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Volatile Sulfur Compounds in Food

EDITED BY
Michael C. Qian, Xuetong Fan,
and Kanjana Mahattanatawee

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Michael C. Qian, Editor
Department of Food Science and Technology
Oregon State University

Xuetong Fan, Editor
Eastern Regional Research Center
Agricultural Research Service
U.S. Department of Agriculture

Kanjana Mahattanatawee, Editor
Department of Food Technology
Siam University

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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 the ACS sponsored symposia based on current scientific research. Occasionally, 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.

As a rule, only original research papers and original review papers are included in the volumes. Verbatim reproductions of previous published papers are not accepted.

ACS Books Department

Preface

Although the importance of sulfur compounds to the flavor and off-flavor characteristics of foods is well known, achieving a complete understanding of how this group of compounds contributes to specific food products has been challenging due to their high reactivity, low sensory thresholds, and low concentration in food systems. Due to the advancement of modern analytical instrumentation with improved sensitivity and reliability, new knowledge on volatile sulfur compounds has been accumulating at a rapid rate. This book brings together intelligent insights and approaches from prominent scientists in the fields of food and flavor to bring a deep understanding about the flavor contributions of sulfur compounds.

It has been more than 15 years since the last ACS book on sulfur compounds in food was published (edited by C. J. Mussinan, et al. 1994). This book has a solid emphasis on volatile compounds, especially sulfur containing compounds that have low boiling points and strong impacts on food odor. A wide range of topics is addressed, including the advances in analytical chemistry for volatile sulfur compounds, occurring of sulfur compounds in food systems, chemical reactions and conversions of sulfur compounds during food processing, as well as sensory and bioactivity aspects of volatile sulfur compounds. This book will be a valuable resource for all scientists and professionals engaging in research, development, and application related to the fields of food and flavor industry. We hope this book will facilitate further research in this important field.

We are grateful to the chapter authors and reviewers who made this book possible.

Michael C. Qian
Department of Food Science and Technology
Oregon State University
100 Wiegand Hall, Corvallis, OR 97330
541-737-9114 (telephone)
michael.qian@oregonstate.edu (e-mail)
541-737-1877 (fax)

Xuetong Fan
Eastern Regional Research Center
Agricultural Research Service
U.S. Department of Agriculture
600 E. Mermaid Lane, Wyndmoor, PA 19038
215-836-3785 (telephone)
Xuetong.Fan@ars.usda.gov (e-mail)
215-233-6445 (fax)

Kanjana Mahattanatawee
Department of Food Technology
Siam University
235 Petkasem Rd., Pharsicharoen, Bangkok 10160, Thailand
662-867-8082 (telephone)
kanjana@siam.edu (e-mail)
662-867-8082 (fax)

Chapter 1

The Significance of Volatile Sulfur Compounds in Food Flavors

An Overview

Robert J. McGorrin

Department of Food Science and Technology, Oregon State University, 100 Wiegand Hall, Corvallis, OR 97331
E-mail: robert.mcgorrin@oregonstate.edu.

Volatile sulfur compounds are important contributors to the characteristic flavors and off-flavors of many foods. As a class, sulfur-containing flavor volatiles have low sensory detection thresholds, are present in low concentration and are often chemically labile, which can present measurement challenges. Advances in analytical separation techniques and instrumentation have enabled understandings of their occurrence and contributions to their relative sensory significance.

Relating the chemistry and sensory contributions of volatile sulfur compounds to food flavor is an ongoing endeavor. The preminent quest in flavor research is to identify and categorize chemical constituents which provide unique sensory characteristics to the aroma and flavor of foods. Sulfur compounds contribute enzymatically-derived flavors in the *Allium* species (garlic, onion, chive) or *Cruciform* families (Brussels sprouts, broccoli, cabbage, cauliflower), and thermally-generated flavors such as roasted meat, chicken, seafood, and coffee. Volatile sulfur compounds play an important role in the aromas of bread, popcorn, nuts, potato products and wine, and contribute subtle flavor characteristics to cheddar cheese, chocolate and tropical fruit flavors, to name a few examples. Additional understandings of biosynthetic pathways, fermentation mechanisms, or thermal processes continue to evolve by which sulfur flavor constituents can be

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produced. As a result, new knowledge of unique sulfur flavor chemicals and their generation pathways facilitates improved quality of food processing, storage, and flavor systems.

Significant progress has been made with investigations of sulfur compounds since the previous ACS Symposium Series book devoted to this topic over 17 years ago (*1*). The intent of this chapter is to provide an updated summary of volatile sulfur compounds that are important contributors to the flavor of meats, seafood, fruits, vegetables and beverages.

Sensory Impact of Sulfur Volatiles

Volatile constituents of food flavors occur as complex mixtures in concentrations ranging from low parts-per-million to parts per trillion. As a class, sulfur flavor compounds are typically present in foods at extremely low levels, often at sub parts-per-billion concentrations. Some of these volatile components provide background sensory nuances to the flavor, while others provide unique flavor characterizing identities to the foods they occur in. The latter unique flavor substances are referred to as "character-impact compounds", and have been recently reviewed (*2*).

When smelled or tasted, a single character-impact chemical or blend of characterizing chemicals contributes a recognizable sensory impression at the typically low concentration levels in natural flavors. Examples of sulfur-containing character-impact compounds include diallyl disulfide (garlic), allyl isothiocyanate (mustard), and 2-furfurylthiol (coffee) (*2*). In some situations, flavor concentration and food context influence sensory perception. For example, at high concentrations dimethyl sulfide conveys an odor reminiscent of cabbage, but when present at reduced levels in the context of heat-treated corn, it conveys the typical flavor impression of canned corn. The relative sensory impact of a flavor compound depends on its individual odor threshold and its concentration in the food. As a class, volatile sulfur compounds exhibit sensory potency at low concentrations due to their low aroma and taste thresholds. In most foods, sulfur compounds contribute appropriate flavor character at low concentrations (< 1 μg/kg), but at higher concentrations, their aromas are perceived as sulfurous and objectionable. The olfactory perception (character and intensity) of sulfur compounds also depends on their diastereomeric and enantiomeric form, if they are able to exist as chiral isomers.

An indirect method to assess whether thiols are contributors to flavor impact is to simply add copper to samples. This methodology was used to assess the organoleptic role of thiols in beer flavor, because copper is selectively able to trap them into odorless chemical complexes (*3*)

Enantiospecific Odor Differences

It is known that the enantiomers of chiral flavor compounds often have different sensory properties (*4*). In some cases, one chiral form may exhibit a lower flavor threshold relative to its epimer. In other situations, the aroma may

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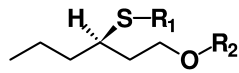
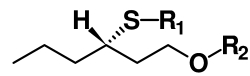
change flavor character between the two enantiomeric forms, or shift in character from odor to odorless. The sensory properties of enantiomers of sulfur volatile compounds have been described and compared (*4*–*6*).

An example of the enantiomeric differences among 3-thio-1-hexanols and their esters, which contribute to the odor and taste properties of yellow passion fruits, is shown in Table I. In general, the (*R*)-enantiomers have a tropical fruit aroma, whereas the (*S*)-enantiomers are sulfur and herbaceous. The stereodifferentiation and odor assessments of naturally occurring chiral sulfur volatiles should provide better insights of their relative flavor impact.

Table I. Sensory Properties for Enantiomeric 3-Thio- and 3-Methylthiohexanols and their Esters. (Adapted from reference (*4*). Copyright 2006 American Chemical Society)

	 (*R*)-isomer	 (*S*)-isomer
	(R)-isomer	***(S)-isomer***
$R_1 = R_2 = H$	intense sulfur odor[a]	intense sulfur odor[a]
$R_1 = H$, $R_2 = COCH_3$	tropical fruit	sulfur, herbaceous
$R_1 = H$, $R_2 = CO\text{-}Pr$	tropical fruit	sulfur, oniony
$R_1 = H$, $R_2 = CO\text{-}Amyl$	herbaceous,fresh sulfur	burnt sulfur
$R_1 = CH_3$, $R_2 = H$	herbaceous, weak	exotic, fruity
$R_1 = CH_3$, $R_2 = COCH_3$	fruity	int. sulfur, herbaceous
$R_1 = CH_3$, $R_2 = CO\text{-}Pr$	fruity, very weak	oniony
$R_1 = CH_3$, $R_2 = CO\text{-}Amyl$	fruity, very weak	weak oniony, roasty

[a] No odor difference.

Analytical Measurements

Sulfur compounds have long been the target of study of flavor chemists, however they have presented unique analytical challenges to surmount during the isolation and identification process (*7*, *8*). Specific issues are the tendency of reduced sulfur compounds (e.g., thiols) to oxidize, rearrange, or isomerize under mild heating conditions or during concentration from food matrices (*8*).

Analytical methods employed to identify volatile sulfur compounds in foods and beverages have been recently reviewed (*9*). Several current isolation and concentration techniques will be discussed.

Extraction of Thiols

p-Hydroxymercuribenzoic acid (*p*HMB) is used for selective trapping of thiols. This organomercury compound has been applied to determine polyfunctional thiols in beer (*10*, *11*), hops (*11*, *12*), and cheese (*13*). Typically, the sodium salt of *p*HMB is used to directly extract thiols from aqueous samples, while *p*HMB is used to concentrate thiols from organic solvent extracts of flavor compounds. The thiol-*p*HMB complexes are loaded on a strongly basic anion-exchanger column, and volatile thiols are released by percolating a hydrochloride L-cysteine monohydrated solution. Elution of beverages (e.g., wine) through a column containing a *p*HMB absorbent is an alternate approach (*14*). Thiols in flavor extracts can also be enriched on agarose gel grafted with *p*-aminophenyl-mercuric acetate, then desorbed with a 10 mM dithiothreitol solution (*15*).

Isolation of Sulfur Volatiles

Selection of flavor extraction and isolation procedures for sulfur compounds depends on the type of matrix and relative volatility of compounds of interest. Consequently, there is not a universal flavor isolation method. Several reviews have summarized analytical techniques for isolation and quantification of volatile flavor compounds (*16–18*). These include solid-phase micro-extraction (SPME) (*19*), stir bar sorptive extraction (SBSE) (*20*), headspace adsorption, vacuum distillation, simultaneous distillation/extraction, and solvent-assisted flavor evaporation (SAFE). Because reduced sulfur compounds are labile to oxidation and thermal rearrangements, care must be taken during isolation procedures to avoid formation of artifacts. It is recommended that borosilicate glass vials and contact surfaces, such as the GC injection liner, should be silylated to deactivate reactive sites prior to chromatographic analysis (*21*).

Multidimensional GC-MS

The separation and identification of trace sulfur compounds in complex food matrices generally requires numerous purification steps to suppress major volatile compounds (*19*, *22*). Multidimensional gas chromatography, especially heart-cut bidimensional gas chromatography, now offers an elegant alternative approach for identifying trace odorants. This hyphenated technique, combined with olfactometry, has recently been used to identify sulfur flavors in wines (*23*) and beer (*11*), thus suggesting new solutions for trace analysis.

Two-Dimensional GC

Comprehensive two-dimensional gas chromatography (GCxGC) is a powerful analytical tool for enhancement of separation capacity and mass spectral resolution (*24*). This hyphenated technique, often used in tandem with mass spectrometry, provides more accurate and sensitive quantification for improved characterization of flavor volatiles in complex mixtures. Advantages to this technique are that it

considerably shortens the identification process and, of particular importance for reactive sulfur compounds, it avoids enrichment steps that might result in artifact formation. GCxGC-TOFMS recently was used to identify a novel *O,S*-diethyl thiocarbonate in Indian cress (*25*).

GC Detectors

Gas chromatography with pulsed flame photometric detection (GC-PFPD) is a sulfur-specific detector with detection limits of 1 pg (10^{-12} g). A comparison of linear range, detection limits and selectivity of sulfur detectors has been described (*9*). The PFPD detector provides enhanced sensitivity relative to flame photometric detectors, and lower cost than atomic emission detection. PFPD has been recently utilized to measure sulfur compounds in cheese (*26*, *27*), wine (*21*), and strawberry (*28*, *29*) flavors.

Olfactometry

In recent studies, potent sulfur aroma compounds have been identified using sensory-focused analytical methods such as gas chromatography-olfactometry (GC-O) (*30*, *31*). Three variants of this technique include Charm Analysis™, aroma extract dilution analysis (AEDA), and OSME analysis. The potent sulfur compounds that are identified by these methods represent significant contributors to the flavors of the foods they represent. In some instances, these sensory-focused analytical techniques have led to the discovery of new character-impact sulfur compounds. However in other situations, important sulfur volatiles have been identified that, while they do not contribute character impact, impart significant nuances to the overall flavor.

Artifacts

Because sulfur flavor compounds tend to be thermally unstable, prone to oxidative reactions and present at low concentrations, often challenges occur during their isolation and identification (*8*). Previously, artifacts have been noted as a consequence of high temperatures encountered during sample concentration or in the gas chromatograph (GC) injector or mass spectrometry transfer column. It has been suggested that many reported "novel" sulfur compounds were actually secondary reaction products of fragile sulfur-containing flavorants (*7*). Volatile sufur compounds (e.g., methane thiol, dimethyl sulfide) can undergo thermal oxidation to form dimethyl disulfide and dimethyl sulfoxide, respectively, unless suitable analytical conditions are employed. Changes in dimethyl sulfide/disulfide/trisufide were reported in strawberry puree as a result of heating (*29*). Thermal reactions were reported to influence formation of sulfur compounds in Welsh onions and scallions during their identification; branched polysulfides were found in volatile distillates (*32*), but were absent in solvent extracts (*32*). Conversely, methyl methane thiosulfinate and dialk(en)yl thiosulfonates were predominant in key volatiles from solvent extracts (*32*). More recently, 3-propyl-1,2-oxothiolane identified in Sauternes botrytized wine was

shown to be produced from thermal oxidative degradation of 3-sulfanylhexanol disulfide in the GC injector (*23*). To minimize these situations, flavor chemists should utilize sulfur standards and analytical calibration curves to verify recovery. Alternative low-temperature isolation methods or cool on-column GC injection can often mitigate artifact formation.

Sulfur Constituents of Food Flavors

Key sulfur-containing aroma compounds have been identified in herbs and seasonings, fruit, vegetable, meat, seafood, dairy, and Maillard-type flavors. The occurrence of volatile sulfur compounds in food flavors is the subject of several recent reviews (*33–41*).

Herbs, Spices and Seasonings

A variety of sulfur aroma compounds are present in the isolates of spices and herbs, which are commonly used as seasonings in foods. The *Allium* family includes garlic, onion, leek, and chive, all of which are comprised of sulfur-containing character impact compounds. The aroma impact constituents of garlic include diallyl disulfide and the corresponding thiosulfinate derivative (allicin), which are enzymatically released from a sulfoxide flavor predursor (alliin) during the crushing of garlic cloves (*35*). A listing of character-impact sulfur compounds found in herb and seasoning flavors is presented in Table II.

The flavor chemistry of sulfur compounds in onion is quite complex (*32*, *43*, *44*). Early reports of polysulfides and thiosulfinates were later demonstrated to be thermal artifacts from gas chromatographic analysis (*44*). Character impact sulfur compounds have been proposed for fresh, boiled, and fried onion. In raw, fresh onion, propyl propanethiosulfonate, propenyl propanethiosulfonate thiopropanal S-oxide, and propyl methanethiosulfonate are impact contributors (*32*, *35*, *43*). Several compounds contribute to the aroma character of cooked onion, of which dipropyl disulfide and allyl propyl disulfide provide key impact (*35*). Fried onion aroma is formed by heating the latter compound, and is characterized by 2-(propyldithio)-3,4-dimethylthiophene, which has an odor threshold of 10-50 ng/l in water. More recently, a new highly potent aroma compound, 3-mercapto-2-methylpentan-1-ol, was identified in raw onions (*45*), with an odor threshold of 0.03 μg/L (*46*). The flavor impact of this mercaptyl alcohol is strongly dependent on concentration; at 50 μg/L, it provides a pleasant meat broth-like, onion, and leek-like flavor, while at high levels it gives a strong, unpleasant onion-like quality. Higher concentrations are formed in cooked onions, and it was also found in other *Allium* species including chives, onions, and leeks but not garlic (*47*). Representative structures for sulfur impact compounds in herbs and seasonings are shown in Figure 1.

Table II. Character-Impact Sulfur Compounds in Herbs and Seasonings

Character impact compound (s)	*Odor description*	*Occurrence*	*Reference*
Benzenemethanethiol	garden cress seed	garden cress	(*50*)
Diallyl disulfide	garlic	garlic	(*35*)
Diallylthiosulfinate (allicin)		garlic	(*35*)
4-Pentenyl isothiocyante	mustard/horseradish	horseradish	(*35*)
O,S-Diethyl thiocarbonate	red fruit, sulfury	Indian cress	(*25*)
Allyl isothiocyanate	mustard-like	mustard	(*42*)
Propyl propanethiosulfonate	roasted alliaceous	onion, raw	(*32*)
Propyl methanethiosulfonate		onion, raw	(*32*)
3-Mercapto-2-methylpentan-1-ol	broth, onion, leek	onion, raw	(*45*)
Allyl propyl disulfide	cooked onions	onion, cooked	(*32*)
Dipropyl disulfide	burnt green onion	onion, cooked	(*32*)
2-(Propyldithio)-3,4-Me_2thiophene		onion, fried	(*35*)
Bis(methylthio)methane	sulfury, truffle	truffle	(*48*, *49*)
1,2,4-Trithiolane		truffle	(*48*)

Unique sulfur compounds were identified as providing key aromas in black and white truffles (*48*, *49*) The predominant sulfur component in black and white truffle aroma is dimethyl sulfide, however bis(methylthio)methane and 1,2,4-trithiolane are reported as unique to white truffle aroma (*48*). 1-(Methylthio)propane and 1-methylthio-1-propene were newly identified in black truffle.

Isothiocyanates are character-impact constituents which provide pungency and typical flavor to mustard (allyl isothiocyanate), radish, *trans*-4-(methylthio)-3-butenyl and *trans*-4-(methylthio)butyl isothiocyanate) and horseradish (4-pentenyl and 2-phenylethyl isothiocyanate) (*35*). Garden cress (peppergrass) is classified in the *Brassica* (*Cruciferae* /mustard) family, and its leaves and seeds were historically used as a spicy condiment to contribute "peppery" flavor. Benzenemethanethiol was identified as the character-impact flavor for the unique flavor of garden cress (*Lepidium sativum*) seed, and also occurs in the volatile extracts of potatoes (*50*). Recently, a novel sulfur volatile, *O,S*-diethyl thiocarbonate was identified in Indian cress (*Tropaeolaceae* family), providing a "red fruity-sulfury" character (*25*). A series of thiocarbonate homologues were synthesized with "sulfury", "fruity" and "pineapple" aromas.

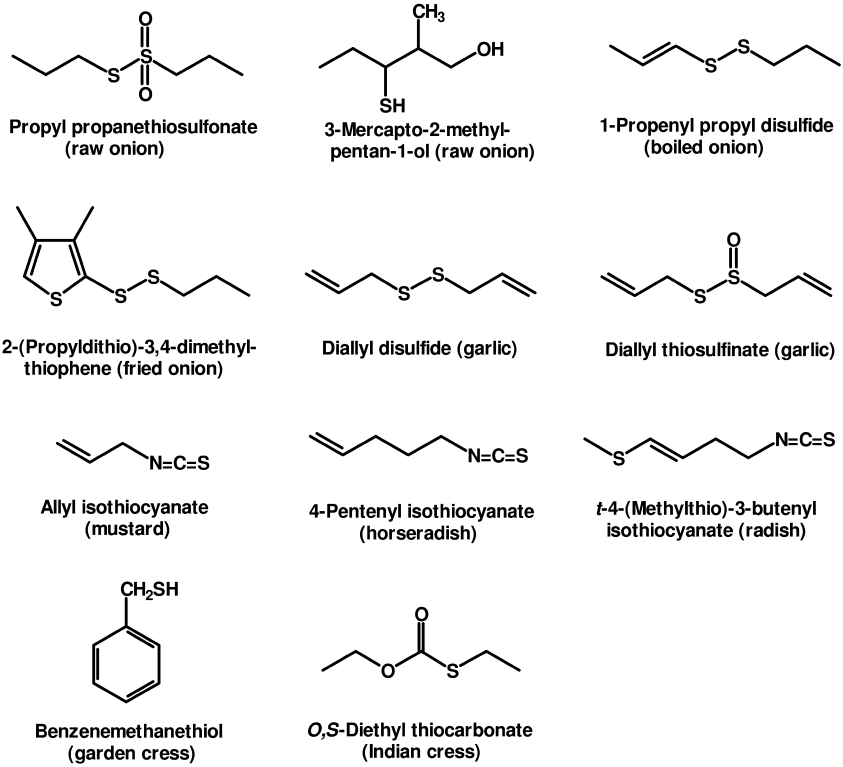


Figure 1. Character-impact sulfur flavor compounds in herbs and seasonings.

Fruit Flavors

The volatile composition of fruit flavors is extremely complex, and non-characterizing volatile esters are common across species. However, trace sulfur volatiles have been identified which play significant roles in the flavor of grapefruit, wine, strawberry, passion fruit, and other tropical fruits. A compilation of character-impact sulfur compounds in fruit flavors is summarized in Table III.

Ethyl 3-mercaptopropionate provides the pleasant fruity fresh grape aroma in Concord grape at low parts-per-million levels (*51*). It is well-established that characteristic wine flavors are produced from secondary metabolites of grapes and yeast during the fermentation process, which increase the chemical and aroma complexity of wine (*52*). For example, in wines, there is no single compound that characterizes its fruity aroma, but rather a complex blend of odorants (*53*). Wines made from certain *vinifera* grape varieties possess unique "fruity, fresh, green" character contributed from polyfunctional thiols, including 2-furfurylthiol, benzyl mercaptan, 3-mercaptohexan-1-ol, and 3-mercaptohexyl acetate in white and rose´ wines ((*54*) *and references cited therein*.). The latter two mercaptans are discussed in more detail below in the context of tropical fruit flavor character. Hydrogen sulfide, methanethiol, and methylthioacetate were measured at low-ppb

levels in Pinot Noir, Cabernet Sauvignon, Pinot Grigio, and Chardonnay wines; methionol was present at ppm levels. At these concentrations, they contribute a positive background impression to wine aroma, however at higher amounts they are responsible for "reduced", "rotten egg" sulfury off-flavors (*21*).

4-Mercapto-4-methyl-2-pentanone (cat ketone) provides "catty" character and typical flavor of Sauvignon blanc wine (and also Scheurebe) at an aroma perception threshold below 3 ppt (*54–57*). It is also a characteristic flavorant in Japanese green tea (sen-cha) (*58*), a main contributor to the "fruity" aroma in beer made with certain hops (*11*), and contributes significantly to the flavor of hand-squeezed grapefruit juice (*59*). Representative chemical structures for sulfur-containing character-impact compounds in fruits are shown in Figure 2.

