*f*
Sublimation
  sulfur compounds, 5
  See also Analytical methods
Sugars
  degradation reactions, 151–152
  See also Oxygen heterocycles
Sulfates
  accumulation by yeast in brewing process, 55–57
  assimilation, 56*f*
Sulfides
  chemical class, 25
  occurrence and formation, 83–84
  odor characteristics, 84*t*
  selected foods, 77*t*
  solid phase micro extraction (SPME), 6
  See also Aroma in heated foods
Sulfites, accumulation by yeast in brewing process, 55–57
Sulfur compounds
  aromas of cooked and uncooked foods, 74
  chemical classes in food flavors, 3
  classes in heated foods, 74–77
  comparison of recoveries from standard mixture of four, for Carboxen–PDMS fibers, 7*f*
  effect of thermal processing, 109–110
  efficiency of diverse ripening bacteria, 68*f*
  foods, 76*t*
  isolation and concentration techniques, 3–11
  literature on occurrence and chemistry, 3
  objectionable odors, 73–74
  odor threshold values in water, 78*t*
  off-flavor generation, 48–50
  See also Analytical methods; Aroma in heated foods; Biogeneration of sulfur compounds; Grapefruit juice; Sensory relevance of sulfur compounds in food
Supercritical fluid extraction (SFE), halogenated aroma compounds, 262–263
Swallowing process
  exhaled ethyl butanoate using exhaled odorant measurement (EXOM) technique, 306, 307*f*
  flavor perception and eating, 296–298
  liquids, 302–304
  mastication of solid bolus in sagittal magnetic resonance imaging (MRI) series, 305*f*
  method for real-time MRI, 299
  real-time MRI, 302–304
  schematic of anatomy in sagittal plane, 297*f*
  solid foods, 304
  stages of swallowing of liquid bolus in sagittal MRI series, 303*f*
  See also In-mouth release of heteroatomic odorants

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T

Taste
  evaluating active compounds, 313–314
  molecules responsible for, 313
  See also Omission tests
Thermal processing, effect on sulfur compounds, 109–110
Thiamin
  breakdown pathways for thermal degradation, 80*f*
  content of foods, 79*t*
  degradation products, 80*f*
  thermal degradation, 78–79
  See also Aroma in heated foods
Thianes, selected foods, 77*t*
Thiapyrans
  alkylthiapyran formation in model systems, 97–99
  beef and lamb, 96–97
  2-ethyl-(*2H*)-thiapyran formation, 98–99
Thiazines, eliciting roasted note, 179
Thiazoles
  2-acetylthiazole in cooked foods, 85
  beef and lamb, 95–96
  chemical class, 25
  occurrence and formation, 84–85, 87
  odor characteristics, 85*t*
  route to alkylthiazoles, 85, 86*f*, 87
  vacuum distillation and solvent extraction, 118
  See also Aroma in heated foods
Thiazolines
  2-acetyl-2-thiazoline (2-AT), 179–180
  beef and lamb, 95–96
  eliciting roasted note, 179
  occurrence and formation, 84–85, 87
  odor characteristics, 85*t*
  See also Aroma in heated foods; Roasted notes
Thiocyanates, formation from glucosinolates, 140–141
Thioesters
  conversion of methionine and acyl CoA, 69*f*
  Geotrichum candidum producing, 65, 67
  See also L-Methionine
Thiolanes, selected foods, 77*t*
Thiols
  artifact formation, 15
  chemical class, 25
  column materials with selective affinity for, 11
  isolation method, 26–27
  isotope dilution analysis of coffee, 21, 22*f*
  low temperature vacuum distillation, 11
  odorants, 3
  odor characteristics, 84*t*
  precipitation, 10
  quantitation by isotope dilution assays, 28–29, 30*f*
  selected foods, 77*t*
  solid phase micro extraction (SPME), 6
  stewed meat juices, 40, 41*t*
  structure-odor activity relationships, 46–48
  trapping with *p*-hydroxymercuric acid (pHMB), 26, 27*f*
Thiophenes
  alkylthiophene formation in model systems, 97–99
  beef and lamb, 96–97
  chemical class, 25
  selected foods, 77*t*
Thiophenones, selected foods, 77*t*
Thiosulfinates
  artifact formation, 15
  white onion, 14*f*
Tomato
  character-impact sulfur compounds, 34*t*
  key aroma volatile constituents, 32*t*

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Publication Date: August 7, 2002 | doi: 10.1021/bk-2002-0826.ix002

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Tomato juice
  composition, 319*t*
  extraction procedure, 315
  omissions of organic acids, sugars, amino acids, mononucleotides, and mineral salts, 325*t*
  omission testing, 324–326
  omission testing method, 317–318
  sample, 314
  See also Omission tests

V

Vacuum distillation
  low temperature for thiols, 11
  nitrogen heterocycles, 117–118
Vacuum sublimation, thiols, 5
Vegetables
  pyridines, 141, 143
  sulfur-containing aroma compounds, 74

W

Wastewater treatment, bromophenols, 276
White onion
  effect of temperature gradient on volatile decomposition, 13*f*
  high performance liquid chromatography (HPLC), 14*f*
  See also Allium
Wine
  aromas, 38–40
  Bordeaux red, 40
  character-impact sulfur compounds, 34*t*
  chromatogram of Sauvignon blanc wine extract, 27*f*
  cork taint, 271–273
  formation of sotolon as function of temperature and alcohol content, 233*f*
  3-hydroxy-4,5-dimethyl-2(5*H*)-furanone (sotolon), 232, 235
  nitrogen depletion in wine fermentation, 57
  perception odor thresholds of constituents, 39*t*
  proposed biogeneration of sotolon in French wine, 233*f*
  Scheurebe wine, 40, 41*f*
  sensory relevance, 39
  structures of volatile S-containing constituents of Sauvignon blanc wine, 38*f*
  thiazolidine derivatization for aldehydes, 198
  volatile S-containing constituents of Sauvignon blanc wine, 38*f*

Y

Yeast
  thiamin content, 79*t*
  See also Biogeneration of sulfur compounds
Yellow passion fruit, thioester detection using flame photometry, 17*f*