Table III. Character-Impact Sulfur Compounds in Fruits

Character impact compound (s)	*Odor description*	*Occurrence*	*Reference*
4-Methoxy-2-methyl-2-butanethiol	blackcurrant, sulfur	blackcurrant	(*35*)
8-Mercapto-*p*-menthan-3-one	blackcurrant, catty	blackcurrant (synthetic)	(*34*)
4-Mercapto-4-methyl-2-pentanone	cat urine	grape, Sauvignon	(*55*)
		grapefruit	(*59*)
1-*p*-Menthene-8-thiol	juicy grapefruit	grapefruit	(*62*)
Ethyl-3-mercaptopropionate	fresh grape, foxy	grape, Concord	(*51*)
3-Methylthiohexan-1-ol	green, vegetable	passion fruit	(*33*)
2-Methyl-4-propyl-1,3-oxathiane	tropical, green	passion fruit	(*63*)
3-Mercaptohexan-1-ol	citrus, tropical fruit	passion fruit, wine	(*54*, *64*)
3-Mercaptohexylacetate	black currant	Riesling, pink guava	(*33*, *70*)
Ethyl 3-(methylthio)propionate	sulfury pineapple	pineapple	(*74*, *75*)
	green, fresh melon	muskmelon	(*72*, *73*)
Methyl thioacetate	cheesy, garlic	strawberry	(*29*)
Methyl thiobutanoate	cheesy, cabbage	strawberry	(*29*)
3,5-Dimethyl-1,2,4-trithiolane	sulfury, onion	durian	(*77*)

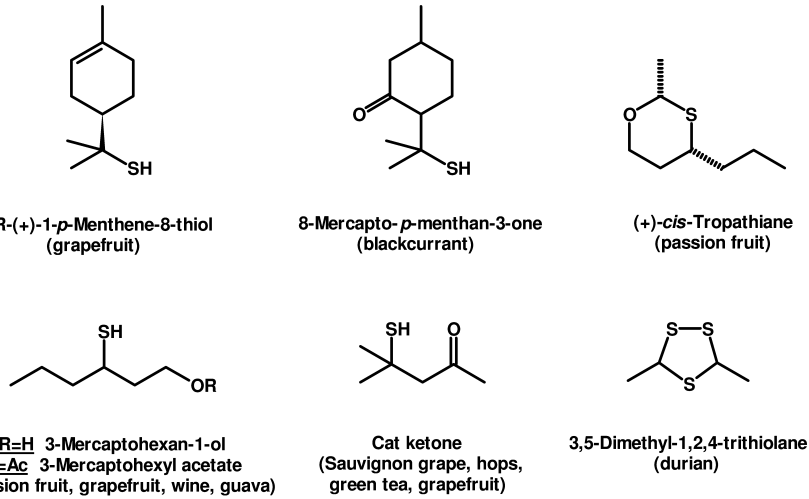


Figure 2. Representative character-impact sulfur flavor compounds in fruits.

Blackcurrant flavor is popular in Europe, and is associated with numerous health-related functional foods and alcoholic drinks (cassis liqueur). The key aroma component in blackcurrant is 4-methoxy-2-methyl-2-butanethiol (*35*). The "catty/ribes" flavor of blackcurrant (*Ribes nigrum*) was earlier attributed to "cat ketone", but it was later shown to be absent during flavor and sensory analysis of blackcurrant juice concentrates (*60*). 2-Methyl-3-furanthiol ("cooked meat") was among the five most potent aroma compounds recently identified by GC-olfactometry in blackcurrant juice (*61*). The flavor chemical 8-mercapto-*p*-menthan-3-one contributes a powerful blackcurrant, cassis and catty-like aroma character, however it has not been identified in the natural fruit (*34*). This unique mercaptan is synthesized by reaction of hydrogen sulfide with (-)-pulegone from buchu leaf oil.

The fresh juicy note of grapefruit juice is attributable to 1-*p*-menthene-8-thiol. This compound has a detection threshold of 10^{-1} parts-per-trillion (ppt), among the lowest values reported for aroma chemicals (*62*). The (+)-R-isomer was found to have a lower aroma threshold in water than the racemic mixture, and it imparts a pleasant, fresh grapefruit juice character, as opposed to the extremely noxious sulfur note contributed by the (-)-S-epimer.

The "tropical fruit" category is one of the most important areas for new discoveries of key impact flavor compounds. Analyses of passion fruit have produced identifications of many potent sulfur aroma compounds (*33*). Among these is tropathiane, 2-methyl-4-propyl-1,3-oxathiane, which has an odor threshold of 3 ppb (*63*). 3-Mercapto-1-hexanol is a powerful odorant reminiscent of citrus and tropical fruit. It was first isolated from passion fruit and contributes to its character impact (*64*, *65*). It has been extensively studied in wine flavor, and identified as one of the key aroma compounds in Sauvignon blanc, Riesling, Gewurztraminer, Cabernet Sauvignon, and Merlot wines (*23*). Its acetate derivative (3-mercaptohexyl acetate) provides a characteristic

"Riesling-type note" (*33*). 3-Mercapto-1-hexanol and its acetate were also reported in fresh grapefruit juice, where they contribute "grapefruit, passion fruit, box tree-like" aromas (*66*). Recent studies have identified a series of C_5-C_7 3-mercaptyl-1-alcohols in wines made from *Botrytis*-infected grapes (*67*). All contribute citrus-like aromas, except the branched 3-mercapto-2-methylbutanol isomer, which is "raw onion". 3-Mercaptohexan-1-ol is especially impactful to tropical fruit aroma in this wine, and its disulfide oxidation product has been identified in passion fruit (*65*) and Sauternes botrytized wine (*23*). In Sauternes wine, polyfunctional thioaldehdyes, (e.g., 3-mercaptoheptanal "fruity, lemon"; 3-methyl-3-mercaptobutanal "petroleum, bacon") were shown to decrease during bottle aging, and most thiols were lost during 1-2 years of storage (*68*). 2-Methylfuran-3-thiol contributes an interesting "bacon" note to this wine (*68*). 3-Mercapto-3-methylbutanol ("cooked leeks") was identified in Sauvignon blanc wine, however it was deemed unimportant to the aroma character since its concentration was significantly below its 1500 ng/L detection threshold (*69*). A new citrus odoriferous component, 3-propyl-1,2-oxathiolane, was shown to be produced by thermal oxidative degradation of the disulfide in the GC injector (*23*). In pink guava fruit, 3-mercapto-1-hexanol ("grapefruit") and 3-mercaptohexyl acetate ("black currant") contribute significantly to the strong and characteristic tropical fragrance of the fresh fruit in a nearly racemic ratio. Both 3-mercapto-1-hexanol enantiomers have extremely low odor thresholds of 70-80 pg/L (*70*). 2-Pentanethiol, which was previously attributed to guava fruit character (*71*), was not detected in these recent studies.

Sulfur esters have been identified in other tropical/subtropical fruits including melon, pineapple, kiwifruit, and durian. For muskmelon, ethyl 3-(methylthio)propionate is an important supporting character impact compound, contributing green, fresh melon notes (*72*, *73*). Additional thioether esters in melons include methyl 2-(methylthio)acetate, ethyl (methylthio)acetate, and the corresponding thioether alcohols. In pineapple, methyl 3-(methylthio)propionate and ethyl 3-(methylthio)propionate provide background green notes, however their contribution to the fruit aroma was considerably lower relative to the most odor-active volatiles (*74*, *75*). Methyl (methylthio)acetate and ethyl (methylthio)acetate have been identified in kiwifruit (*76*) and Indonesian durian in which sulfur volatiles dominate the overall aroma perception (*77*). From flavor extract dilution analysis, 3,5-dimethyl-1,2,4-trithiolane was identified as the most potent odorant in durian.

Freshly-picked lychee (var. litchi) fruit has a distinct sulfurous aroma character which diminishes upon storage. Sulfur volatiles and their individual sensory contribution in three lychee cultivars were identified as hydrogen sulfide (sulfur), dimethyl sulfide ("cabbage"), diethyl disulfide ("moldy, sulfur"), 2-methyl thiazole ("fresh garlic spice"), 2,4-dithiopentane ("burnt tire, cabbage"), dimethyl trisulfide ("cabbage, sulfur"), methional ("boiled potato"), and 2-acetyl-2-thiazoline ("nutty-woody") (*78*).

Methyl thioacetate ("cheesy, garlic") and methyl thiobutanoate ("cheesy, garlic, cabbage") were reported in strawberry as aroma-active sulfur volatiles with very low thresholds at relatively high concentrations (*29*). Recently, a series of novel thioesters were reported in fresh strawberry, including methyl

(methylthio)acetate, ethyl (methylthio)acetate, methyl 2-(methylthio)butyrate (fruity, floral, garlic), methyl 3-(methylthio)propionate, ethyl 3-(methylthio) propionate, methyl thiopropanoate, methyl thiohexanoate, and methyl thiooctanoate (fruity, pineapple) (*28*). Except as indicated, the aroma character of these esters was principally "cheesy/onion/garlic/sulfurous".

Table IV. Character-Impact Sulfur Compounds in Vegetables

Character impact compound (s)	*Odor description*	*Occurrence*	*Reference*
Dimethyl sulfide		asparagus	(*85*)
	cabbage	cabbage	(*80*)
	canned corn	corn	(*81*)
	sulfury, tomato	tomato paste	(*79*)
2-iso-Butylthiazole	tomato leaf	tomato (fresh)	(*79*)
1,2-Dithiacyclopentene		asparagus, heated	(*80*)
4-Methylthiobutyl isothiocyanate	cabbage, radish	broccoli	(*81*)
Methyl methanethiosulfinate		Brussels sprouts	(*82*)
		sauerkraut, cabbage	(*83*)
Allyl isothiocyanate	horseradish	cabbage, raw	(*81*)
	sulfur, garlic	cauliflower, cooked	(*84*)
3-(MeS)propyl isothiocyanate	pungent, horseradish	cauliflower	(*81*)
2-Acetyl-2-thiazoline	popcorn, roasty	corn, fresh	(*81*)
3-Methylthiopropanal	cooked potato	potato (boiled)	(*35*)
2-Heptanethiol	bell pepper, fruity	bell pepper	(*86*)
(E)-3-Heptene-2-thiol	sesame, green	red bell pepper	(*15*)
(E)-4-Heptene-2-thiol	sesame, coffee	red bell pepper	(*15*)

Vegetable Flavors

In vegetables, the contribution of sulfur flavor impact compounds depends considerably on how they are prepared (cutting, blending), and the form in which they are consumed (raw vs. cooked). For example, the character impact of fresh tomato is delineated by 2-*iso*-butylthiazole and (Z)-3-hexenal, with modifying effects from β-ionone and β-damascenone (*79*). Alternatively, dimethyl sulfide is a major contributor to the flavor of thermally-processed tomato paste (*79*, *80*). Dimethyl sulfide is also a flavor impact compound for sweet corn, while hydrogen

sulfide, methanethiol and ethanethiol may further contribute to its aroma due to their low odor thresholds (*81*). A summary of sulfur character-impact compounds for vegetable flavors is outlined in Table IV.

Among the Cruciform vegetables, cooked cabbage owes its dominant character impact flavor to dimethyl sulfide. In raw cabbage flavor, allyl isothiocyanate contributes sharp, pungent horseradish-like notes (*81*). Methyl methanethiosulfinate was observed to provide the character impact of sauerkraut flavor, and occurs in Brussels sprouts and cabbage (*82*, *83*). Compounds likely to be important to the flavor of cooked broccoli include dimethyl sulfide and trisulfide, nonanal, and erucin (4-(methylthio)butyl isothiocyanate) (*80*). Cooked cauliflower contains similar flavor components as broccoli, with the exception that 3-(methylthio)propyl isothiocyanate is the characterizing thiocyanate (*80*). Allyl isothiocyanate, dimethyl trisulfide, dimethyl sulfide, and methanethiol were the key odorants of cooked cauliflower "sulfur" odors (*84*). Key volatile sulfur compounds were recently identified in the flavor of cooked asparagus (*85*).

2-Heptanethiol was recently reported as a new flavor compound in cooked red and green bell peppers (*86*). Subsequently, a series of newly-reported thiols, mercapto ketones, mercapto alcohols, and methylthio-thiols were identified in red bell pepper which cover a palette of organoleptic properties (*15*). Their flavor descriptors range from "berry/tropical fruit", to "green/vegetable-like, meaty, onion-like, sesame, peanut, coffee, and roasted." Characteristic bell pepper notes could be attributed to (*E*)-3-heptene-2-thiol ("sesame, green, bell peppers, fresh") and (*E*)-4-heptene-2-thiol ("sesame, coffee, bitterness of peppers"). It was reported that few of the new compounds identified have unpleasant "rubbery/rotten" notes that are notorious for sulfur compounds. Representative structures for character-impact sulfur compounds in vegetables are presented in Figure 3.

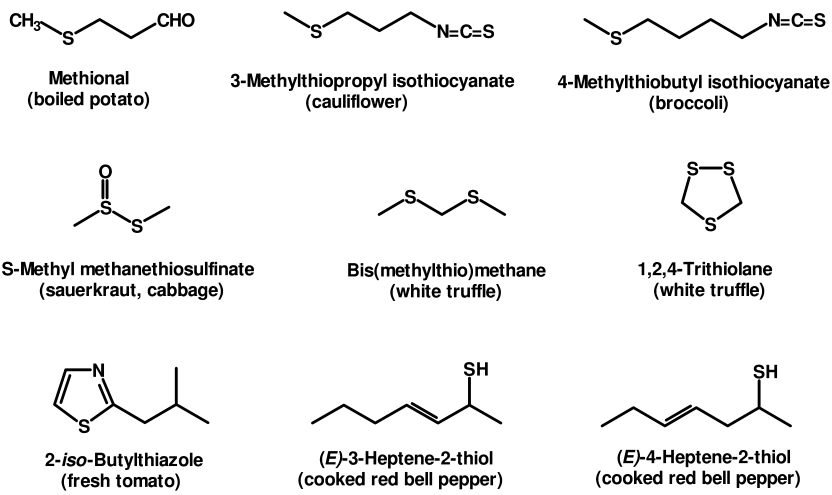


Figure 3. Representative character-impact sulfur flavor compounds in vegetables.

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Maillard-Type, Brown and Cereal Flavors

Characteristic heated flavor compounds arise via the Maillard pathway, the thermally-induced reaction between sulfur amino acids (cysteine/cystine, methionine) and reducing sugars. Sulfur heterocyclic constituents that contribute aromas of coffee, toasted cereal grains, popcorn and roasted meats are products of Maillard reactions (*87*). 2-Furfurylthiol is the primary character impact compound for the aroma of roasted Arabica coffee (*88*). It has a threshold of 5 ppt and smells like freshly brewed coffee at concentrations between 0.01 and 0.5 ppb (*89*). At higher concentrations it exhibits a "stale coffee, sulfury" note. Other potent odorants in roasted coffee include 5-methylfurfurylthiol (0.05 ppb threshold), which smells meaty at 0.5-1 ppb, and changes character to a sulfury mercaptan note at higher levels (*89*). Furfuryl methyl disulfide has a sweet mocha coffee aroma (*63*). While other sulfur compounds such as 3-mercapto-3-methylbutyl formate and 3-methyl-2-buten-1-thiol were previously thought to be important factors for coffee aroma, recent studies have confirmed that the flavor profile of brewed coffee is primarily contributed by 2-furfurylthiol, 4-vinylguaiacol, and "malty"-smelling Strecker aldehydes, among others (*88*, *90*). In the volatile fraction of roasted hazelnuts, both 2-furfurylthiol and 2-thienylthiol contribute "coffee-like, sulfury" notes with high odor activities (*91*). For wines and champagnes aged in toasted oak barrels, 2-furfurylthiol and 2-methyl-3-furanthiol have been identified as providing their "roast-coffee" and "cooked meat" aroma characters, respectively (*14*). A summary of character-impact sulfur compounds for thermally generated flavors is outlined in Table V.

Thiols of branched and linear C_5-C_6 alcohols and ketones are produced during fermentation of fresh lager beers, including 3-mercaptohexanol, 2-mercapto-3-methylbutanol, and 3-mercapto-3-methylbutanol (*10*) These hop-derived thiols contribute subtle background characters such as "rhubarb", "onion/sulfur" and "broth/onion/sweat", respectively. 2-Methyl-3-furanthiol contributes a "meaty/nutty" background note in lager beer (*10*). It is formed via reactions between H_2S and thermal degradation products of sugars.

Cereal grains including corn and rice have characteristic thermally-derived "toasted, nutty" flavors that are generated through Maillard pathways (*87*). Two novel, highly intense "roasty, popcorn-like" aroma compounds were identified in Maillard model systems: 5-acetyl-2,3-dihydro-1,4-thiazine (0.06 ppt odor threshold) from a ribose-cysteine reaction (*92*); and N-(2-mercaptoethyl)-1,3-thiazolidine (3-thiazolidineethanethiol) (0.005 ppt odor threshold) from reaction of fructose with cysteamine (*93*). Neither of these sulfur flavor compounds have been reported in food aromas to date. Representative chemical structures for thermally-generated sulfur flavor impact compounds are shown in Figure 4.

While pyrazines typically contribute "roasted potato" odors, the combination of methional with other key aromatics (2-acetyl-1-pyrroline, phenylacetaldeyde, butanal) provides important flavor character for extruded potato snacks (*50*). A minor but potent flavor component identified in potato chip aroma is 2-acetyl-2-thiazoline which contributes a "roasty, popcorn" character (*94*).

Table V. Character-Impact Sulfur Compounds in Cooked Flavor Systems

Character impact compound (s)	*Odor description*	*Occurrence*	*Reference*
2-Furfurylthiol	fresh brewed coffee	coffee	(*88*)
	coffee-like, sulfury	hazelnut, roasted	(*91*)
	roast-coffee	wine (barrel-aged)	(*14*)
5-Methylfurfurylthiol	meaty	coffee	(*89*)
2-Thienylthiol	coffee-like, sulfury	hazelnut, roasted	(*91*)
Furfuryl methyl disulfide	mocha coffee	coffee	(*63*)
2-Methyl-3-furanthiol	cooked meat	wine (barrel-aged)	(*14*)
	meaty, nutty	beer, lager	(*10*)
3-Mercaptohexanol	rhubarb, fruity	beer, lager	(*10*)
2-Mercapto-3-methylbutanol	onion, sulfur	beer, lager	(*10*)
3-Mercapto-3-methylbutanol	broth, onion, sweat	beer, lager	(*10*)
2-Acetyl-2-thiazoline	roasty, popcorn	potato chip	(*94*)
5-Acetyl-2,3-dihydro-1,4-thiazine	roasty, popcorn	Maillard model system	(*92*)
3-Thiazolidineethanethiol	roasty, popcorn	Maillard model system	(*93*)

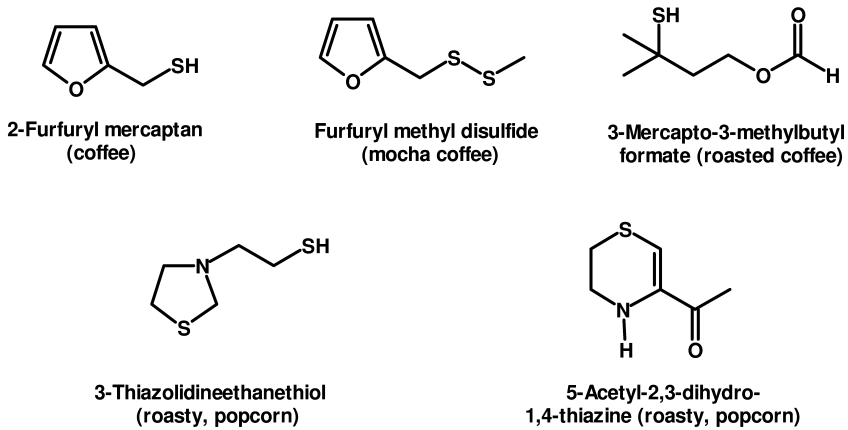

Figure 4. Representative character-impact sulfur flavor compounds in cooked foods.

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Meat and Seafood Flavors

Sulfur-containing heterocyclic compounds are associated with meaty characteristics. Two compounds with the most potent impact include 2-methyl-3-furanthiol (1 ppt) and the corresponding dimer, bis-(2-methyl-3-furyl) disulfide (0.02 ppt) (*35*). Both substances have been identified in cooked beef and chicken broth, and have a strong meaty quality upon dilution. 2-Methyl-3-furanthiol also occurs in canned tuna fish aroma (*95*). The disulfide has a recognizable aroma character of "rich aged-beef, prime-rib" (*63*). Both compounds are produced from the thermal degradation of thiamin (*96*). A related sulfur volatile, 2-methyl-3-(methylthio)furan, is the character impact compound for roast beef (*35*). Besides 2-methyl-3-furanthiol, additional potent sulfur-containing odorants 2-furfurylthiol, 2-mercapto-3-pentanone, and 3-mercapto-2-pentanone were identified in heated meat (*97*). A summary of character-impact sulfur compounds for meat and seafood flavors is shown in Table VI.

Table VI. Character-Impact Sulfur Compounds in Meat and Seafood

Character impact compound (s)	*Odor description*	*Occurrence*	*Refer-ence*
Dimethyl sulfide	stewed clam	clam, oyster	(*108*)
Methional	boiled potato	boiled clam	(*102*)
		crustaceans	(*104*)
Pyrrolidino-2,4-(Me_2)dithiazine	roasted	boiled shellfish	(*107*)
2-Acetylthiazole	popcorn	boiled clam	(*102*)
2-Acetyl-2-thiazoline	roasty, popcorn	roasty (beef)	(*98*)
		cooked chicken	(*99*)
	nutty, popcorn	crustaceans	(*104*)
4-Me-5-(2-hydroxyethyl)thiazole	roasted meat	Maillard reaction	(*63*)
2-Methyltetrahydrofuran-3-thiol	brothy, meaty	Maillard reaction	(*63*)
2-Methyl-3-furanthiol	roasted meat	meat, beef	(*35*)
	meat, fish, metallic	canned tuna fish	(*95*)
Bis-(2-methyl-3-furyl) disulfide	rich aged-beef	aged, prime-rib	(*35*)
2-Methyl-3-(methylthio)furan	beefy, coffee	roast beef	(*35*)
2,5-Dimethyl-1,4-dithiane-2,5-diol	chicken broth	Maillard reaction	(*63*)

In cooked meats and other thermally-processed foods, the key aroma compound 2-acetyl-2-thiazoline imparts a potent "roasty, popcorn" note that enhances meaty and roast flavors. It was first identified in meat systems among the flavor volatiles of beef broth, and later reported as one of the character-impact compounds of roasted beef (*98*). 2-Acetyl-2-thiazoline was subsequently detected as a potent odorant of chicken broth and cooked chicken (*99*). Representative structures for meat and seafood sulfur flavor impact compounds are shown in Figure 5.

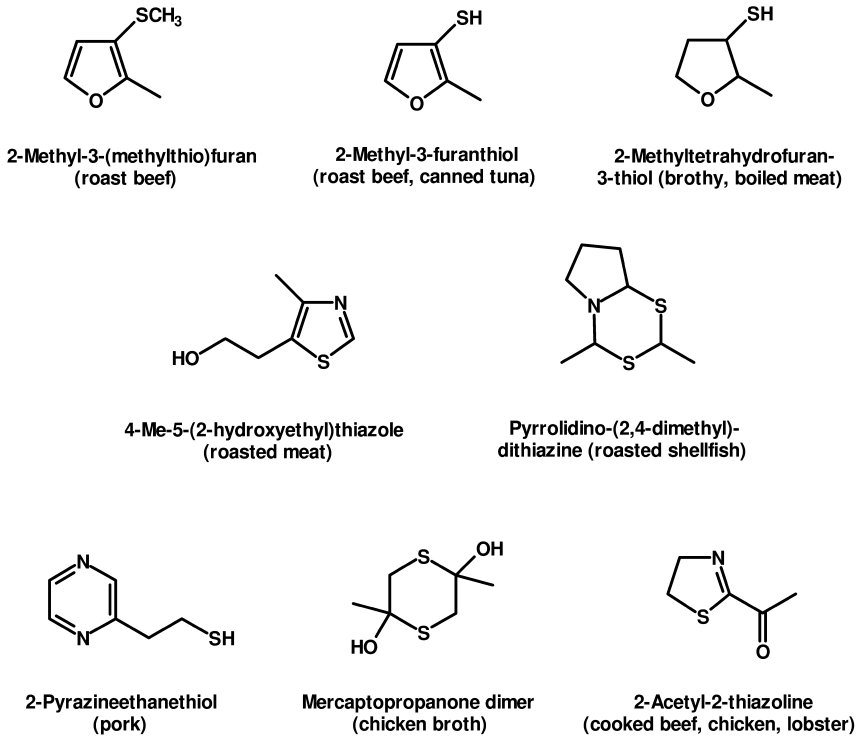

Figure 5. Representative character-impact sulfur flavor compounds in meat and seafood.

A brothy compound associated with boiled beef, 4-methyl-5-(2-hydroxyethyl)lthiazole (sulfurol), is a "reaction flavor" product from hydrolysis of vegetable protein. It is suspected that a trace impurity (2-methyltetrahydrofuran-3-thiol) in sulfurol is the actual "beef broth" character impact compound (*63*). Another reaction product flavor chemical, 2,5-dimethyl-1,4-dithiane-2,5-diol (the dimer of mercaptopropanone), has an intense chicken-broth odor. A series of C_4-C_9 3-(methylthio)aldehydes have been reported in cooked beef liver (*100*) and other highly thermally processed foods (fried potatoes, tomato paste, dried squid) (*101*). Unlike methional, which is derived from the amino acid methionine, these sulfur compounds are likely formed via reaction of methyl mercaptan with unsaturated aldehydes. Volatile

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isolation employed techniques such as Likens-Nickerson for identification of these 3-(methylthio)aldehydes, so the possibility that they are artifacts needs to be further investigated.

Fish flavors are primarily composed of noncharacterizing "planty" or melon-like aromas from fatty acid-derived unsaturated carbonyl compounds. Three notable sulfur volatiles in boiled clam were determined to be principal character-impact compounds by AEDA: 2-acetyl-2-thiazoline ("roasted"), 2-acetylthiazole ("popcorn") and 3-methylthiopropanal (methional) ("boiled potato") (*102*). Because 2-acetyl-2-thiazoline is readily degraded by heating in aqueous media (*103*), it is presumed that acetylthiazoline is initially produced at low temperature, and then oxidized to 2-acetylthiazole. Methional and 2-acetyl-2-thiazoline also contribute to the "meaty" and "nutty/popcorn" aroma notes in cooked crustaceans such as crab, crayfish, lobster, and shrimp (*104*, *105*). 2-Acetyl thiazoline was also identified in boiled trout, cooked mussels, turbot, and boiled carp fillet. (*106*).

A potent character-impact odorant in cooked shellfish, including shrimp and clam, was identified as pyrrolidino[1,2-*e*]-4*H*-2,4-dimethyl-1,3,5-dithiazine (*107*). This dithiazine contributes a roasted character to boiled shellfish, and has the lowest odor threshold recorded to date, 10^{-5} ppt in water. Dimethyl sulfide is reported to contribute the character aroma of stewed clams and oysters (*108*).

Cheese and Dairy Flavors

Key odor-active compounds in milk and dairy flavors have been recently reviewed (*109–113*). With a few exceptions, many of the known important flavors in dairy products do not provide characterizing roles. This is especially true for milk, cheddar cheese and cultured products, such as sour cream and yogurt. Sulfur compounds including methanethiol, hydrogen sulfide, and dimethyl disulfide contribute to the strong garlic/putrid aroma of soft-smear or surface-ripened cheeses. Key aroma compounds in Parmigiano Reggiano cheese were recently reported, including dimethyltrisulfide and methional (*114–116*). A summary of sulfur compounds for cheese and dairy flavors is presented in Table VII.

By a wide margin, cheddar is the most popular cheese flavor in North America. While its flavor is described as "sweet, buttery, aromatic, and walnut," there is no general consensus among flavor chemists about the identity of individual compounds or groups of compounds responsible for cheddar flavor.

At present, it is thought to arise from a unique balance of key volatile components, rather than a unique character impact compound. Sensory-guided flavor studies concluded that the important sulfur contributors to cheddar cheese aroma are methional, dimethyl sulfide, dimethyl trisulfide, and methanethiol (*117*, *118*). Two recent studies confirmed that hydrogen sulfide and dimethyl disulfide only increased during initial stages of cheddar cheese aging, whereas dimethyl sulfide, dimethyl trisulfide, and methanethiol continued to develop throughout the aging process (*25*, *26*). A desirable "nutty" flavor supports "sulfur" and "brothy" characters in a quality aged cheddar cheese sensory profile. 2-Acetyl-2-thiazoline (*117*, *119*) contributes "roasted, corny" flavors in synergy with 2-acetyl-1-pyrroline, which were suggested to be related to the "nutty"

flavor. In cheddar cheese powders, dimethyl sulfide imparts a desirable "creamed corn" flavor (*120*). Representative structures of significant sulfur volatiles in cheese are shown in Figure 6.

A thiolester, ethyl 3-mercaptopropionate, was reported for the first time in Munster and Camembert cheeses (*13*). This sulfur volatile was described at low concentrations as having pleasant "fruity, grapy, rhubarb" characters. It has previously been reported in wine and Concord grape (*51*) but was never mentioned before in cheese.

Novel polyfunctional thiols were recently reported in aged cheddar cheese. Among these were 4-mercapto-4-methyl-2-pentanone ("catty") and 4-mercapto-3-methyl-2-pentanone ("cooked milk, sweet") (*121*). Other tentatively identified thiols include 4-mercapto-2-pentanol, 4-mercapto-3-methylpentan-2-ol, 5-methyl-4-mercapto-hexan-2-one, and 5-methyl-4-mercaptohexan-2-ol (*121*).

Table VII. Significant Sulfur Compounds in Cheese and Dairy

Character impact compound (s)	*Odor description*	*Occurrence*	*Reference*
Dimethyl sulfide	creamed corn	cheese, cheddar	(*117*)
Dimethyl trisulfide	putrid	cheese, cheddar	(*117*)
Methanethiol	sulfury	cheese, cheddar	(*117*)
Methional	boiled potato	cheese, cheddar	(*117*)
		Parmigiano Reggiano	(*114–116*)
Ethyl-3-mercaptopropionate	grapy, rhubarb	cheese, Camembert	(*13*)
2-Acetyl-2-thiazoline	roasted, corny	cheese, cheddar	(*117*, *119*)
4-Mercapto-4-Me-2-pentanone	catty	cheese, cheddar	(*121*)
4-Mercapto-3-Me-2-pentanone	cooked milk, sweet	cheese, cheddar	(*121*)
2-Methyl-3-furanthiol	brothy, burnt	whey protein	(*122*)

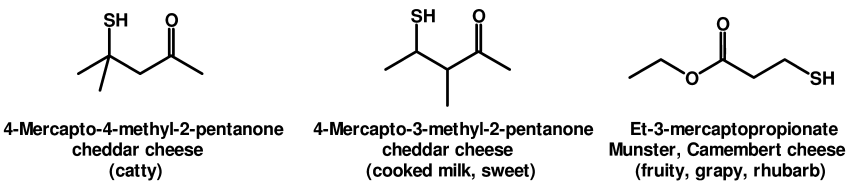


Figure 6. Representative sulfur flavor compounds in cheese.

Key aroma-active compounds have been reported in dried dairy products including nonfat milk and whey powders. A supporting role was provided by the sulfur flavor impact compound. 2-methyl-3-furanthiol ("brothy/burnt") identified in whey protein concentrate and whey protein isolate (*122*).

Sulfur Volatile Contributions to Off-Flavors and Taints

Exposure of beer to light generates 3-methyl-2-butene-1-thiol, which provides a skunky off-flavor in "sun-struck" or "light-struck" ales (*123*, *124*). This mercaptan has a sensory threshold of 0.05 ppb in beer. It derives from complex photo-induced degradations of isohumulones (hop-derived, bitter iso-acids) to form free-radical intermediates, which subsequently react with the thiol group of cysteine. Lightstruck off-flavor can be controlled in beer through packaging technology (colored glass bottles), use of chemically- modified hop bitter acids, antioxidants, or its precipitation with high molecular weight gallotannins and zinc salts (*125*). In addition to dimethyl sulfide, thioesters have been reported to contribute a "cabbagy, rubbery" off-note that sometimes are derived from hops in beer, the most significant being S-methyl hexanethioate, which has a detection threshold of 1 ppb (*126*). A summary of off-flavor impact sulfur compounds in foods and beverages is presented in Table VIII.

Another staling off-flavor in beer is described as "catty/ribes", whose character comes from 4-mercapto-4-methyl-2-pentanone (cat ketone). This off-flavor can develop rapidly in beers that are packaged and stored with significant quantities of air in the headspace (*127*). A similar "catty/ribes" off-flavor in aged lager beer was attributed to 3-mercapto-3-methylbutyl-formate (*128*). 2-Mercapto-3-methylbutanol was detected in a Finnish beer characterized by an intense "onion-like" off flavor and suggested that it likely was derived from hops (*129*).

Sulfur compounds present in wine can have a detrimental effect on aroma character, producing odors described as "garlic, onion and cauliflower," the so-called Boeckser aromas. This sulfurous character is correlated with 2-methyl-3-hydroxythiophene, 2-methyl-3-furanthiol and ethanethiol. Their concentrations in wine are influenced by winery practices and the use of certain winemaking yeasts (*130*). Off-flavors in European wines were associated with the non-volatile bis(2-hydroxyethyl) disulfide, a precursor to the "poultry-like" character of 2-mercaptoethanol and hydrogen sulfide (*131*). Examples of off-flavor sulfur compounds in foods and beverages are shown in Figure 7.

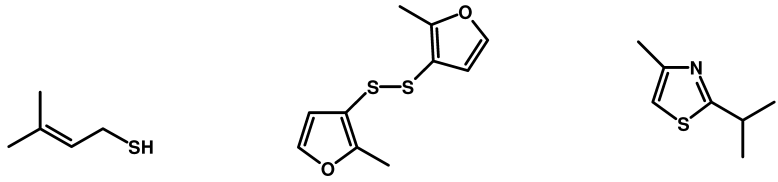
Strecker aldehydes are a frequent source of off-flavors in fermented products. Development of oxidized off-flavors in white wines typically marks the end of shelf life. Methional (3-methylthiopropionaldehyde) was identified as producing a "cooked vegetables" off-flavor character in a young white wine that had undergone spontaneous oxidation (*132*). Methional levels increased in wines spiked with methionol or methionine, suggesting its formation via direct peroxidation or Strecker degradation of methionine. The importance of methional in the development of characteristic oxidation notes in white wine was further demonstrated (*133*). Methional and 2-methyl-3-furanthiol are purported off-flavor

components in stored orange juice (*134*). Methanethiol, 1-*p*-menth-1-ene-8-thiol, 2-methyl-3-furanthiol, and dimethyl trisulfide contributed atypical "tropical fruit/grapefruit" character to canned orange juice (*135*). Methional was shown to impart a "worty" off-flavor in alcohol-free beer, with more sensory significance than was previously attributed to 3-methyl- and 2-methylbutanal for this taint (*136*).

A vitamin-derived off-odor problem was described in which a pineapple fruit juice beverage was fortified with riboflavin. The "vitamin, cabbage, brothy, vegetable soup" off-odor was characterized as 4-methyl-2-isopropylthiazole, which resulted from riboflavin-sensitized Strecker degradation of valine, cysteine and methionine, followed by reaction of the resulting aldehydes with ammonia and hydrogen sulfide (*137*).

Table VIII. Off-Flavor Impact Sulfur Compounds in Foods and Beverages

Impact compound (s)	*Off-flavor description*	*Occurrence*	*Reference*
3-Methyl-2-butene-1-thiol	skunky, plastic	beer (light-struck)	(*123*, *124*)
S-Methyl hexanethioate	cabbagy, rubbery	beer	(*126*)
3-Mercapto-3-methylbutyl-formate	cat urine, ribes	beer, aged	(*128*)
4-Mercapto-4-methyl-2-pentanone	cat urine, ribes	beer, aged	(*127*)
2-Mercapto-3-methylbutanol	onion-like	beer	(*129*)
Methional	worty	beer (alcohol-free)	(*136*)
	cooked vegetables	oxidized white wine	(*132*)
	cooked potato	orange juice, aged	(*134*)
	potato	UHT-milk	(*142*)
Dimethyl disulfide	sunlight off-flavor	milk	(*138*)
2-Methyl-3-furanthiol	meaty/vitamin B	orange juice, aged	(*134*)
Benzothiazole	sulfuric, quinoline	milk powder	(*139*)
Bis(2-methyl-3-furyl)disulfide	vitamin B_1 odor	thiamin degradation	(*140*)
4-Methyl-2-isopropylthiazole	vitamin, cabbage	orange juice (vit B_2)	(*137*)



3-Methyl-2-butene-1-thiol
lightstruck beer

Bis-(2-methyl-3-furyl)disulfide
vitamin B_1 (thiamin)

4-Methyl-2-iso-propylthiazole
vitamin B_2 (fort. orange juice)

Figure 7. Representative off-flavor impact sulfur compounds in foods and beverages.

Sunlight off-flavor in milk ("cardboard-like") can result from milk exposed to high intensity fluorescent light or sunlight, which generates dimethyl disulfide from photooxidation of methionine (*138*). Part of a characteristic off-flavor in spray-dried skim milk powder is contributed by benzothiazole (sulfuric, quinoline) as low ppb levels (*139*).

While bis(2-methyl-3-furyl)disulfide contributes a desirable aged, prime rib flavor in beef, it is the principal "B-vitamin" off odor resulting from thiamin degradation (*140*).

Character compounds which contribute positive flavor impact at low levels can become off-flavors when they occur at higher concentrations. For example, dimethyl sulfide provides an appropriate "corn-like" background character to beer flavor at low levels, however it contributes a highly undesirable "cooked vegetable" or "cabbage-like" malodor when present at levels significantly above its sensory threshold (30-45 ppb) (*141*). Similarly for cheddar cheese flavor, it imparts a rotten vegetable taste when present at high levels (*118*).

In ultra-high temperature (UHT) processed milk, the "UHT milk flavor" character is contributed by methional plus pyrazines (*142*). Additionally, dimethyl sulfide was reported as one of the significant flavor components of UHT milk off-flavor (*143*). Methional, methanethiol, and dimethyl sulfide are the source of "sulfur" and "cooked" off flavors in ultrahigh-temperature (UHT) processed soy milks (*144*).

Conclusion

In the 17 years since the previous review, there have been significant strides in the flavor chemistry of volatile sulfur compounds due to analytical advancements in sulfur-specific detectors and limits of detection. Greater awareness and care is being taken to prevent isomerization of reactive sulfur flavor compounds during their analysis. New key aroma compounds containing sulfur have been discovered in fruits, vegetables, dairy products, wine, beer, and heated foods. Traditionally, sulfur compounds have been associated with unpleasant, noxious off-flavors, however recent discoveries indicate that a positive perspective is developing towards the role of sulfur volatiles in tropical, fruity, and savory character of food flavors. Undoubtedly, flavor research on sulfur volatiles will

continue to accelerate given these recent discoveries and a new appreciation of their sensory significance in foods.

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Chapter 2

Challenges and Artifact Concerns in Analysis of Volatile Sulfur Compounds

Eric Block*

Department of Chemistry, University at Albany, State University of New York, Albany, NY 12222
***E-mail: eb801@albany.edu.**

Various sensitive techniques are available to assist in the identification of volatile sulfur compounds (VSCs) from food and beverages. However, not all of the VSCs found by these techniques are originally present: some are formed enzymatically from non-volatile precursors during processing and some are artifacts of the analytical techniques used. Artifacts can arise from thermal breakdown or reaction in the injection port of a gas chromatograph (GC), during use of solid phase microextraction fibers, or from oxidative or metal-catalyzed processes. Examples, chosen from analyses of genus *Allium* and *Brassica* plants and wine, involve both achiral and chiral VSCs as well as mixed selenium-sulfur compounds. These analyses employ achiral and chiral GC and liquid chromatography (LC) methods with various detectors, as well as those based on direct analysis in real time mass spectrometry (DART-MS), coupled ultraperformance LC-silver coordination ion spray mass spectrometry (UPLC-(Ag^+)CIS-MS), and microwave, nuclear magnetic resonance, and X-ray atomic spectroscopy.

Introduction

Volatile sulfur compounds are of considerable interest, prominently contributing to the enjoyment of food and beverages, but also signaling spoilage or quality control problems. Furthermore, they may be responsible for unpleasant breath or body odor through metabolic or disease processes, which may have

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a dietary connection. The significance of the VSCs is due in part to the very low (femtogram!) levels often detectable by the human nose. While low levels of some VSCs are perceived as pleasant, as is the case with 2-furfurylthiol, a roasted coffee and roasted sesame seed flavor note (*1*), and 3-mercaptohexan-1-ol and 1-*p*-menthene-8-thiol from fruits and wine, higher levels can be unpleasant smelling "off-flavors" (*2*). Furthermore, enantiomers of chiral VSCs can differ in their aromas. The chemist needs to be especially vigilant for artifact formation given the above considerations, the common natural occurrence of sulfur compounds in fresh and processed food, and the wide range of chemical reactions possible for these compounds caused by heat, light, metals or their ions, oxidants, and reactions with each other.

Artifacts are false or inaccurate results, caused by the technology used in the experimental investigations. More specifically, compounds not naturally present but produced during the course of an analysis can be considered as artifacts. However, the distinction between what is an artifact and what is real at times can become blurred. Thus, a thermally unstable compound produced upon crushing a plant, when subjected to gas chromatography (GC) may decompose giving several new compounds, not initially present immediately after crushing. However, upon standing, this unstable compound can undergo slow decomposition leading to the same mixture of new compounds formed during GC analysis after crushing. These new compounds are therefore at the same time artifacts when formed under analytical conditions from freshly crushed samples, as well as genuine "secondary" natural products when formed by slow decomposition of the unstable initial products of crushing. Specific examples of this situation will be given below.

It is worthwhile to remind readers of the distinction between the terms "unstable" and "reactive" (*3*). "Unstable," and its opposite "stable," are thermodynamic properties, measured by relative molar standard Gibbs energies. For example, an "unstable" chemical species has a higher molar Gibbs energy than a "stable" standard. "Reactive," and its opposite "unreactive," are kinetic properties. A species is said to be more reactive or have a higher reactivity than some other species if the former has a larger rate constant for a specified reaction. "Reactive" and "unstable" are sometimes incorrectly used interchangeably, although more reactive species are frequently also more unstable. However, a relatively more stable chemical species may at the same time be more reactive than some reference species toward a given reaction partner. Methanethiol, a very reactive VSC due to the ease with which it undergoes oxidation to dimethyl disulfide and nucleophilic addition and displacement processes, is actually a very stable compound. Very unstable chemical species tend to undergo exothermic unimolecular decomposition. Sometimes the term "reactive" is used more loosely as a phenomenological description and then may reflect not only rate but also equilibrium constants.

When working with unstable and reactive food-derived compounds it is best to prepare pure samples of these compounds and examine their behavior under a variety of analytical conditions to identify those conditions leading to artifact formation. Mechanistic studies are also very useful to identify favorable decomposition pathways and to predict the likely structures of the products

ultimately formed. Examples of this approach will be also be illustrated in this chapter. Finally, it is important that the topic of "artifacts in analysis" be incorporated into academic curricula and in the training and mentoring of new coworkers performing or interpreting analytical results.

This chapter updates a 1994 ACS chapter on artifact formation in *Allium* (garlic/onion) chemistry (*4*) as well as a more current, brief treatment of this subject (*5*). Related reviews have appeared (*6*, *7*). Recent examples from the author's work and the literature have been added, which more broadly examine the challenges chemists face in the analysis of VSCs in food. In particular, newer instrumental methods will be described that minimize artifact formation associated with extraction and heating, and that maximize sensitivity toward compounds otherwise undetected due to limitations of current analytical techniques.

Types of Sulfur Compounds Whose Analysis Can Result in Artifacts

Thiols are among the VSCs with the lowest detection levels, e.g., 0.00004 ppb for methanethiol (*8*) and 0.00002 ppb (the equivalent of one gram in 10 million metric tons of water!) for (*R*)-(+)-1-*p*-menthen-8-thiol (*9*). Thiols can be formed through enzymatic as well as chemical processes from stable precursors in food and can rapidly disappear through oxidation to disulfanes (disulfides). Thus, rapid sampling is important when it is necessary to catch thiols before they disappear. For some purposes, the kinetics of formation and disappearance can also be of interest. At the same time, due to their sensitivity to oxidation and other processes, care must be taken to avoid reactions of thiols associated with the analytical procedures themselves. Disulfanes and polysulfanes, important VSCs that can also have strong odors, can be thermally unstable, particularly when 1- or 2-propenyl (allylic) groups are present, and when more than three linked sulfur atoms are present. Various compounds with sulfur–oxygen bonds such as thiosulfinates, thiosulfonates, and sulfenic and sulfinic acids are also important in food science. Once again, several of these may be both unstable and reactive, leading to artifact formation under a variety of conditions.

Chemistry in a Salad Bowl: VSCs from Genus *Allium* Plants

Allicin and Other Sulfur Compounds from Garlic (*Allium sativum*) and Other Alliums

When garlic and other alliums are crushed, diverse reactive, biologically active organosulfur compounds form. In 1844, Wertheim in Germany suggested that distilled oil of garlic contained "allyl sulfur," e.g., diallyl sulfide. One hundred years later, in 1944, Cavallito in Rensselaer, New York, characterized a compound from crushed garlic he named allicin, the presumed precursor of the garlic oil diallyl polysulfanes (*5*). In 1951, Stoll in Basel postulated that the non-protein amino acid alliin (**1**) from intact garlic underwent alliinase enzyme-catalyzed cleavage to 2-propenesulfenic acid (**2**) and α-aminoacrylic acid, the former condensing to give allicin (**3**), and the latter hydrolyzing to ammonium pyruvate

(Figure 1). A detailed mechanism has been proposed (*5*) for conversion of allicin to diallyl trisulfane (**5**) involving the intermediacy of trisulfane *S*-oxide (**6**) and 2-propenesulfinic acid (**7**). Despite the extensive published work on *Allium* chemistry, direct observation of intermediates **2**, **6** and **7** from crushed garlic has not proven possible until recently, as will be discussed below.

In order to rapidly volatilize injected samples, GC inlet ports are heated to 250 °C. Unfortunately, many *Allium* compounds decompose at this temperature. For example, when a fresh extract of garlic was subjected to GC-MS analysis, major and minor unknown *m/z* 144 ($C_6H_8S_2$) peaks appeared. Injection of a synthetic sample of allicin (**3**; *m/z* 162, $C_6H_{10}S_2O$) gave the same two peaks, which were assumed to result from dehydration of allicin, and were assigned the structures 3-vinyl-[4*H*]-1,3-dithiin (**9**) and 3-vinyl-[6*H*]-1,2-dithiin (**10**), for the major and minor peaks, respectively (Figure 2) (*10*). Compounds **9** and **10** were assumed to be artifacts and not components of garlic, and were not formed when synthetic allicin decomposed at 20 °C, the only products being diallyl disulfane (**4**) and trisulfane (**5**) and sulfur dioxide. Mechanism-based studies by the author (*4*) showed that the minor product had the structure 3-vinyl-[4*H*]-1,2-dithiin (**11**) rather than **10**, and that **9** and **11** are not allicin-dehydration products but rather dimers of thioacrolein (**12**), formed from allicin by an intramolecular elimination process, which also affords 2-propenesulfenic acid (**2**).

Further studies revealed that **9** and **11** are in fact formed by the decomposition of allicin in organic solvents, even though they are not formed when allicin slowly decomposes in water. Compounds **9** and **11**, found in some commercial garlic supplements, show anticoagulant activity in vitro (*5*). Since hydrogen bonding ties up the electron pairs on the oxygen of allicin, retarding their hydrogen-abstracting ability (*11*), allicin is substantially more stable in water than it is neat or in organic solvents, and can be stored for extended periods as a frozen aqueous solution (*12*, *13*). Some garlic preparations, when analyzed by GC-MS, also show a peak at *m/z* 104, identified as the heterocycle 3*H*-1,2-dithiole (**13**), suggested to be formed by a rearrangement-elimination process that also produces allyl alcohol (**8**) (*5*).

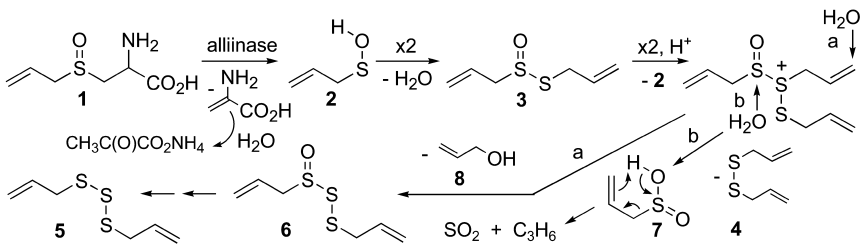

Figure 1. Formation and hydrolysis of allicin (3).

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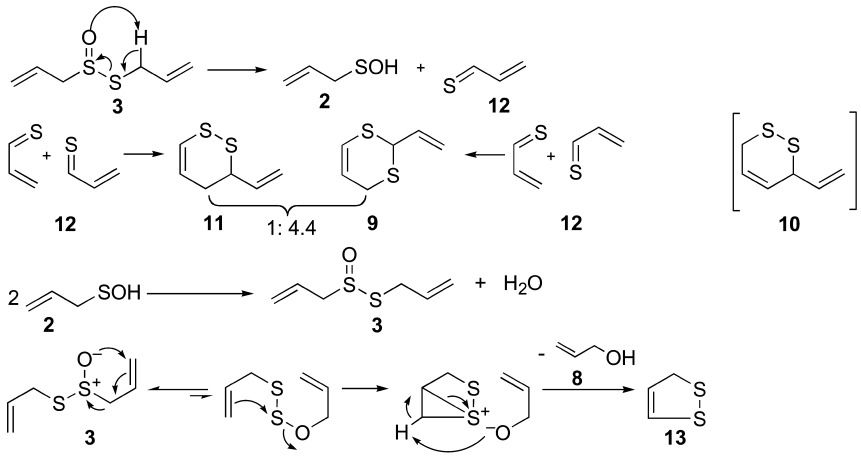

*Figure 2. Formation of heterocycles **9**, **11** and **13** from allicin (3).*

Studies of the process involved when leeks (*A. porrum*) are attacked by the leek moth, *Acrolepiopsis assectella*, showed that propyl propanethiosulfinate (PrS(O)SPr) is the most attractive volatile substance for the moth (*5*, *14*). While this compound decomposes during GC analysis, except on very short columns (*15*), it is in fact stable in the gas phase (*16*). These observations led Auger to conclude "the majority of sulfur volatiles identified by GC-MS in *Allium* spp. are thus artifacts produced during the isolation of the sample and during chromatography" (*15*). Similarly, comparative studies of different *Allium* spp., including ramps (*A. tricoccum*), using HPLC, LC-MS, GC-MS with short columns and low injection port temperatures, and supercritical fluid extraction and chromatography (SFE and SFC, respectively) showed that thiosufinates, the predominant components found with milder analytical methods, decompose in a GC with a hot injection port and long GC columns, giving polysulfanes as artifacts (*17–23*). Interestingly, MeSSMe, PrSSPr and MeSSPr, artifacts from decomposition of leek thiosulfinates on a GC, are the volatiles from the leek moth frass (the fine powdery material the moths pass as waste after digesting plant parts) that attracts the wasp, *Diadromus pulchellus*, which in turn parasitizes the leek moth (*24*).

Artifact problems occur in the analysis of volatiles from onion (*A. cape*) and other alliums using solid-phase microextraction (SPME) techniques. For example, in the combined analysis of the onion lachrymatory factor (LF) and thiosulfinates from onions it was reported: "SPME accelerates the degradation of labile thiosulfinates but the lachrymatory factor remains intact. The identification of *Allium* thiosulfinates is only obtained on juice extracted by diethyl ether using a fast GC-MS analysis on a 10 m × 0.3 mm column of 4 μm coating, with routine splitless injection. The lachrymatory factor is best analysed directly on fresh onion juice by SPME with the same chromatographic conditions" (*25*, *26*).

Several recent papers treat the polysulfane artifacts from dynamic headspace GC-MS and SPME/GC-MS analysis of alliums as if the peaks were genuine components from the plant preparations (*27*, *28*). In a study of onion aphid

attractants, SPME and GC-MS were used to identify the main headspace components of *A. fistulosum* (Welsh onion or Japanese bunching onion) and *A. tuberosum* (Chinese chives). The author reports that "the main volatile components of *A. fistulosum* were dipropyl disulphide (relative contents: 67%), 1-propenyl propyl disulphide (23%) and dipropyl trisulphide (6%). In the headspace of *A. tuberosum*, diallyl disulphide was detected as the main component (58%)" (*29*). This work used a polydimethylsiloxane SPME fiber, a 30 m × 0.25 mm GC column temperature programmed to 200 °C and a 220 °C injector temperature. In this author's opinion, the above volatiles are likely artifacts of the SPME and GC analysis; it is unlikely that they are the true attractants of the onion aphid.

In studies of volatiles from "tearless" (reduced-LF) onions, where GC-MS analysis was conducted with a 150 °C injection port temperature, 2-mercapto-3,4-dimethyl-2,3-dihydrothiophene (**15**)(Figure 3) was found: "the dihydrothiophenes detected in reduced-LF plants are likely to be formed from di-1-propenyl disulfide in thermally severe SPME-GC-MS analysis. The results presented here indicate that the di-1-propenyl thiosulfinate and its corresponding di-1-propenyl disulfide are thermally very unstable and as such difficult to assess quantitatively, despite the use of standard GC-MS protocols. Thus, while the disulfides and dihydrothiophenes may not be present in raw reduced-LF onion, they are likely to be produced in a cooked reduced-LF onion" (*30*). Compound **15**, formed on heating di-1-propenyl disulfide (**14**), loses H_2S when further heated, forming 3,4-dimethylthiophene (**16**), present in distilled onion oil (*31*).

Microwave (MW) Spectroscopy in the Characterization of *Allium* VSCs

Microwave spectroscopy is a specialized but useful technique, particularly when dealing with stereochemistry, tautomerism, position of deuterium incorporation, and structures of highly reactive molecules (e.g., CH_3S–O–H) not easily obtained by other methods (*5*). Detailed study (Figure 4) of the onion LF, using the technique of pulsed-beam Fourier transform microwave spectroscopy, showed it to be a mixture of (*Z*)-propanethial *S*-oxide ((*Z*)-**19**; major) and (*E*)-propanethial *S*-oxide ((*E*)-**19**; minor), in a 98:2 ratio. Further microwave studies established the site of incorporation of deuterium when onions are cut in D_2O, as well as the structure of the lowest energy rotamer of the major isomer. The structure of the LF-dimer was also determined (**20**) and a mechanism proposed for its formation (*32*). NMR spectroscopy was also very helpful in these structure determinations.

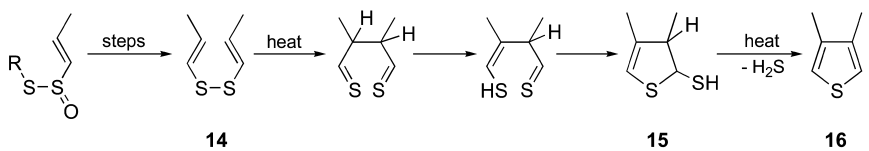

*Figure 3. Rearrangement of **14** to **15**; conversion of **15** to **16**.*

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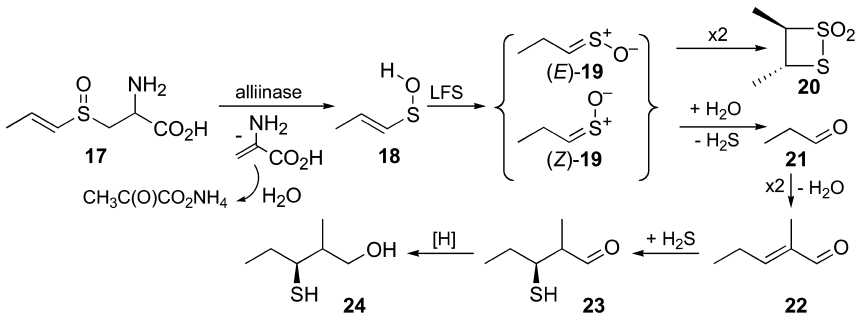

Figure 4. Formation, dimerization and hydrolysis of the onion LF.

Chiral but Racemic *Allium* VSCs

While all *Allium* thiosulfinates are chiral, GC-MS analysis of a mixture of the most volatile thiosulfinates from onion extracts, methyl methanethiosulfinate (MeS(O)SMe) and the isomeric methyl propyl thiosulfinates, MeS(O)SPr and MeSS(O)Pr, on a chiral 30 m × 0.32 mm γ-cyclodextrin GC column showed them to be completely racemic, as would be expected based on their non-enzymatic formation from condensation of achiral sulfenic acids (*4*).

Onion preparations contain 3-mercapto-2-methylpentan-1-ol (**24**), thought to be formed from H_2S addition to aldol product **22**, followed by reduction (*33*). The four stereoisomers of **24** have the same general odor that is concentration dependent. At 1 ppm in 5% saltwater, the odor is "sulfuric, burnt gum, sweaty, onion," while at 0.5 ppb, it is "meat broth, sweaty, onion, leek." While the odor threshold for the mixture of stereoisomers in water was 0.15 ppb, the values for the separate stereoisomers is somewhat different: (2*R*,3*S*) *anti*, 0.04 ppb; (2*S*,3*R*) *anti*, 0.03 ppb; (2*R*,3*R*) *syn*, >12 ppb; (2*S*,3*S*) *syn*, >30 ppb. Analysis of raw onion extracts by GC showed an *anti*:*syn* diastereomer ratio of 4:1 whereas enantioselective GC showed the ratio of enantiomers of each diastereomer to be 1:1, e.g., only racemates are present naturally (*34*).

What Is the True Odor of Cut *Allium*?: Use of DART-MS

Ferary and Auger pose a question in the title of their 1996 paper, "What is the true odor of cut *Allium*?" (*20*). This is a profound question, since within a very brief period of time following cutting, alliums undergo a rapid cascade of enzyme-initiated reactions, posing special challenges for studying the natural products chemistry of these plants. Standard methods of extraction and analysis could well give a false picture of this chemistry if the initial reactions are sufficiently fast, and if the compounds formed are sufficiently reactive and/or unstable. Fortunately, a new analytical technique, direct analysis in real time mass spectrometry (DART-MS), has become available. DART-MS is one of several popular methods used for ambient ionization mass spectrometry. Because of its ability to directly analyze gases, liquids, and solids in open air, without prior treatment, it has attracted considerable attention (*35*, *36*). Used

with a high-resolution (HR) time-of-flight mass spectrometer, DART is a "soft ionization" method, which for most compounds gives simple mass spectra that are easily obtained by momentarily holding the sample in the gas stream. Ionization under positive ion (PI-DART) conditions gives species formed when analytes collide with protonated water clusters $[(H_2O)_n + H]^+$, producing an $[M + H]^+$ ion for analytes having high proton affinities (*35*). If used under negative ion conditions, e.g., NI-DART-MS, $[M - H]^-$ ions are formed from analyte molecules containing acidic functional groups through proton abstraction by gaseous $[O_2]^{\bullet-}$ (*37*). The ability to perform HR-DART-MS without the need for sample preparation or solvent presents unique opportunities in food and natural products chemistry (*36*), allowing the direct observation of the rapid, complex cascade of enzymatically induced flavor-releasing processes following the wounding of plant cells. We have used DART-MS under both PI and NI conditions to search for intermediates and reactive organosulfur compounds when a variety of *Allium* species are crushed (*5*, *38–40*). These studies provide a solid experimental basis to evaluate the likelihood of artifact formation under harsher analytical conditions.

DART Identification of VSCs from Crushed Garlic

Analysis of a peeled garlic clove by PI-DART at room temperature showed that the predominant products are adducts of allicin (**3**) with a proton $[All_2S_2O + H]^+$ (*m/z* 163) and an ammonium ion $[All_2S_2O + NH_4]^+$ (*m/z* 180), from the stoichiometric ammonia released on hydrolysis of aminoacrylic acid, and dimeric species $[(All_2S_2O)_2 + H]^+$ (*m/z* 325) and $[(All_2S_2O)_2 + NH_4]^+$ (*m/z* 342) (Figure 5; only major products shown). Formula identification here and below was confirmed by HR-MS in all cases. While we had shown in 1994 that the mass spectrum of protonated allicin could be determined (*19*), the DART studies were of particular interest due to the minor products seen, including diallyl trisulfane *S*-oxide (**6**; $[C_6H_{10}S_3O + H]^+$; *m/z* 195), allyl alcohol (**8**; $[C_3H_5OH + H]^+$; *m/z* 59), isomeric allyl methyl thiosulfinates ($[AllMeS_2O + H]^+$; *m/z* 137), methyl methanethiosulfinate ($[Me_2S_2O + H]^+$; *m/z* 111), mixed dimers $[(All_2S_2O)(AllMeS_2O) + H]^+$ (*m/z* 299), a bis-sulfine ($[O{=}S{=}CHCHMeCHMeCH{=}S{=}O + H]^+$; *m/z* 179) and propene ($[C_3H_6 + H]^+$; *m/z* 43). The methyl group-containing products and the bis-sulfine are thought to arise from small amounts of *S*-methyl cysteine sulfoxide (methiin) and *S*-(1-propenyl) cysteine sulfoxide (**17**, isolalliin), respectively, also present in garlic.

At room temperature there is no evidence in the PI-DART spectra from garlic for disulfanes or polysulfanes, which is consistent with these compounds being secondary products, e.g., artifacts, associated with decomposition of the thiosulfinates. However, when the DART measurements are made with a gas heater temperature of 250 °C, additional, significant decomposition peaks are seen including diallyl disulfane (**4**) and diallyl trisulfane (**5** as well as alliin (**1**).

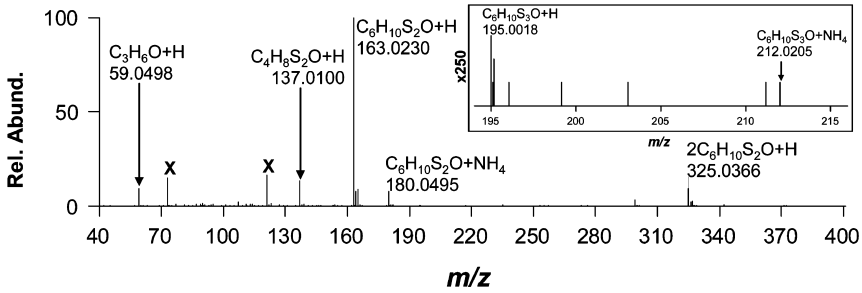

Figure 5. PI-DART mass spectrum from crushed garlic. Reproduced from reference (39). Copyright 2010 ACS.

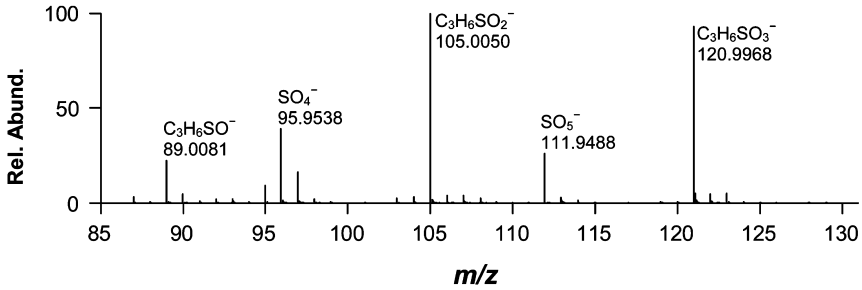

Figure 6. NI-DART mass spectrum from crushed garlic. Reproduced from reference (39). Copyright 2010 ACS.

The products seen in the PI-DART mass spectrum of crushed garlic are fully consistent with the mechanism shown in Figure 1. It is particularly satisfying to see the small peaks for the proton and ammonium ion adducts of **6** as well as a small peak for allyl alcohol (**8**) and a very small peak (not shown) for propene. Furthermore, under negative ion conditions, the NI-DART spectrum (Figure 6) shows the presence of anions of both 2-propenesulfenic acid (**2**; *m/z* 89) and 2-propenesulfinic acid (**7**; *m/z* 105). The intensity of the 2-propenesulfenate peak at *m/z* 89 rapidly decreases relative to that of the other NI peaks, with a half-life of <1 s, eventually disappearing completely (Figure 7). At the same time the *m/z* 87 signal for pyruvate rapidly increases.

The short lifetime for **2** is similar to gas-phase results for methanesulfenic acid as determined using microwave spectroscopy (*5*, *41*). Cysteine sulfenate, sulfinate and sulfonate anions have recently been identified by NI electrospray mass spectrometry following interfacial ozonolysis (*42*).

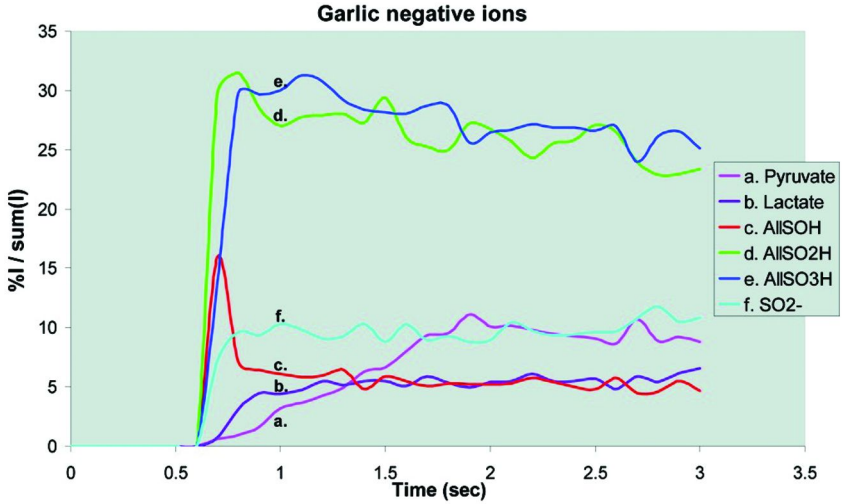

Figure 7. NI-DART of crushed garlic kinetic plot. Reproduced from reference (39). Copyright 2010 ACS.

Limitations of the DART technique should be mentioned. From other work it is known that while the 2-propenyl (allyl) group is the dominant C_3H_5 fragment found in garlic, lesser amounts of the isomeric 1-propenyl group also occur (*5*). Indeed, 1-propenyl thiosulfinates are the likely precursors to the bis-sulfine that is found. The 1-propenyl group cannot be distinguished from the allyl group by HR-MS, requiring additional MS-MS studies. Thus both the *m/z* 163 and 137 species and related NH_4 adducts formed from garlic are likely to contain both allyl and 1-propenyl fragments. While the precise structural formula for the small peaks corresponding to $[C_6H_{10}S_3O + H]^+$ and $[C_6H_{10}S_3O + NH_4]^+$ cannot be determined from the MS data alone, theoretical calculations (*43*) and synthetic studies (*44–48*) indicate that compounds of type RS(O)SSR are favored over isomeric compounds RSS(O)SR. Thus, we suggest that the structure of the $C_6H_{10}S_3O$ species is **7,** consistent with earlier mechanistic studies of Kice (*49*) for self-reaction of diaryl thiosulfinates.

DART Identification of VSCs from Crushed Elephant Garlic (A. ampeloprasum)

We previously reported, on the basis of HPLC analysis, that the thiosulfinates from crushed elephant garlic show an allyl/methyl/1-propenyl ratio of ca. 65:33:2 (*17*). When compared to the alliin/methiin/isoalliin cysteine precursor ratios of 60:17:20 in the intact plant (*50*), the levels of 1-propenyl thiosulfinates from the crushed plant seem surprisingly low. This observation is explained by the PI-DART-MS trace (Figure 8), which reveals the presence of significant concentrations of the onion LF, propanethial *S*-oxide (**19**), in addition to allicin, methyl/allyl thiosulfinate and/or methyl/1-propenyl thiosulfinate, and the bis-sulfine along with traces of diallyl trisulfane *S*-oxide (**6**) (*39*). Most of the

1-propenyl compounds wind up in the form of LF 19 rather than thiosulfinates. As discussed below, the presence of **19** and *bis*-sulfine was confirmed by NMR analysis. Crushing elephant garlic results in an easily observed, mild lachrymatory effect. The presence of **19** in elephant garlic has not been previously reported and makes this plant unique in having both allicin and the onion LF.

DART Identification of VSCs from Crushed Leek

In contrast to the case of garlic and onion, where alliin and isoalliin are the respective dominant precursors, in leek, propiin, methiin, and isoalliin are all present in comparable amounts, leading to the mixture of thiosulfinates seen by PI-DART-MS (Figure 9) (*39*). No evidence was found for the presence of thiosulfinates containing ethyl groups, as suggested by Doran and coworkers (*51*). Furthermore, in our studies disulfanes and polysulfanes were absent, which is consistent with earlier work indicating that such compounds are secondary decomposition products of thiosulfinates.

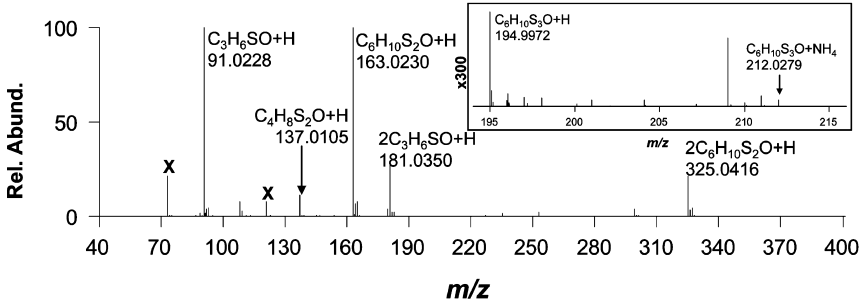

Figure 8. PI-DART mass spectrum from crushed elephant garlic. Reproduced from reference (39). Copyright 2010 ACS.

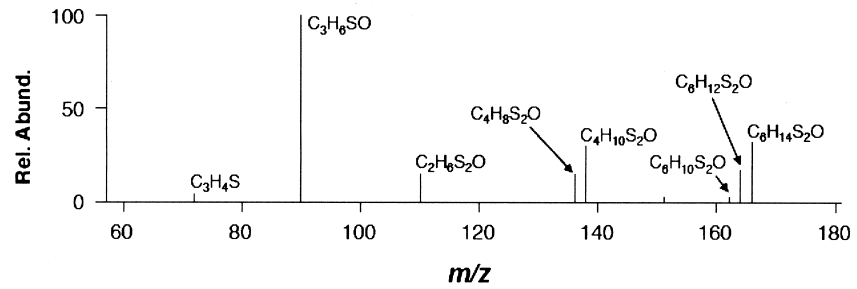

Figure 9. PI-DART mass spectrum from crushed leek. Reproduced from reference (39). Copyright 2010 ACS.

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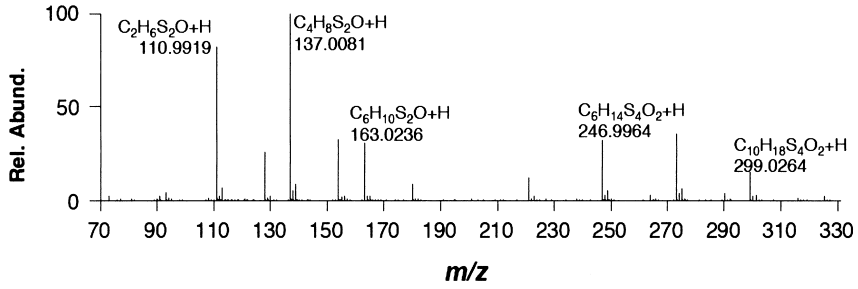

Figure 10. PI-DART-MS of Chinese chive. Reproduced from reference (39). Copyright 2010 ACS.

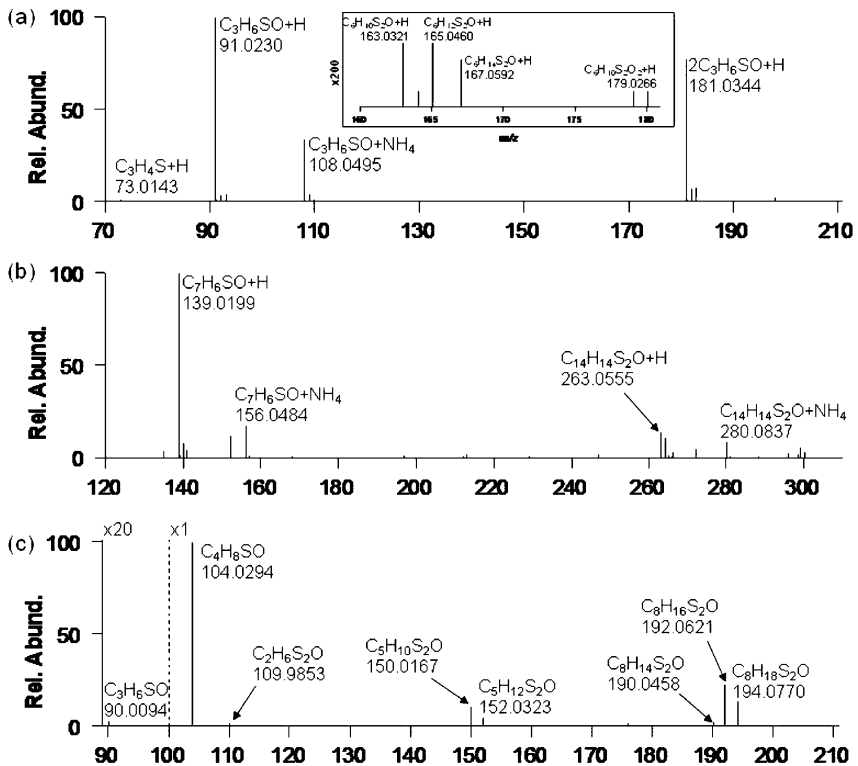

Figure 11. PI-DART-MS from (a) cut onion, (b) P. alliacea and (c) A. siculum (for (c), simplified summed trace combining H^+ and NH_4^+ adducts shown as neutral parents). Reproduced from reference (38). Copyright 2010 ACS.

DART Identification of VSCs from Crushed Chinese Chive (A. tuberosum)

On the basis of the PI-DART-MS data (Figure 10), the ratio of alk(en)yl groups in crushed Chinese chive is 65% methyl and 35% allyl. Traces of 1-propenyl groups are present as indicated by detection of propanethial *S*-oxide (LF); *n*-propyl groups were absent (*39*).

DART Identification of VSCs from Crushed Onion

The PI-DART-MS for onion shows a predominant amount of LF **19**, seen as a set of three intense ions at *m*/*z* 91 ($[C_3H_6SO + H]^+$), *m*/*z* 108 ($[C_3H_6SO + NH_4]^+$), and *m*/*z* 181 ($[(C_3H_6SO)_2 + H]^+$), as shown in Figure 11a. Minor peaks are identified on the basis of their HR-MS and the following assumptions: (1) allyl compounds are present in onions at best at extremely low levels so that a single C_3H_5 group is most likely (*E*)-1-propenyl; (2) on the basis of prior studies of onion preparations, compounds of formula $C_6H_{10}S_2O$ and $C_6H_{10}S_2O_2$ are most likely zwiebelanes and bis-sulfine, respectively. On this basis, onions show as minor components 0.2% zwiebelanes, 1% of mixed 1-propenyl propyl thiosulfinates (PrS(O)SCH=CHMe, PrSS(O)CH=CHMe), 1% of PrS(O)SPr, and lower levels of bis-sulfine (*38*).

DART Identification of VSCs from Crushed Petiveria alliacea

Another sulfine, phenylmethanethial *S*-oxide (**27**; Figure 12), was isolated from extracts of *P. alliacea*, a tropical weed extensively used in traditional medicine (*52*). Sulfine **27** presumably originates from action of a LF synthase on phenylmethanesulfenic acid **26** (*53*), in turn formed via alliinase cleavage of precursor **25** (*54*). Alternatively, **26** can self-condense, giving thiosulfinate **28** (*55*). The woody root of *P. alliacea* was abraded with a knife blade in the DART source region with a heated gas flow until a strong garlic-like odor was released from the plant. Under PI-DART conditions both **27** and **28** were seen as their protonated and ammoniated adducts (Figure 11b). The identification of thiosulfinate **28** was confirmed with a synthetic standard. A small peak corresponding to dibenzyl disulfide (**29**) was also found. The higher temperatures required to volatilize **28** are presumably responsible for the formation of **29** because di- and polysulfides were not seen when *Allium* samples were examined by DART at room temperature.

DART-MS Identification of VSCs from Crushed Mediterranean Bells (A. Siculum)

A. siculum, an ornamental bulbous plant, is a member of a small *Allium* subgenus *Nectaroscordum*, which also includes *A. tripedale*. Also called Sicilian honey garlic or Mediterranean bells, *A. siculum* is native to Asia Minor, southern France, and Sicily, and is used as a seasoning in Bulgaria. The skunky odor

released when the plant is cut is attributed to the presence of butyl thiosulfinates, thought to originate from *S*-*n*-butylcysteine *S*-oxide (butiin) (*56*). Thiosulfinates containing methyl and 1-propenyl groups were also reported to be present (*56*).

Samples examined using PI-DART showed the presence of butanethial *S*-oxide (**32**) as the major component (Figures 11c, 13). The identity of **32**, only the fourth sulfine known to occur naturally, was confirmed by synthesis. By NMR methods (see below) **32** was found to be a mixture of 94% (*Z*)-**32** and 6% (*E*)-**32**. PI-DART also indicated the presence of moderate levels of 1-butenyl/ methyl, 1-butenyl/butyl, dibutyl and dimethyl thiosulfinates (**33**), and the higher homologue of zwiebelanes, along with traces of onion LF **19**. All formulas for the volatiles, which have two, four, five, or eight carbon atoms, reflecting various combinations of compounds with one or four carbon atoms, were confirmed by HR-MS. Significant quantities of disulfanes or polysulfanes were not detected by PI-DART and are therefore assumed not to be primary products. Homoisoalliin **30** was isolated from *A. siculum* and *A. tripedale* (*38*, *57*). NI-DART showed peaks for the anion of 1-butenesulfenic acid (**31**) as well as 1-butenesulfinic and 1-butanesulfinic acids (Figure 14) (*38*).

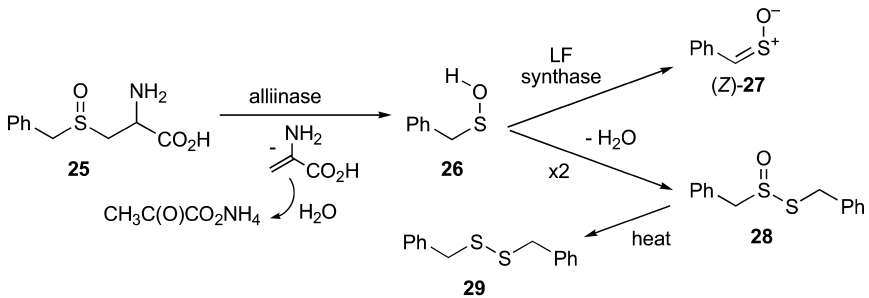

Figure 12. Formation of (Z)-phenylmethanethial S-oxide and dibenzyl thiosulfinate upon cutting P. alliacea.

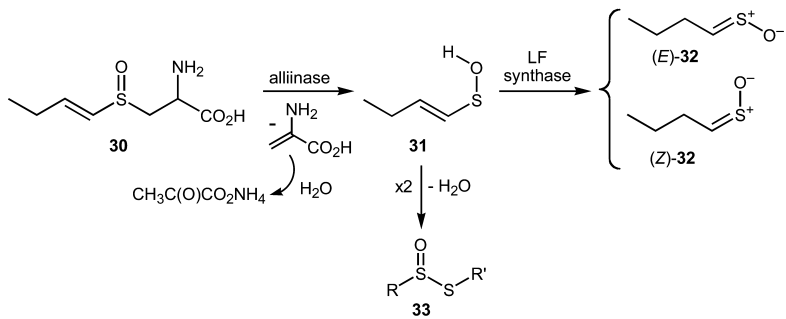

Figure 13. Formation of (E/Z)-butanethial S-oxide and thiosulfinates upon cutting A. siculum.

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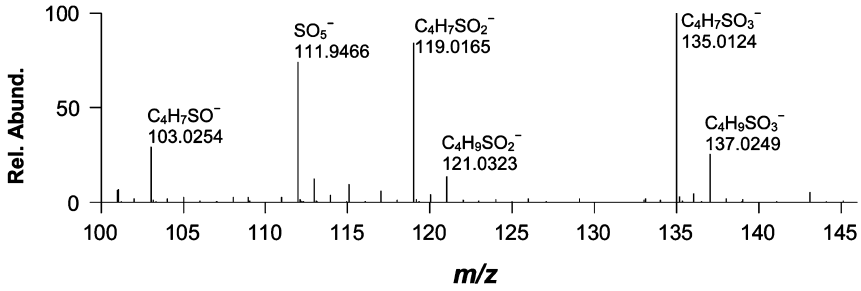

Figure 14. NI-DART-MS from crushing A. siculum (RSO_3^- is probably $RS(O)OO^-$). Reproduced from reference (38). Copyright 2010 ACS.

Mixed Volatile Sulfur-Selenium Compounds from Alliums

Selenium, the 66th most abundant element, occurs naturally at levels ca. 12,000 times lower than those of analogous sulfur compounds and is of interest as an essential trace element (micronutrient) (*5*). Early studies by Finnish Nobel Laureate Arturri Virtanen suggested that there might be a selenium-based flavor chemistry in *Allium* species parallel to that based on sulfur, e.g., originating from soil selenate or selenite rather than sulfate (*5*). Initial efforts examining volatile compounds from cut alliums used headspace-gas chromatography-atomic emission detection (HS-GC-AED) (*58–60*). Headspace-GC reduces or eliminates sample preparation and is ideal for trace analysis of volatiles. Atomic plasma spectra emission provides powerful element-specific detection in the form of GC-AED, and allows simultaneous multi-element analysis. It can flag compounds in the GC effluent containing specific elements, even if these compounds co-elute with other much more abundant compounds (*5*).

When homogenized elephant garlic was examined using HS-GC-AED, the sulfur and carbon channels showed the expected thiosulfinate decomposition products, namely MeSAll, MeSSMe, AllSAll, MeSSAll, AllSSAll, MeSSSAll, AllSSSAll. Thiosulfinates themselves were not detected by the HS-GC methods due to their water solubility and diminished volatility. The Se channel showed eight peaks, namely MeSeMe, MeSeSMe, MeSeSeMe, MeSSeSMe, MeSeAll, MeSeSAll, MeSeSCH=CHMe, and MeSSeSAll. Structures were established by MS as well as by comparison with spectra of synthetic materials (*58*, *59*). Human garlic breath was analyzed using GC-AED with breath samples collected using 1.5 L Tedlar (polyvinylfluoride, PVF) bags. The sulfur channel showed the presence of AllSMe (major), MeSSMe, AllSSMe, AllSAll, and AllSSAll (major), while the Se channel showed the presence of MeSeMe (largest peak), AllSeMe, MeSeSMe, MeSeSAll, and MeSeSeMe. 2-Propenethiol was present when human garlic breath was sampled immediately after garlic ingestion but disapeared after one hour (*60*). Subsequent studies using both GC-AED and LC-ICPMS showed that the volatile Se compounds originated from selenoamino acids (*5*, *61*).

Analysis of the volatiles from Se-enriched *A. fistulosum* using SPME fibers together with GC-ICPMS and GC-TOF-MS showed the presence of MeSeSMe, PrSSeMe, MeCH=CHSSeMe, MeSSeSMe, MeSSSeMe, PrSSeSMe

and PrSSeSPr (*62*). By headspace analysis of the *Bacillus* species LHVE using a SPME fiber with GC with a sulfur chemiluminescence detector (GC-SCD) as well as GC-MS, showed the presence of dimethyl diselenenyl sulfide, MeSeSeSMe, and dimethyl selenenyl sulfide, MeSeSMe, along with MeSH, MeSMe, MeSSMe and MeSSSMe (*63*). Dimethyl selenenyl disulfide, MeSSSeMe, has been reported in the headspaces of bacterial cultures (*64*). In some of these analyses, dimethyl polysiloxane appeared as an artifact from the SPME fiber.

Use of NMR Spectroscopy in the Analysis of *Allium* VSCs

Both ^{1}H and ^{13}C NMR spectroscopy can be extremely useful as a complement to other analytical techniques in characterizing VSCs. ^{1}H NMR was used to confirm the presence of both (*Z*)-propanethial *S*-oxide ((*Z*)-**19**) and (*Z,Z*)-*d,l*-2,3-dimethyl-1,4-butanedithial 1,4-dioxide (bis-sulfine) detected by PI-DART-MS in fresh homogenates of elephant garlic through a characteristic triplet at δ 8.20 (*J* = 7.8 Hz) and doublet at δ 8.11 (*J* = 9.6 Hz), respectively (*39*). Under these same conditions the ^{1}H NMR spectrum of authentic (*Z*)-**19** shows δ 8.17 (t, *J* = 7.9 Hz) while the bis-sulfine shows δ 8.09 (d, *J* = 9.6 Hz) (*32*).

Analysis of an extract of a whole *A. siculum* plant by ^{1}H NMR showed major and minor triplets at δ 8.16 (*J* = 8.0 Hz; 94%) and 8.82 (*J* = 9.3 Hz; 6%), corresponding to (*Z*)- and (*E*)-butanethial *S*-oxide, respectively (*38*). Peak assignments were confirmed with an authentic sample of the sulfine. Similar results have been obtained showing (*Z*)-propanethial *S*-oxide and (*Z*)-phenylmethane *S*-oxide to be the major VSCs from homogenized onion (*32*) and *P. alliaceae* (*52*), respectively.

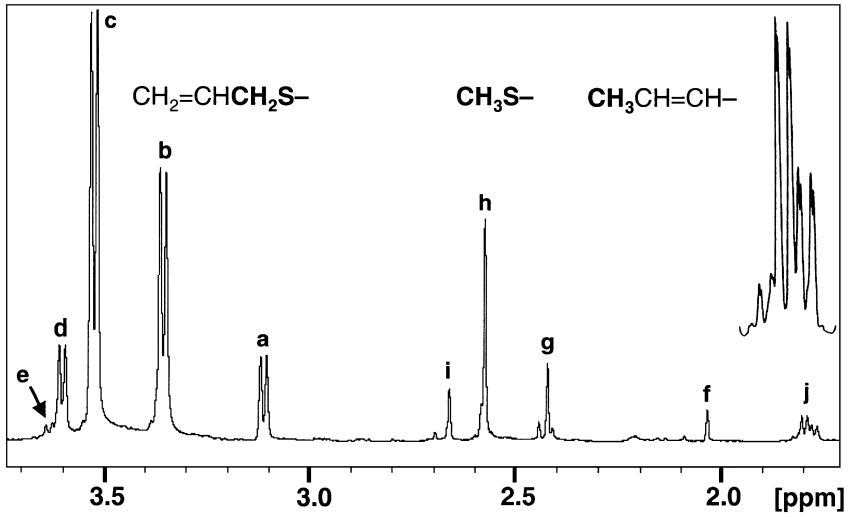

Figure 15. The 1.8-3.7 ppm region of the ^{1}H NMR spectrum (500 MHz, $CDCl_3$) of distilled oil of garlic. Reproduced from reference (66). Copyright 2004 ACS.

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ACS Symposium Series; American Chemical Society: Washington, DC, 2011.

NMR spectroscopy has also been used in the analysis of a series of allyl polysulfanes from the distilled oil of garlic (*5*, *65*). For a complex mixture of naturally derived compounds, garlic oil shows a surprisingly simple ^{1}H NMR spectrum (Figure 15). It consists of a well separated series of doublets (J = 7.3 Hz) from 3.1 to 3.7 ppm for the thioallylic protons (CH_2=CHC***H***$_2$S), a similarly well separated series of singlets from 2.0 to 2.7 for the CH_3S groups, a weak set of doublets of doublets at 1.8 ppm ($J \cong 7$ and 1) for the (*E*)- and (*Z*)-1-propenyl groups (2:1 *E*:*Z* ratio), along with 5-6 ppm olefinic multiplets. There is virtually no absorption in the 0-1.8 ppm region nor in the 2.7-3.1 and 3.7-5.0 ppm regions. It is notable that the intensity patterns of the four major singlets (peaks f, g, h, i) parallel the pattern of the four major doublets (peaks a, b, c, d), which is consistent with these sequences of peaks reflecting the relative abundances of the families of compounds of formula RS_nAll (n = 1-4, R = Me or All). The ^{1}H NMR chemical shifts of the thioallylic protons and mixed methyl allyl polysulfanes (***Me***$AllS_n$) and dimethyl polysulfanes (***Me***$_2S_n$) methyl protons progressively shift downfield as the number of sulfur atoms increases: (All_2S [7%], δ 3.11; All_2S_2 [26%], δ 3.36; All_2S_3 [33%], δ 3.52; All_2S_4 [6%], δ 3.60; All_2S_5 [5%], δ 3.63; All_2S_6 [tr], δ 3.67; ***Me***AllS [2%], δ 2.04; ***Me***$AllS_2$ [5%], δ 2.42; ***Me***$AllS_3$ [11%], δ 2.58; ***Me***$AllS_4$ [2%], δ 2.70; ***Me***$_2S_2$ [0.4%], δ 2.44; ***Me***$_2S_3$ [1%], δ 2.59; ***Me***$_2S_4$ [tr], δ 2.67). The dimethyl polysulfane (***Me***$_2S_n$) signals appear as downfield shoulders on the ***Me***$AllS_n$ peaks. Relative mole%, in brackets, is calculated from the integration as described in the original report (*65*). The changes in ^{13}C NMR chemical shifts for these same series of compounds, not shown here, are not as regular but are still useful. The NMR method is useful for determination of the composition of garlic oil since the higher polysulfanes are thermally unstable, precluding use of GC for quantification. Use of HPLC requires peak calibration if a UV detector is used, since polysulfane extinction coefficients increase with the number of contiguous sulfur atoms.

Use of X-ray Absorption Spectroscopy in the Analysis of *Allium* VSCs

Real time, in situ information on sulfur biochemistry as it occurs in *Allium* species, for example producing VSCs, is difficult to obtain because of a lack of biophysical techniques that have sufficient sensitivity to molecular form. This is in part due to the fact that sulfur lacks a well-established spectroscopic probe, and is often called a spectroscopically silent element. For example, the low natural abundance, weak magnetic moment, and significant nuclear electric quadrupole moment of ^{33}S combine to make ^{33}S NMR challenging, and it is infrequently used. However, it is possible to use sulfur K-edge X-ray absorption spectroscopy (XAS) as a direct probe of the sulfur biochemistry of living cells to generate maps of different chemical forms of sulfur, taking advantage of the chemical shift range of more than 14 eV. In particular, onion cell samples can be scanned in a microfocus X-ray beam at a number of different incident energies, providing sensitivity to different sulfur chemical forms. The sulfur X-ray fluorescence is monitored, and with information about the spectra of standard species, the data can be converted to quantitative maps of the different chemical forms, e.g.: organic disulfides at 2469.88 eV; organic sulfides at 2470.55 eV; organic sulfoxides at 2473.59 eV;

sulfate at 2479.58 eV (Figure 16). The sulfur K-edge XAS of a pure sample of the onion lachrymatory factor **19** (LF) was also measured. In intact onion cells X-ray fluorescence spectroscopic imaging using an X-ray microprobe in parallel with an optical microscope showed elevated levels of sulfoxides (e.g., the LF-precursor isoalliin, **17**) in the cytosol and elevated levels of reduced sulfur in the central transport vessels and bundle sheath cells. XAS of onion sections showed increased levels of LF **19** and thiosulfinates, along with decreased levels of LF-precursor **17**, following cell breakage (Figure 17) (*5*, *66*, *67*).

Ultra-Performance-(Ag^+)-Coordination Ion Spray-Mass Spectrometry (UPLC-[Ag^+]CIS-MS) in the Analysis of *Allium* VSCs

Analysis of complex mixtures of non-polar components found in distilled alliums oils, valued as flavorants, poses special challenges. Separation of mixtures of structurally similar compounds is difficult to achieve, requiring reversed phase columns and long elution times. Sensitivity is low using standard LC-ESI-MS conditions due to the low Lewis basicity of divalent sulfur. Fortunately, as described below, solutions are available for both of these problems.

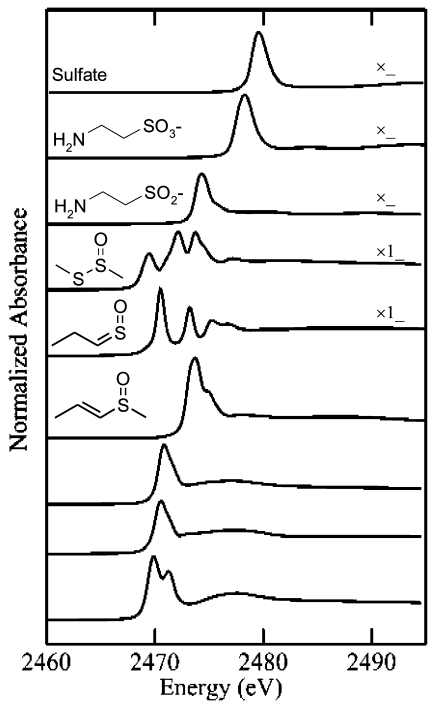

Figure 16. Normalized sulfur K-edge X-ray absorption spectra of sulfur species relevant to onion. Reproduced from reference (66). Copyright 2009 ACS.

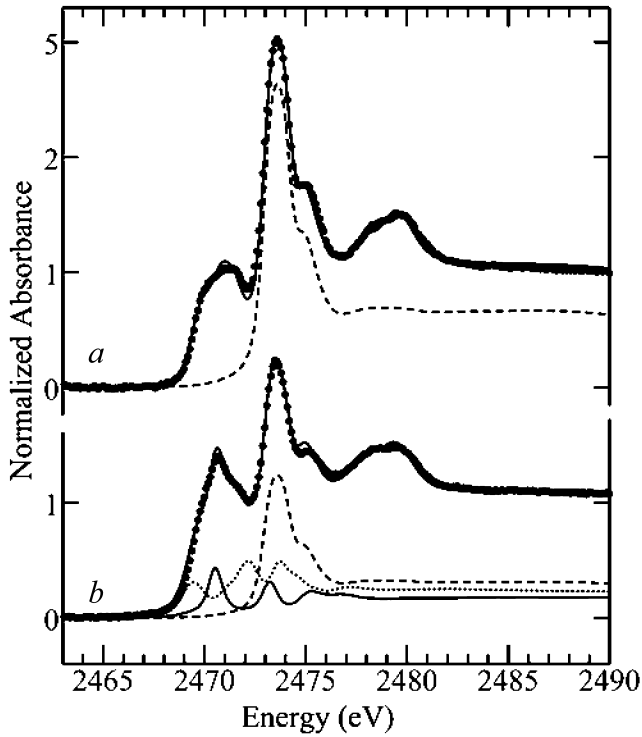

*Figure 17. Sulfur K-edge X-ray absorption spectra of onion tissue (a) before, and (b) after, rubbing to induce cell breakage, showing linear combination fits (solid lines) to the experimental data (points, •). The dashed lines in (a) and (b) indicate sulfoxide components. In (b) LF **19** and thiosulfinates are indicated by the thin line and the dotted lines, respectively. Reproduced from reference (66). Copyright 2009 ACS.*

HPLC-(Ag^+)CIS-MS, employing post-separation infusion of a $AgBF_4$ solution, is useful for analysis of various nonpolar and poorly ionized substances but has seen limited application to organosulfur compounds (*40*, *68*, *69*). Reports have appeared on post-column use of silver salts as coordinating ions in HPLC-ESI-MS analysis of diallyl polysulfanes from "garlic powder" (*68*) and LC-CIS-MS analysis of polysulfanes with two to eight sulfur atoms in rubber vulcanization model systems (*69*). A related technique, extractive electrospray ionization quadrupole time-of-flight mass spectrometry (EESI-QTOF-MS), used a $AgNO_3$/ water solution to generate an electrospray to facilitate detection of nonpolar VSCs in human garlic breath, captured as their $^{107}Ag/^{109}Ag$ adducts (*70*).

Ultra performance liquid chromatography (UPLC), employing smaller chromatographic support particle size and higher pressures, has an advantage over HPLC in significantly reducing the elution times with a resultant sharpening of peaks, important for MS analysis of late-eluting trace components (*71–73*). Application of UPLC-(Ag^+)CIS-MS to a sample of garlic oil led to rapid separation (13 min) of a series of peaks identified by selective ion monitoring

as $^{107}Ag/^{109}Ag$ adducts of diallyl disulfide through nonasulfide; liquid sulfur treatment further increased the garlic oil sulfur content (Figure 18).

In this work, immediately following chromatographic separation, a solution of $AgBF_4$ is introduced into the liquid sample. This same technique was used to characterize other families of polysulfanes in garlic oil, allowing identification of several previously unknown compounds (*5*, *40*, *74*). In the above studies, elemental sulfur (S_8) was sometimes found. It can form through decomposition of organosulfur compounds, such as diallyl polysulfanes or isothiocyantes, or through bacterial action. Sometimes it can occur as an artifact, fooling the analyst into thinking that it is a carbon-containing compound. Elemental sulfur elutes during GC or HPLC like a non-polar organic compound, for example co-eluting with octadecane on a GC column (*75*). Its mass spectrum shows an M^+ at *m/z* 256 with a strong M+2 ion at *m/z* 258 (35.2% of the abundance of M^+), while the fragmentation pattern consists of a series of peaks separated by 32 amu. While an S_8 peak is seen with GC-MS and electron-capture GC, it may not be detectable using a flame ionization detector.

Chemistry in a Salad Bowl: VSCs from Genus *Brassica* Plants

Isothiocyanates from Brassica Plants

Glucosinolates (**34**; Figure 19) are secondary metabolites from plants of the order Capparales, particularly the genus *Brassica* (cruciferae), and are derived from protein and nonprotein amino acids. There are more than 120 different glucosinolates, distinguished from one another by their different aglycons (organic side chains). They occur in all parts of the plant and can degrade by both enzymatic as well as nonenzymatic hydrolysis (*76*). The most important degradation pathway for glucosinolates at neutral pH affords isothiocyanates. Allyl isothiocyanate is the volatile sulfur compound responsible for the pungent taste of mustard, horseradish and wasabi. It is released during the decomposition of leaf tissues and seeds of black mustard (*Brassica nigra*) or brown Indian mustard (*B. juncea*). It is formed by action of the myrosinase (thioglucosidase) enzyme on the plant glucosinolate sinigrin.

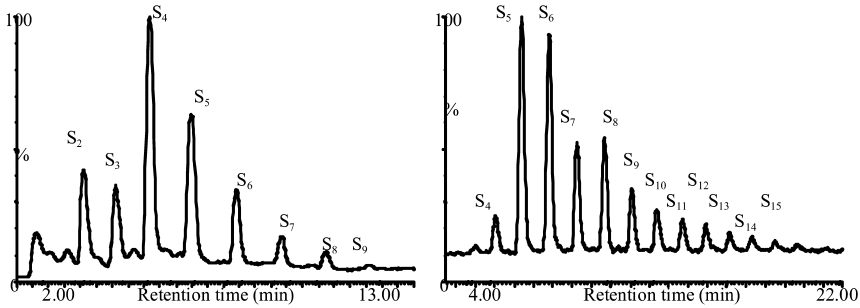

Figure 18. UPLC-(Ag^+)CIS-MS of diallyl polysulfanes in garlic oil before (left), and after (right), liquid sulfur treatment (74).

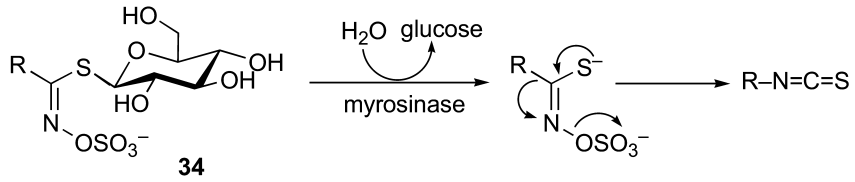

*Figure 19. Conversion of glucosinolates **34** to isothiocyanates via myrosinase catalysis.*

At low pH, glucosinolates can degrade giving nitriles and elemental sulfur (see above comments on detection of sulfur). Some glucosinolates, particularly the 2-propenyl, benzyl and 4-methylthiobutyl, can also afford thiocyanates, while others afford oxazolidines. If the R group of the thiocyanate contains a terminal alkenyl group, the thiocyanate may be converted into an epithionitrile (*76*). Most of the above degradation products have insecticidal, nematicidal and herbicidal properties that, at least in the case of the isothiocyanates, can involve bonding to protein sulfhydryl groups (*76*). Allyl isothiocyanate from sinigrin showed particularly strong nematocidal action (*77*).

The main volatile species present in the *B. juncea* headspace are allyl isothiocyanate and 3-butenyl isothiocyanate, found in a ratio of 2:1 using HS-SPME-GC-ICPMS and GC-MS methods (*78*). 3-Butenyl isothiocyanate is formed from the glucosinolate gluconapin. The isothiocyanates are believed to act as a plant defense mechanism during invasion by pathogens or insect pests. The roots of the rapeseed plant *(B. napus*) when plowed in the field, produce 2-phenylethyl glucosinolate allelochemical degradation products that may be useful for the control of soil-borne pests (*76*).

Like the thiosulfinates from genus *Allium* species, some of the seondary metabolites from *Brassica* species decompose in the injection ports of GC or GC/MS equipment. Sulforaphane, $MeS(O)(CH_2)_4N{=}C{=}S$, from broccoli and cabbage, was found to undergo thermal degradation at temperatures as low as 50 °C giving MeSSMe, MeS(O)SMe, $MeSO_2SMe$, $MeSCH_2SSMe$, and 1,2,4-trithiolane, among other products (*79*).

Brassica oleracea (kale) VSCs have been analyzed using HS-SPME on a DVB/PDMS fiber combined with GC/IT-MS, with the GC injection port at 220 °C and the 30 or 60 m capillary column heated in the GC oven programmed to a maximum of 220 °C (*80*). Major volatiles included allyl isothiocyanate, 3-butenyl isothiocyanate, and 3-methylthiopropyl isothiocyanate along with lesser amounts of other isothiocyanates, thiocyanates, and small amounts of dimethyl disulfane and trisulfane (*80*). Similar studies have been conducted with *B. juncea* using HS-GC-MS, with parallel LC-TOF-MS analysis of the precursor glucosinolates (*81*).

Artifact Formation with SPME Fibers

Solid-phase microextraction (SPME), first introduced to the scientific community in 1993, is widely used for the analysis of volatile compounds as an alternative to adsorbent tubes. The syringe-like device is easy to use and can

be performed in a standard GC injection port. Polydimethylsiloxane/Carboxen (PDMS/CAR) is the most commonly used SPME fiber, leading to sub-µg m^{-1} detection limits. The most efficient adsorbents for low molecular weight molecules are usually micro-porous, in order to increase the specific surface area for adsorption. Unfortunately, such materials require high temperature to achieve quantitative desorption of analytes. Artifact formation can occur during the thermal desorption step (typically 250-280 °C) catalyzed by metals, e.g., iron and nickel, found as fine stainless steel particles in the fibers and thought to be produced by the SPME stainless steel needle. Some samples of the Carboxen coating used in SPME fibers are reported to contain up to 0.9 mg g^{-1} of Fe (*82*).

In a study of artifact formation in the analysis of VSCs using SPME fibers, it was found that the fibers sometimes released small amounts of sulfur dioxide, which was presumed to originate from oxidation of residual elemental sulfur present in the Carboxen coating. It has also been reported that during SPME analysis, thiols can be partially oxidized to disulfides, and dimethyl sulfide to dimethyl sulfoxide, with methanethiol being especially sensitive to oxidation. When mixtures of thiols are analyzed, mixed disulfides can result (*82*). The extent of artifact formation can be substantial. Under conditions of thermal desorption, iron compounds could serve as catalysts for both oxidation as well as dehydrogenation (*83*). 2-Methylpropanethiol (*i*-BuSH) has been reported to undergo surface-catalyzed loss of hydrogen sulfide during thermodesorption in studies involving SPME with GC-AED analysis (*84*). Other artifacts sometimes formed with SPME fibers include nitrogen compounds from the glue used to hold the SPME fiber in the fiber assembly, and siloxanes, which can be released by the vial septum. The latter problem can be avoided by baking the septum in a 150 °C oven overnight (*85*). Other recommended precautions include deactivation of the GC injection port with bis(trimethylsilyl)-trifluoroacetamide (BSTFA) and flushing of all sample vials with inert gas (*2*).

Relative humidity can be a problem with SPME analysis of VSCs because water significantly lowers the capacity of adsorbents, can clog cryogenic traps and can cause baseline perturbations and retention time shifts in chromatography. It has been noted that more water is absorbed by SPME fibers above 40 °C, causing baseline shift in the chromatogram. To avoid this problem, a short extraction time (15 min) and a low temperature (30 °C) were used (*2*). Drying agents have been found to be suitable for analysis of many VSCs and are therefore recommended (*83*). Another problem with SPME analysis on PDMS/CAR fibers is that higher molecular weight compounds in a sample can displace lower molecular weight compounds from the fiber as a consequence of competition for active sites on the fiber, e.g., the suppression of MeSH, Me_2S, and to a lesser extent Me_2S_2 adsorption by the presence of CS_2, itself barely detectable (*86*). The relative proportions of the components adsorbed onto the fiber depend on their ratio in the headspace. It is noted "as their relative concentrations change from sample to sample, the varying interactions result in irregular analytical responses, reflectic in erratic calibration curves. Standards containing single components are not valid; only a standard containing all components found in the sample to be analyzed, and at the same relative concentrations, is appropriate. In practice, this may preclude the use of fibers for quantitative analysis of multicomponent mixtures"

(*86*). Another researcher concludes that PDMS/CAR SPME is unsuitable for time weighted average (TWA) sampling of H_2S, MeSH, EtSH, and Me_2S, since the uptake rates of these compounds vary greatly with humidity, temperature, and time; for H_2S and MeSH, concentration also has significant effects (*87*). In the analysis of wine, SO_2 present at levels as high as 50 ppm can interfere with the detection of VSCs when sulfur-specific detectors are used. Acetaldehyde, which reacts with SO_2, has been shown to be effective in eliminating interference due to SO_2 (*2*).

In connection with analysis of grapefruit VSCs using SPME and GC with pulsed flame photometric detection (GC-PFPD), it was found that artifact formation could be avoided by using nitrogen instead of air for headspace purging. The nitrogen headspace is thought to decrease oxidation of the VSCs (*88*) and prevent cyclization of 1-*p*-menthen-8-thiol to 2,8-epithio-*cis*-*p*-menthane. The latter cyclization is also promoted by light at room temperature. Thus, direct exposure to light should be avoided in such analyses. Significant differences were found among different SPME fibers with regard to their ability to concentrate 1-*p*-menthen-8-thiol: PDMS/CAR failed to concentrate this compound, in contrast to the triphase DVB/PDMS/CAR fibers. It was also found that slower fiber sorption occurred with the later eluting compounds, requiring a minimum of 15-30 min of SPME fiber exposure time; 45 min SPME exposure time was selected as optimum. Very long exposure times may allow displacement and secondary reactions to occur. Increasing sample extraction temperature improved the extraction efficiency of late eluting volatiles up to a point (e.g., 40 °C). At higher temperatures (e.g., 60 °C), evidence of thermally generated artifacts was seen: "the sample extraction temperature of 40 °C was chosen as it represented the best compromise of maximum headspace concentrations with minimal artifact formation for most sulphur volatiles" (*88*). Other researchers noted similar problems, but needed slightly higher temperatures (50 °C) for most satisfactory results (*89*). SPME fibers can also suffer from saturation with high analyte concentrations, for example as occurs with isothiocyanates produced from mustard seeds (*90*).

In some cases, it can be difficult to distinguish artifact formation under thermal desorption conditions caused by the SPME fiber from processes occurring in the hot GC injection port. For example, in analytical studies involving CH_3SCH_2SH, $CH_3SCH{=}S$ was sometimes detected, in amounts which varied with the analytical conditions (*91*, *92*). This latter compound was postulated to arise by metal-catalyzed dehydrogenation of the thiol at the elevated temperatures employed, analogous to processes seen with amines.

Chemistry in a Wine Glass: VSCs in Wine

Among the more than 1000 volatile compounds present in wine, 3-mercaptohexan-1-ol (**35**/**36**; 3-MH), 3-mercaptohexyl acetate (**37**/**38**; 3-MHA) and 4-mercapto-4-methylpentan-2-one (**39**; 4-MMP) (Figure 20) have attracted considerable attention as VSCs that can impart pleasant, varietal aromas. First identified in fruits such as black currant, grapefruit, passion fruit, or guava, these

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compounds have since been found in wine made from many different cultivars of *Vinis vinifera*. These wine thiols are extremely potent and have some of the lowest odor detection thresholds (*ca.* 3 ng/L in wine) of any compounds found in food or beverages (*93*). The perception thresholds of (*R*)-3-MH (**35**) and (*S*)-3-MH (**36**) are very similar. However, these two enantiomers have different aromas, with the (*R*)- and (*S*)-forms being reminiscent of grapefruit and passion fruit, respectively (*93*). The perception threshold of (*S*)-3-MHA (**38**) is ca. four times lower than that of (*R*)-3-MHA (**37**). The (*S*)-enantiomer is also three times more abundant in wine than the (R)-enantiomer. (*R*)-3-MHA (**37**) has a passion fruit odor in contrast to the more herbaceous, boxwood odor for (*S*)-3-MHA (**38**) (*93*). Recent work has shown that compounds of structures related to those of **35**-**39**, are found in sweet wine made from *Botrytis*-infected grapes (2-methyl-3-mercaptobutan-1-ol [**40**], 3-mercaptopentan-1-ol [**41**], and 3-mercaptoheptan-1-ol [**42**]; the first two have citrus aromas while the third is reminiscent of raw onion) (*94*) and in beer brewed using the New Zealand hop cultivar Nelson Sauvin (3-mercapto-4-methylpentan-1-ol and 3-mercapto-4-methylpentyl acetate; these VSCs have a grapefruit-like and/or rhubarb-like odor) (*95*).

4-Mercapto-4-methylpentan-2-one and 3-mercaptohexan-1-ol are not present *per se* in grape juice but rather are found as their odorless glutathione conjugates, undergoing cleavage to the free thiols by enzymes present during fermentation. The glutathione conjugates presumably arise from conjugate addition of glutathione to 2-hexenal, both present in grapes, followed by reduction (*96*, *97*). The thiols are isolated from wine using *p*-hydroxymercuribenzoate with separation of the chiral molecules by GC on a cyclodextrin capillary column using GC-MS-SIM detection (*93*). Alternatively, thiols are isolated using covalent chromatography on mercuric bounded agarose gel, followed by elution with 1,4-dithio-DL-threitol and subsequent analysis by GC with ion trap tandem mass spectrometry (GC-ITMS/MS) (*98*). With regard to the use of mercury salts in the isolation of trace thiols from wine, it is interesting to note the use of metallic copper as an adsorber of thiols in qualitative testing for wine thiols (*95*).

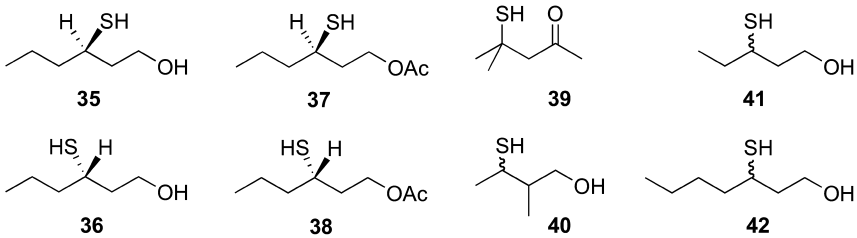

Figure 20. Wine thiols.

The above work clearly demonstrates that the enantiomer distribution of chiral thiols must be taken into account is assessing their olfactory impact in wine. Other studies indicate the disproportionate impact of **39** on the aroma of wine despite its low concentration among numerous other aroma compounds; **39** is also remarkable in that "it does not communicate to the wine its primary aromatic characteristics (boxwood, mango), but gives the wine a citric and fruity note" (*99*).

The Nose Knows: Chiral Non-*Allium* Derived VSCs

Since it is well known that enantiomers of chiral compounds can possess differences in odor (*100*), it is not surprising that the differences in odors shown by enantiomers of chiral VSCs play an important role in food chemistry. Thus, (*R*)-(+)-1-*p*-menthen-8-thiol (**43**), present in wine and grapefruit juice, possesses the pleasant odor of fresh grapefruit juice with an extremely low threshold value for detection (Figure 21). On the other hand, the enantiomer, (*S*)-(+)-1-*p*-menthen-8-thiol (**44**), at low levels has only a weak, nonspecific odor. In earlier studies, compound **44** was described as having an extremely obnoxious sulfur note, but this was found to be due instead to a cyclization product of **44**, 2,8-epithio-*cis*-*p*-menthane (**45**). This illustrates the point that in assigning odor, purity is essential, and that the presence of artifacts, such as **42**, can lead to an incorrect interpretation of odors.

A second example involves chiral cyclic VSCs 2-methyl-4-propyl-1,3-oxathianes **46-49**, found in yellow passion fruit. These stereoisomers can be separated by GC on a chiral nickel(II) bis[3-(heptafluorobutyryl)-1(R)-camphorate] capillary column (*101*). Compound **47** was found to correspond to the natural *cis*-2-methyl-4-propyl-1,3-oxathiane in yellow passion fruit. Stereoisomer **46** has a fatty, fruity-green, tropical fruit, grapefruit odor; **47** has a sulfurous, herbaceous green, roasty linseed oil-like, onion odor; **48** has green-grass root, earthy red radish note; **49** has a sulfurous, slight bloomy-sweet odor, less intense than **48**. The authenticity of natural materials or the question of natural versus nonnatural can sometimes be evaluated based on the enantiomeric composition. Chiral GC columns, such as those involving cyclodextrins or chiral metal complexes, can be used to evaluate the enantiomeric composition of VSCs (*100*).

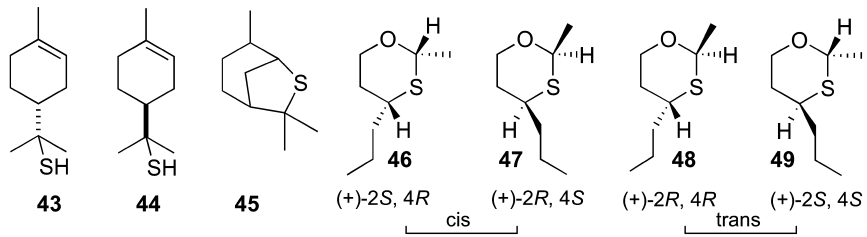

Figure 21. Chiral VSCs.

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Conclusions

Application of a variety of mild analytical methods to the initial products formed by cutting *Allium* plants established that disulfanes and polysulfanes are artifacts or secondary decomposition products of the first-formed sulfur-oxygen compounds. Trace amounts of families of structurally-related thiols in wine, thought to be formed from glutathione conjugates in grape skins, are found to have a substantial impact on wine aroma despite their very low concentrations. Enantiomer ratios need to be determined in the case of chiral VSCs, since individual enantiomers can have different odors and threshold levels.

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Chapter 3

The Role of Separation in the Identification of Trace Aroma Compounds

J. Lin,[*,1] Y. Wang,[1] P. L. Perry,[1] E. Frerot,[1,2] A. Rada,[1,3] and J. Impellizzeri[1]

[1]Firmenich Inc., North America R&D, P.O. Box 5880, Princeton, NJ 08543, USA
[2]Current Address: Firmenich SA, Corporate R&D Division, Route des Jeunes 1, CH-1211 Geneva 8, Switzerland
[3]Current Address: Firmenich Inc., North America Flavors, P.O. Box 5880, Princeton, NJ 08543, USA
[*]E-mail: Jianming.lin@firmenich.com.

Diverse motivations drive the analysis of food aromas and the sensory-directed aroma analysis, namely gas chromatography–olfactometry (GC–O), represents a valuable technique in the detection of trace aroma compounds. Even though the sensitivity of mass spectrometry has significantly improved, the complexity of food aromas and the trace levels of many food aroma compounds demand the development of new methodology for more efficient and unequivocal food aroma analysis. In this paper, the development of two selective solid phase extraction methods for basic volatile compounds and acidic volatile compounds will be presented. The effectiveness of these selective methods will be demonstrated by their application in real samples. The usefulness of two dimensional GC/O/MS in the identification of trace aroma compounds is also illustrated. The combination of traditional fractionation with 2D GC/O/MS analysis enabled the identification of trace aroma-active compounds in a complex mint oil. Finally, examples are used to prove that Amdis deconvolution software is a powerful data mining tool to remove interfering signals or background noises to obtain valuable mass spectral information for unambiguous identification of trace aroma compounds.

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Introduction

Diverse motivations, such as discovery of novel chemicals with interesting organoleptic properties, reconstruction of complex food aromas, flavor quality control, off-flavor problem-solving, or simple intellectual curiosity, drive the analysis of food aromas. The sensory-directed aroma analysis, namely gas chromatography–olfactometry (GC–O) represents a valuable technique to detect trace aroma compounds (*1*, *2*). On one hand, the sensitivity of mass spectrometry, especially tandem mass spectrometry allows us to identify compounds at very low levels (*3*, *4*). On the other hand, aroma extracts often require further fractionation to reduce the complexity of the whole extract and to enrich trace aroma compounds to facilitate their identification by MS. A variety of sample preparation methods for GC/O analysis have been developed and recently reviewed (*5*). But new development in analytical methodology is constantly needed to increase the chance of discovering new chemicals, to improve the speed and throughput of analysis, or to carry out analyses in greener ways. Confronted by the challenges of complex food aroma analyses, we developed several separation techniques, ranging from selective solid phase extraction by chemical functionality to deconvolution by Amdis software. The role of these separation techniques in the identification of trace aroma compounds is demonstrated through a range of concrete examples in this chapter.

Results and Discussion

Selective Solid Phase Extraction by Chemical Functionality

Selective SPE of Basic Volatile Compounds

In the aroma characterization of a Peanut Butter, several roasty, nutty notes were detected in its aroma extract by GC/O. However, they couldn't be identified by GC/MS because they occurred at trace levels and co-eluted with other volatile compounds present in much higher amount. It is well known that roasty, nutty-smelling compounds are most likely N-containing heterocyclic compounds. A method was therefore developed to selectively extract basic volatile compounds based on SPE using Waters Oasis® MCX cartridges. In this method, all volatile compounds in an aroma hydrodistillate sample are initially retained on the sorbent based on hydrophobic interaction. A solution of formic acid is passed through to acidify the basic volatile compounds and to lock them on the sorbent based on strong ionic interaction. Acidic and neutral compounds are then washed away with organic solvent. The basic compounds are eventually released by neutralization with ammonia and eluted with organic solvent in one step. A schematic representation of the procedure is shown in Figure 1.

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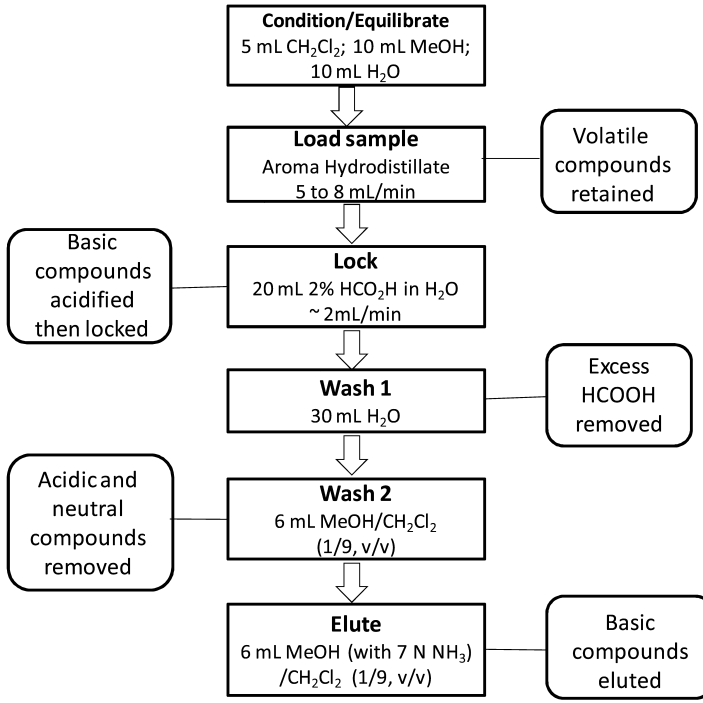

Figure 1. Procedure for Selective SPE of Basic Volatile Compounds from Aroma Hydrodistillates using Oasis® MCX Cartridges (6 cc/500 mg, 60 µm).

When this method was applied to the Peanut Butter sample, a basic volatile extract eliciting strong popcorn, roasty, nutty, pyrazine aroma was obtained. GC/MS analysis of the extract confirmed the sole presence of pyrazines, pyridines and other N-containing heterocyclic compounds. A GC/MS chromatogram of the extract with peak identification is shown in Figure 2. Five potent roasty compounds were easily identified in the basic extract by GC/O/MS. They are 2-acetyl-1-pyrroline, 2-propanoyl-1-pyrroline, 2-acetyl pyrazine and the two forms of acetyl tetrahydropyridine (Figure 2). More than 300 volatile compounds had been identified in roasted peanuts (*6*). Seveal aldehydes, pyrazines, pyrroles and other compounds were isolated from peanut butter by purge-and-trap techniques (*7*) and a list of dithiazines had been reported in peanut butter (*8*). However, these potent odorants were first time identified in peanut products due to the selective extraction method used. As recently reviewed by Adams and De Kimpe (*9*), these compounds are present in a great variety of processed and cooked food products.

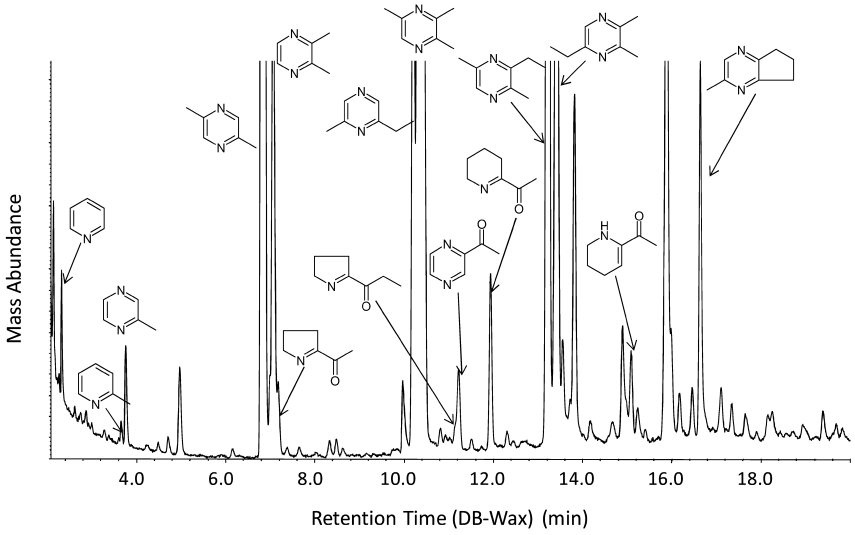

Figure 2. GC/MS Identification of Many N-Containing Compounds in a Basic Volatile Extract of Peanut Butter.

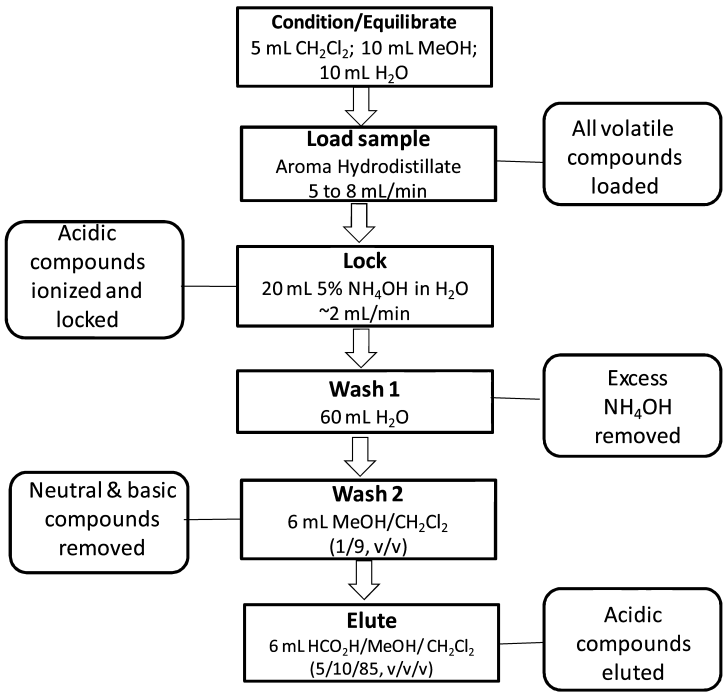

Figure 3. Procedure for Selective SPE of Acidic Volatile Compounds from Aroma Hydrodistillates using Oasis® MAX Cartridges (6 cc/500 mg, 60 µm).

Similarly, several sweet, lactonic notes and a few smoky, phenolic odors were detected by GC/O in the aroma characterization of toasted oat flakes, but couldn't be identified by GC/MS. Smoky notes could be from phenolic compounds and sweet notes were likely due to lactones. A SPE method using Oasis® MAX cartridges was then developed to selectively extract acidic volatile compounds. The schematic procedure of this method is shown in Figure 3. In this procedure, all volatile compounds in an aroma hydrodistillate sample are initially retained on the sorbent based on hydrophobic interaction. A solution of aqueous ammonia is passed through to ionize the acidic volatile compounds and to lock them on the sorbent based on strong ionic interaction. Basic and neutral compounds are then washed away with organic solvent. And the acidic compounds are eventually released after neutralization with formic acid and eluted with organic solvent in one step. Recovery of different acidic compounds with this procedure was investigated with an aqueous model aroma solution. Carboxylic acids were almost quantitatively recovered, recoveries for phenolic compounds were good (> 80 %), while the recoveries for lactones were acceptable (60 to 70 %).

Applying this procedure to a hydrodistillate of toasted oat flakes, a volatile extract containing only acidic compounds was obtained. GC/MS analysis of the extract revealed that short chain fatty acids were the main constituents of this fraction (Figure 4). Four of them, namely, (E)-2-hexenoic acid, (E)-2-heptenoic acid, decanoic acid and (E)-2-decenoic acid, appeared as small peaks. Six phenols, i.e. guaiacol, phenol, p-cresol, 4-vinylguaiaol, 4-vinylphenol and vanillin were straightforwardly identified in the extract based on mass spectra and retention indice (Figure 4 and Table 1). These phenolic compounds are known to have impacts on many food aromas (*10–12*). Most excitingly, ten lactones were unambiguously identified due to this selective extraction method (Figure 4 and Table 1). Lactones are widely present in foods and they are one of the most important class of aroma-impact compounds (*13*).

These two procedures enabled the removal of interferering components and the enrichment of targeted compounds. Liters of aroma hydrodistillate can be enriched onto a small cartridge and this step can be carried out unattended with a pump to control the flow. With SPE, a very small amount (12 mL) of organic solvent is used. Most importantly, the target compounds are eluted into a 6 mL fraction, which requires minimum further concentration. Trace levels of labile volatile compounds may disappear during a long concentration process to remove a large volume of solvent due to physical losses or chemical changes. Furthermore, the entire sample preparation starting from hydrodistillation and ending at a concentrated sample for GC/O/MS analysis can be carried out within one day. The short time span of the entire sample preparation reduces the possibility of losing trace aroma compounds or moderately unstable compounds.

Separation by Two Dimensional GC

In the characterization of complex aromas, two dimensional GC/O/MS system is very helpful in the identification of potent aroma-impact compounds which often occur at trace levels. Determination of aroma-impact regions is carried out on the first dimension by GC/O analysis. Individual aroma regions are sent to the second column by heartcutting with or without cryotrapping for further separation. The eluent of the second column is split between a sniff port and a mass detector, which allows the detection of the odorants and concurrent MS identification of them. Taking coffee oil as an example, as shown in Figure 5A, two interesting aroma events, the nutty-pyrazine and sweet-honey smell around 14.0 min, the coffee-sulfury note right before 17.0 min, were detected in regions showing no peak above the baseline. Each of the interesting aroma events was then heartcut and sent to the second dimension for further separation. Surprisingly, a narrow window of about 0.2 min heartcut could be further separated into 20 to 30 peaks on the second column as shown in Figure 5 (B&C). GC-O of these separated peaks was used to determine which one of the peaks was responsible for the odor of interest and the mass spectra revealed the identities of the peaks as phenylacetaldehyde for the honey note, 6,7-dihydro-5H-cyclopenta[B]pyrazine for the nutty-pyrazine smell and furfuryl methyl disulfide responsible for the coffee odor.

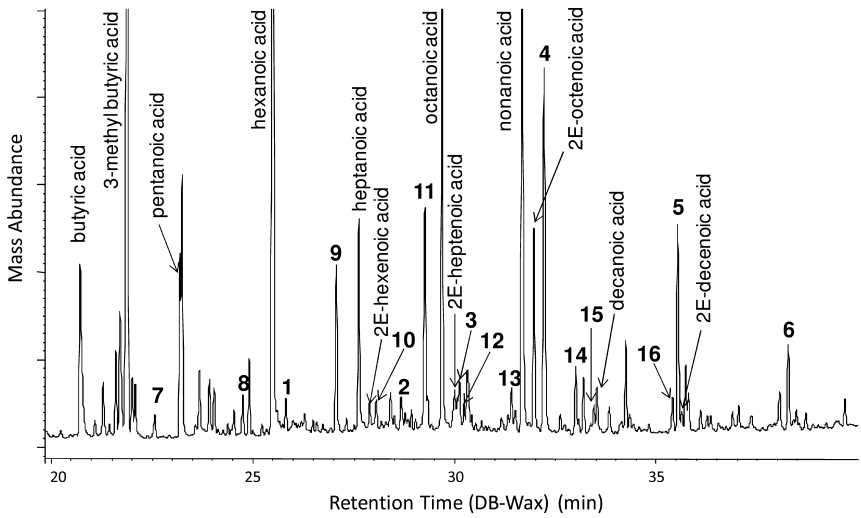

Figure 4. GC/MS Identification of Carboxylic Acids, Phenols and Lactones in an Acidic Volatile Extract of Toasted Oat Flakes (the labeling numbers correspond to the compounds in Table 1).

Table 1. Phenols and Lactones Identified in an Acidic Volatile Extract of Toasted Oat Flakes based on MS and RI

No.	*Compounds*	*RT (min)*	*RI (DB-Wax)*
	Phenols		
1	guaiacol	25.83	1858
2	phenol	28.68	2000
3	p-cresol	30.13	2077
4	4-vinylguaiacol	32.22	2191
5	p-vinylphenol	35.52	2382
6	vanillin	38.27	2553
	Lactones		
7	gamma-hexalactone	22.58	1705
8	gamma-heptalactone	24.75	1806
9	gamma-octalactone	27.08	1919
10	delta-octalactone	28.07	1969
11	gamma-nonalactone	29.26	2031
12	delta-nonalactone	30.25	2083
13	gamma-decalactone	31.41	2146
14	2-decen-5-olide	32.99	2234
15	gamma-undecalactone	33.45	2260
16	gamma-dodecalactone	35.39	2375

Fractionation Combined with Two Dimensional GC Separation

In the GC-O analysis of a Peppermint oil, some intense odorant regions clearly arose from minor components. Due to the low levels and the complexity of the minor components, these aroma events couldn't be unambiguously perceived by GC/O, not mentioning the identification of the molecules responsible for the odors. Fractionation of the essential oil was found necessary. Fractional distillation was used to remove the low-boiling major components, flash chromatography was used to further fractionate the residue based on polarity. Fifteen fractions were obtained and four of them were found interesting by aroma evaluation (Table 2). These four fractions were further analyzed by two dimensional GC/O/MS analysis.

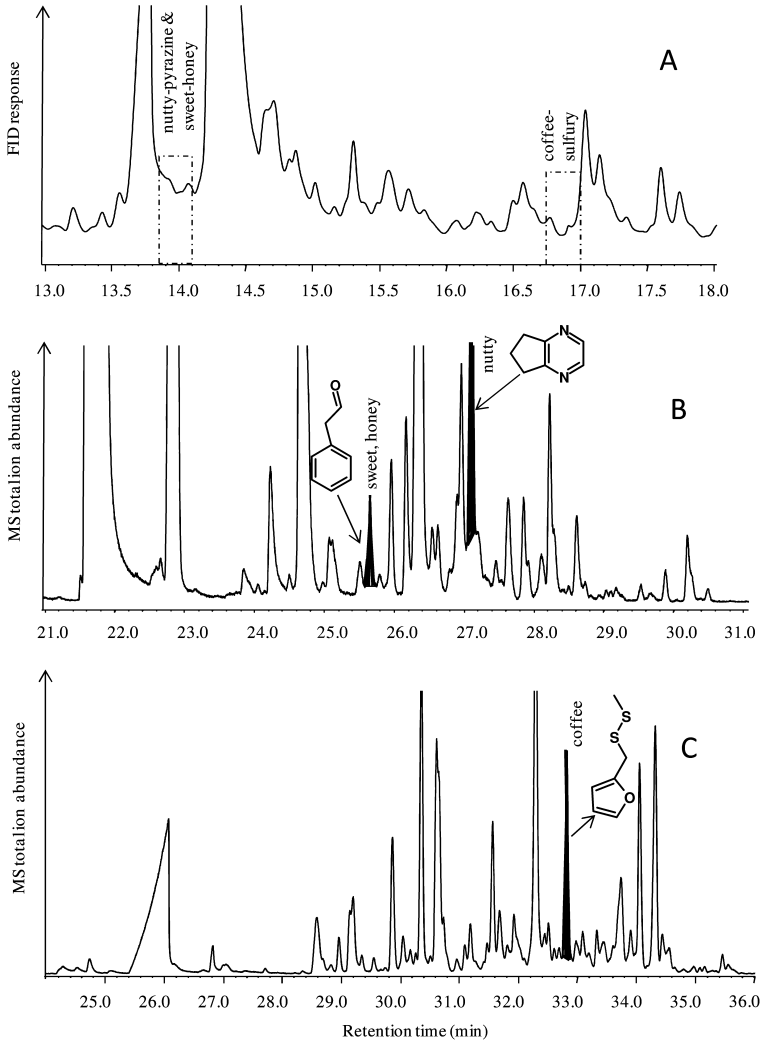

Figure 5. Identification of Trace Aroma Compounds in Coffee Oil by 2D GC/O/MS Analysis. A. Partial GC-FID Chromatogram Showing Two Aroma Regions; B & C. GC/MS Chromatograms Showing the Separation of the Heartcuts on the Second Column and the Identification of the Compounds.

Table 2. Organoleptically Interesting Peppermint Flash Chromatographic Fractions Revealed by Aroma Evaluation

Fraction	*Aroma description*	*Comments*
B	fruity, fresh, slightly aldehydic, good lift, dry pineapple, sweet, very nice, lasting	the most interesting, selected for GC-O analysis
C	lactonic, hay-like, sweet, minty, coconut	interesting, selected for GC-O analysis
H	woody,dry, fruity note, citronella, citral	very interesting, selected for GC-O analysis
I	coconut, ketone, hay-like note, lactonic	interesting, selected for GC-O analysis

Table 3. Three Interesting Aroma Events Detected in Fraction B along with Their Aroma Qualities, Intensities and Heartcut Windows

Aroma Event	*Descriptor*	*RT (min)*	*Heartcut Window*	*Intensity*
1	citrus	5.53	5.50-5.60	61
2	citrus peel	6.36	6.30-6.45	61
3	lactonic, sweet	6.88	6.80-7.00	82

Taking fraction B as an example, a list of aroma events were detected on the first dimension by GC/O screening, three of which are listed in Table 3. Heartcutting of Aroma Event **2** led to the detection of two aroma-active peaks at the second dimension by GC/O. They are fruity-green at 12.78 min and citrus, floral, sweet at 13.32 min. Two distinctive second dimension GC/MS peaks corresponded nicely to the two notes as shown in Figure 6 Top. The peak eluting at 12.78 min was tentatively identified as (Z,Z)-8-ocimenyl acetate based on its mass spectrum (Figure 6 Bottom). The identification was subsequently confirmed by the synthesis of the reference compound. The peak eluting at 13.32 min was found to be 1-p-menthen-9-yl acetate based on its mass spectrum and RI. Both compounds had been reported as minor components in Peppermint oil (*14*).

Deconvolution with Amdis Software

During the GC/O/MS analysis of a honeydew melon aroma extract (*4*), the identification of several aroma-impact compounds could not be confirmed by their mass spectra when we used the manual way of manipulating the MS with the vendor's software. However, Amdis deconvolution software (*15*) was able to detect these trace components either automatically or manually, thus led to their unambiguous identification. For instance, a soapy-fatty aroma event was tentatively identified as (Z)-2-nonenal based on aroma quality and RI. Amdis

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deconvolution software automatically detected 2 components, 2-nonenal and 2-hepten-1-ol with 0.014 min difference in retention time. The scan spectrum at the retention time is the fusion of the two spectra, which does not resemble either of the two.

In another case, a mushroom-like odor perceived by GC-O was tentatively identified as 1-octen-3-one based on aroma quality and RI. Running automatic Amdis deconvolution did not detect this compound. Extracting two intense ions of 1-octen-3-one (i.e. m/z 70 and m/z 55) suggested the presence of the compound (Figure 7 Top). Performing manual Amdis deconvolution using m/z 70 as model ion gave rise to a good EI spectrum identified as that of 1-octen-3-one, which was hidden in the scan spectrum (Figure 7 Bottom).

Similarly, a cabbage note was detected at a retention time corresponding to a well resolved GC-MS peak. The mass spectrum of the peak indicated the coelution of 3-hexen-1-ol with a compound having m/z 126 (Figure 8). Using m/z 126 as a model ion, manual Amdis deconvolution was carried out. The resulting deconvoluted spectrum was that of dimethyltrisulfide. In conjuction with the aroma quality and retention index, the trace aroma compound was unequivocally identified as dimethyltrisulfide.

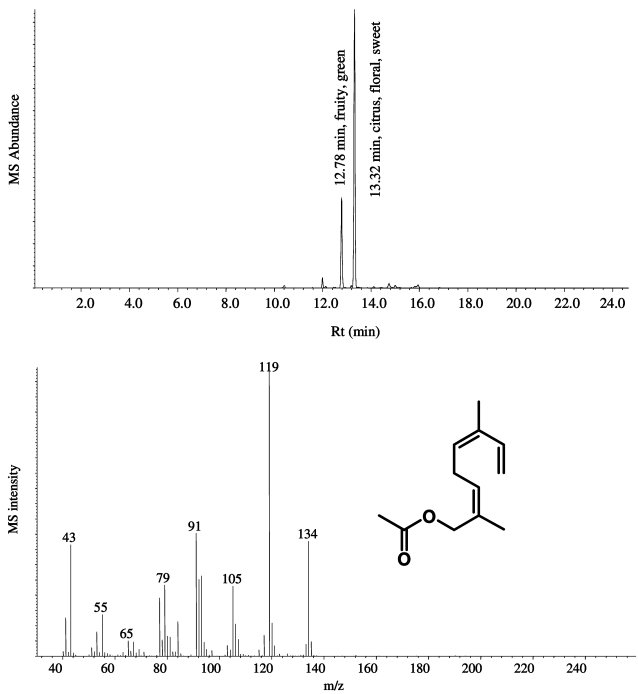

Figure 6. Two Dimensional GC/O/MS Analysis of a Peppermint Flash Chromatographic Fractions. Top: Second Dimension GC/MS Chromatogram of a Heartcut, Bottom: EI Spectrum of the Compound Eluting at 12.78 min.

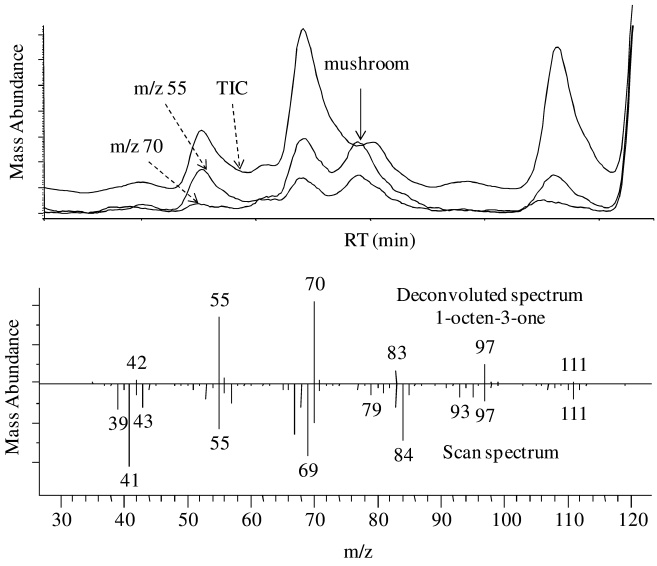

Figure 7. Confirmation of the Identification of 1-Octen-3-one by Its Mass Spectrum Obtained via Manual Amdis Deconvolution.

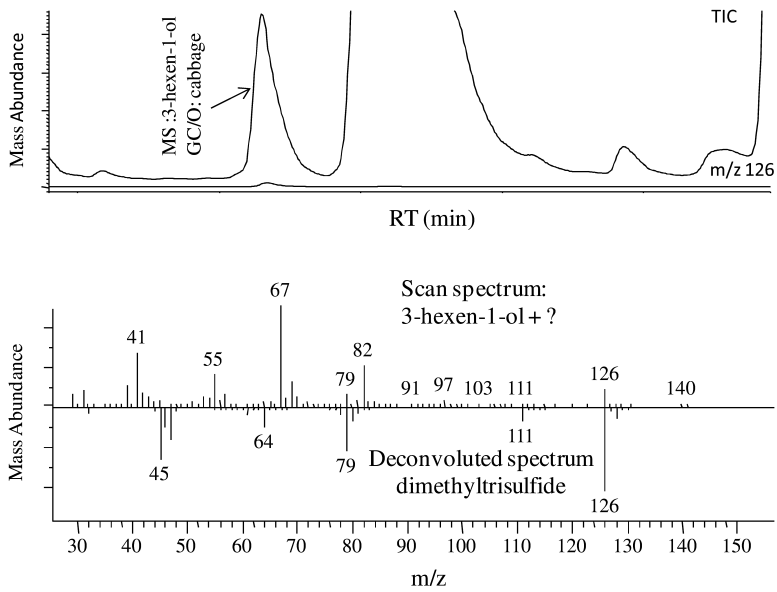

Figure 8. Identification of Dimethyltrisulfide by Its Mass Spectrum Obtained from Manual Amdis Deconvolution.

Conclusions

In the GC/O/MS characterization of complex aromas, the identification of trace aroma compounds can be realized or confirmed by their mass spectra after fractionation and enrichment. Separation techniques, such as selective SPE extraction based on chemical functionalities and two dimensional GC separation have been developed and successfully applied. Combination of them with conventional fractional distillation and flash chromatography allowed us to succeed in even more demanding challenges. Finally, Amdis deconvolution software proved to be a powerful data mining tool to remove interfering signals or background noises to obtain valuable mass spectral information for confident trace aroma compound identifications.

Acknowledgments

We would like to thank Dr. Jose Matiella and Gio Cipolla for their excellent training of the Amdis Deconvolution Software for us.

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Chapter 4

Emerging Analytical Techniques for the Assessment of Aroma Relevant Sulfur Compounds in Coffee

Luigi Poisson,[*,a] Christine Hug,[a,b] Juerg Baggenstoss,[a] Imre Blank,[a] and Josef Kerler[a]

[a]Nestle Product Technology Centre Orbe, Nestec LTD., CH-1350 Orbe, Switzerland
[b]Technical University of Munich, D-85748 Garching, Germany
[*]E-mail: luigi.poisson@rdor.nestle.com.

Aroma extraction by solid phase micro extraction (SPME) was combined with comprehensive two-dimensional gas chromatography (2D-GC) using two column configurations and detection by time-of-flight mass spectrometry (TOF-MS) for the quantitative assessment of trace sulfur compounds in coffee. Based on the optimization of 2D separation and TOF-MS detection parameters, quantification assays were established for 3-methyl-2-butene-1-thiol (MBT) and 3-(methylthio)propionaldehyde (methional). The quantification of these compounds in roast and ground (R&G) coffee as well as in filter coffee brew resulted in satisfactory detection limits and repeatability of the method. Furthermore, quantitative results highlighted the importance of MBT in the aroma above a freshly ground coffee. Data indicated that it is almost quantitatively lost during preparation of filter coffee beverage due to evaporation and degradation. In contrast, methional was found quite abundant in R&G coffee and is highly recovered in the final beverage. In addition, identification was compared to standard methods comprising aroma isolation by high vacuum transfer and solvent aroma extraction. The evaluation of the solvent extract by 2D-GC-TOFMS as well as GC-olfactometry resulted in fewer sulfur compounds identified, thus indicating high degradation rates of reactive sulfur compounds during

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sample work-up. The study revealed that 2D-GC-TOF-MS is a powerful tool to identify and quantify trace sulfur compounds in coffee. Combined with SPME aroma isolation, it represents a rapid, sensitive, and ecological alternative to conventional methods.

Introduction

The characterization of coffee aroma is a challenging task as many of the important odorants are just present in trace amounts and/or are reactive and unstable. This is valid in particular for sulfur compounds, such as thiols, that are known to be susceptible to oxidative degradation reactions (*1*, *2*). The trapping of thiols with *p*-hydroxymercuri benzoate, adsorption of the resulting product on an anion exchange column, and subsequent release with cysteine has been proven as an efficient method for the enrichment of trace amounts of thiols in food (*3*). Several sulfur compounds could be detected for the first time in coffee by combining this methodology with GC-olfactometry (GC-O) and GC-MS analysis (*4*). As examples, quantitative analysis in various coffee brews revealed 3-mercapto-2-pentanone, 2-mercapto-3-pentanone and 4-methoxy-2-methylbutan-2-thiol contents that are significantly above their odor thresholds in water (*4*).

The approaches for the quantification of trace aroma compounds described above generally involve an excess amount of sample and require labor-intense and time-consuming extraction and pre-separation steps, often in conjunction with the use of large quantities of solvents. Furthermore, co-elution phenomena in GC separation and insufficient sensitivity in MS detection limit their assessment in "routine" analysis and, thus, represent further constraints for a closer evaluation of the role of sulfur compounds in coffee aroma.

Besides the aspect of exploring rapid and sensitive methodologies, the development of environment-friendly procedures has become increasingly important. In recent studies, our group demonstrated the potential of SPME aroma isolation combined with the emerging GC×GC-TOF-MS technique as a relatively rapid and less labor-intense tool to analyze key aroma compounds in coffee beverages. As an example, trace sulfur compounds such as 2-acethylthiazole, 3-(methylthio)propionaldehyde, and 2-methyl-3-furanthiol were detected and positively identified, which could not be achieved using linear GC-MS or heart-cut GC/GC-MS (*5*).

The aim of the present work was to develop a quantification method based on comprehensive GC×GC-TOF-MS using the isotope dilution assay (IDA) method with emphasis on trace sulfur compounds like 3-(methylthio)-propionaldehyde (methional) and 3-methyl-2-butene-1-thiol (MBT).

Experimental

Sample Preparation

Coffee Brews

60 g of roast and ground (R&G) coffee (Arabica, Colombia; 85%; Robusta, Indonesia, 15%) were brewed over a filter paper with 1000 mL tap water. After cooling down to room temperature, 7 mL of the brew was pipetted into a 20 mL headspace vial.

Quantification by Isotope Dilution Assay (IDA)

R&G Coffee

R&G coffee was suspended in hot water to get a slurry and after cooling spiked with defined quantities of isotope labeled analogues of the analytes.

Filter Coffee Brews

The filter coffee was prepared as described above. After cooling down of the brew to room temperature an aliquot of the brew was spiked with defined quantities of labeled isotopes of the analytes.

Isolation of Aroma Compounds by High Vacuum Transfer (HVT) and Solvent Extraction

200 mL of filter coffee were distilled under vacuum at 40 °C using the so-called "Solvent Assisted Flavour Evaporation" apparature (SAFE), in order to separate the volatiles from the non-volatile material. After phase separation, and extraction with solvent, the organic phase was washed with a saturated NaCl-solution and dried over Na_2SO_4. The extract was concentrated on a Vigreux-column (60 cm) at 40 °C to about 5 – 10 mL and then further to 1 mL by means of microdistillation.

Isolation of Aroma Compounds by Solid Phase Micro Extraction (SPME)

The prepared coffee solutions were equilibrated for 60 min at 20 °C in the sealed vials and the aroma compounds were then extracted from the headspace during 10 min at 40 °C using SPME (2 cm fiber coated with PDMS/DVB/Carboxen; Supelco, Bellefonte, PA, USA). Aroma compounds were thermally desorbed into the split-splitless injector (in splitless-mode) heated at 240°C.

2D-Gas Chromatography Time-of-Flight Mass Spectrometry (GC×GC-TOF-MS)

The system consisted of a Agilent 7890A GC (Agilent, Palo Alto, CA, USA) equipped with a split-splitless injector and a CTC-PAL autosampler (CTC, Brechbühler, Switzerland). Column setup A: 1st column DB-FFAP (30 m × 0.25 mm; film thickness, 0.25 µm; J&W Scientific; Folsom; CA, USA) and 2nd column DB-1701 (2 m × 0.1 mm; film thickness, 0.1 µm; J&W Scientific; Folsom; CA, USA). Column setup B: 1st column Equity-1701 (30 m × 0.25 mm; film thickness, 0.25 µm; Supelco; Bellefonte, PA, USA), and 2nd column DB-WAX (2 m × 0.1 mm; film thickness, 0.1 µm; J&W Scientific, Folsom, CA, USA). Helium was used as carrier gas with a constant flow of 1.2 mL/ min. Same oven program was applied for both column setups: initial temperature of 40 °C was held for 2 min; raised to 140 °C at 4 °C/min, and then raised to 240 °C (for column setup A) or 235 °C (for column setup B) at 10 °C/min and held for 10 min.

Modulation was performed with a four-jet thermal modulator (LECO, St. Joseph, MI, USA) using liquid nitrogen for cooling. The modulation period was set at 5 s in column setup A and at 10 s in column setup B. The modulation temperature was kept 15 °C above the oven temperature for column setup A and 20 °C for column setup B, respectively.

Mass spectrometry was performed on a Pegasus 4D TOFMS (LECO, St. Joseph, MI, USA). The mass spectrometer was operated at a spectrum storage rate of 200 Hz and a detector (multi channel plate) voltage of 1500 V to 1800 V. Chromatograms were processed using the LECO ChromaTOF™ software.

GC-O

This was performed on a Fisons gas chromatograph (Type HRGC MEGA SERIES) using two different fused silica thin-film capillaries; DB-FFAP (J&W Scientific; Folsom, CA, USA) and ZB-1701 (Phenomenex, Aschaffenburg, Germany), each 30 m × 0.25 mm; film thickness, 0.25 µm. Samples were applied by the "cold on-column" injection technique at 40 °C. After 2 min, the temperature of the oven was raised by 6°C/min to 240 °C and held for 10 min. The Kovats retention indices were calculated by co-chromatography of *n*-alkanes. The identification of the compounds was based on retention indices on two columns of different polarity (DB-FFAP and OV-1701), co-chromatography of references and odor quality on the sniffing port (Sniffer 9000 System, Brechbuehler, Switzerland).

Results and Discussion

Identification of Trace Sulfur Compounds

In 2D GC the overall separation is influenced by the type and combination of the two columns, their dimensions (length and diameter), the thickness of the stationary phases, the carrier gas velocity, the temperature regime for both columns, and the modulation time. Hence, the analysis of a complex food

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matrix such as coffee requires optimization in method development to achieve the identification of targeted trace aroma compounds. Therefore, the analysis methodology has to be designed in such a way that the combined first- and second-dimension separation allows both the identification and quantification of the analytes of interest. The major part of coffee aroma compounds ranges in a broad spectrum from semi-polar to polar. As a consequence, a combination of semi-polar and polar columns for an efficient separation in 2D GC seems to be reasonable. Indeed, the results of a former study (*5*) revealed that a better separation was obtained with a polar/ medium polar column set (SolGel-Wax × DB-1701) as compared to the apolar/medium polar configuration (ZB-5MS × DB-1701). Analyte peaks were more efficiently distributed along the primary dimension, resulting also in a better separation in the second dimension. Combined with SPME aroma isolation, sulfur compounds like 2-methyl-3-furanthiol (MFT), 2-furfurylthiol (FFT), and 3-(methylthio)propionaldehyde (methional) could be identified. The identification of 3-methyl-2-butene-1-thiol (MBT) was also targeted, but not achieved by any of column setups mentioned. Therefore, in the present study special emphasis was put on the identification and quantitative evaluation of MBT in R&G coffee powder and the filter coffee beverage. Optimization work particularly focused on the column setup, the two-dimensional separation as well as the detection parameters. Two combinations of polar and semi-polar phases were applied for the assessment of coffee aroma, i.e. hyphenation of a polar with a medium polar column (column setup A: DB-FFAP × OV-1701) in comparison to the medium polar-polar configuration (column setup B: Equity-1701 × DB-Wax).

As shown in the 2D contour plot (Figure 1), separation by the column setup A led to a good distribution of the analytes in the first dimension; whereas, the second dimension was less well performing. Nevertheless, the resolution was satisfactory in both dimensions and resulted in the detection of methional (Table 1). Despite of the co-elution with abundant compounds furfural and acetic acid (Figure 2**,** retention at 1360 s), methional was well separated on the second column (1.905 s), and a clean mass spectrum was obtained. The deconvoluted mass spectrum was compared with the NIST library and sufficient similarity was found.

The optimization of the 2D-GC-TOFMS system for the detection of methional started first with the assessment of the optimal carrier gas flow. Different constant carrier gas flow rates (from 0.8 to 2.0 mL/min) were tested, and best flow was found at 1.2 ml/min when a 30 m DB-FFAP with a diameter of 0.25 mm was applied. An optimal flow rate is not possible in 2D-GC since two columns with different inner diameters are connected in series. This means that the volumetric flow through both columns is the same but linear velocities in each of them differ. But due to the increased pressure level in the first column provoked by the high flow resistance of the narrow-bore second-dimension column, diffusion in the first column is slower and, thus, optimum velocity is far lower than in 1D-GC. Therefore, Beens et al. (*6*) stated that in the combination of two columns with different internal diameters, one column should be operated close to its optimum flow conditions, while the second column is operated at sub-optimal conditions. Hence, the optimal flow in 2D evaluation is always a compromise between the flow in 1st and 2nd dimension for given column types and dimensions.

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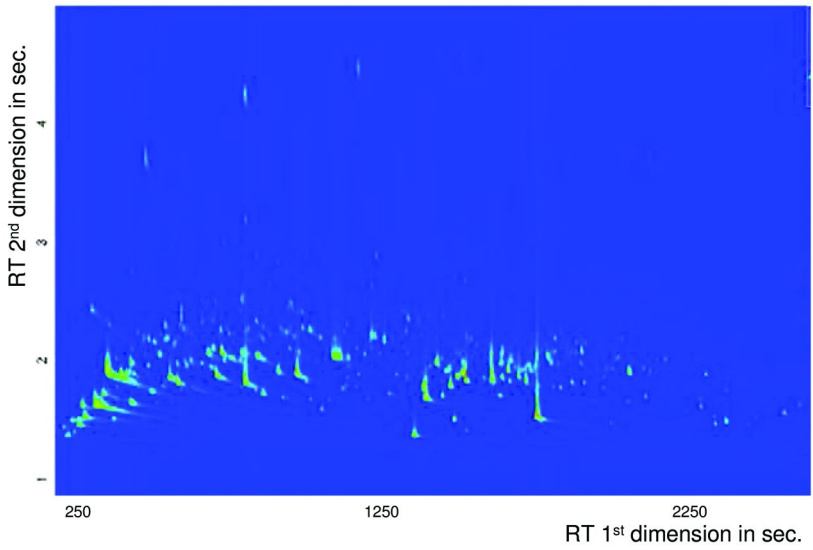

Figure 1. 2D diagram for the column setup A (DB-FFAP/OV-1701), coffee brew extracted by SPME (40 °C for 10 min.); heating rate up to 180 °C: 4 K/min, 15 K offset, modulation time 5 sec; carrier gas flow of 1.2 ml/min. (see color insert)

Table 1. Identification of trace sulfur compounds by different analytical methods and column configurations

	SPME-GC×GC-TOFMS		*HVT GC×GC-TOFMS*	*HVT GC-O*
S-Compound	*A: DB-FFAP × DB-1701*	*B: Equity-1701 × DB-Wax*	*A: DB-FFAP × DB-1701*	*DB-FFAP*
3-Methyl-2-butene-1-thiol	+	-	-	+
3-(Methylthio)-propionaldehyde	+	+	+	+

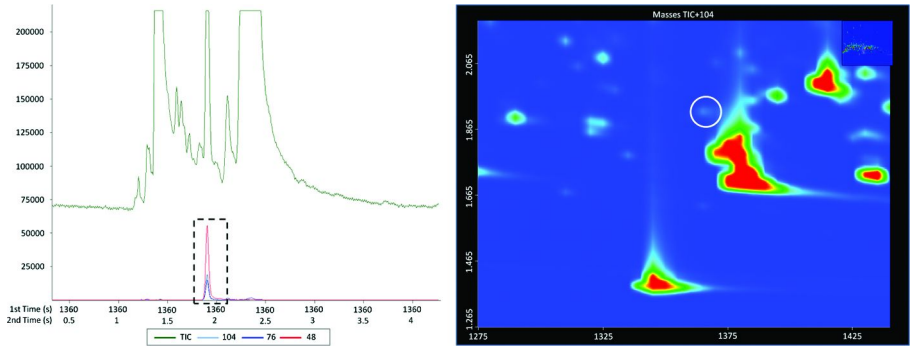

Figure 2. Identification of 3-(methylthio)propionaldehyde in coffee brew using SPME-GC×GC-TOFMS in combination with column setup A; position of the analyte in 1D chromatogram (black frame, left) and in 2D plot (circle, right). (see color insert)

In GC×GC, next to the separation in the first-dimension column, the temperature-programming rate also influences the retention times in the second column, and consequently have to be optimized together with the carrier gas flow. For the polar/medium-polar column setup (setup A) an oven heating rate of 4 °C/min was found adequate when above designated carrier flow rate was applied. The optimization of modulation frequency of the first dimension peak is important for preserving the separation achieved in first dimension and improving sensitivity through peak refocusing. In general, the modulation time should be set slightly higher than the retention time of the analyte with the highest retention in the second dimension. Together with the modulation time the offset-temperature, the temperature difference between first oven and the second oven, has to be optimized in parallel. The combination of modulation time (2-10 s) and off-set temperature (5-15 °C) was tested with given column program of 4 °C/min and column flow of 1.2 ml/min. The described approach resulted in an effective combination setting for column setup A with a modulation time of 5 s and an offset-temperature of 15 °C.

Despite of the above mentioned extensive optimization efforts, the targeted sulfur odorant MBT could not be detected. Thiols such as MFT, FFT, and MBT are known to highly interact with the coffee matrix (i.e. melanoidins) leading to rather low extractable amounts present in the gaseous phase (*7*) above the coffee beverage. Therefore Milo et al. (*8*) suggested the release of reversible bound thiols by the addition of cysteine. In the present study, release of thiols by cysteine was also applied in combination with an increase of the detector voltage from 1500 V to 1700 V to enhance sensitivity of detection.

Figure 3 illustrates the chromatogram with the total ion current (TIC) and the specific masses for MBT m/z 69 and m/z 102. The m/z of MBT are highly overlaid by the TIC of co-eluting 2,3-pentanedione. Actually, the LECO Pegasus software can deconvolute peaks whose apexes are only three data points apart. One illustrative example that demonstrates the need for deconvolution was observed with MBT. However, by deconvolution the specific masses m/z 69 and m/z 102

at 715 s in 1st D and 2.4 s in 2nd D were found to be unique masses, and the MS spectra was identified as that of MBT (Figure 4).

For the medium polar/polar column setup (setup B: Equity-1701×DB-FFAP) oven heating rate and first column flow were set equal to setup A, namely 4 °C/ min and a capillary gas flow of 1.2 ml/min in primary dimension, respectively. In contrast to setup A, the best conditions for the modulation were found at an increased modulation time of 10 s combined with an enlarged offset temperature of 20 °C. Figure 5 shows that the 2nd dimension is fully charged in the rather large range of 10 s. However, the chromatogram showed a better spread of the components in 2nd dimension. But, a significant tailing of the major peaks resulted in the 2nd dimension accompanied by an inferior resolution. At first dimension methional is co-eluting with acetic acid at a retention time of 1070 s, but it is well separated from acetic acid at a retention time of 3.345 s in the 2nd dimension, resulting in a clear MS spectrum due to a considerable improved signal to noise ratio.

In contrast to the column setup A, MBT could not be detected in the application of column setup B. The addition of cysteine into the coffee sample to release bound thiols did not result in its detection either. The reasons therefore may be found in the rather high modulation period that causes a decreased resolution in the 2nd dimension, and thus lower detection sensitivity.

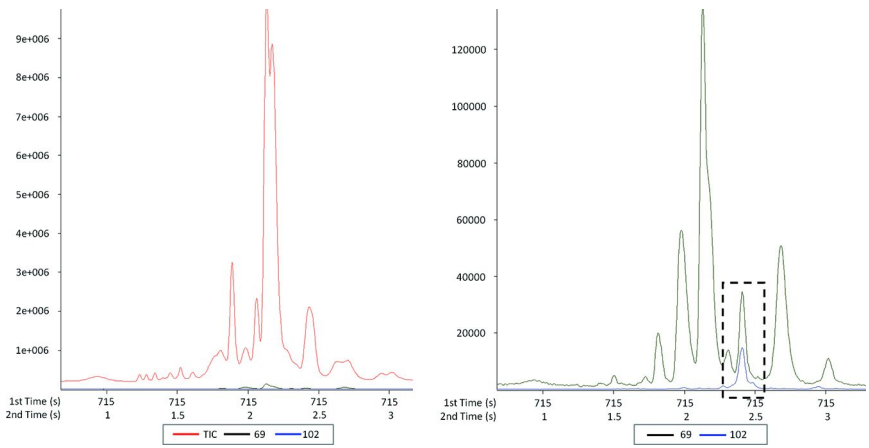

Figure 3. Identification of 3-methyl-2-butene-1-thiol (MBT) in coffee brew using SPME-GC×GC-TOFMS (TIC, left) and (m/z 69 and 102, right); Column setup A. (see color insert)

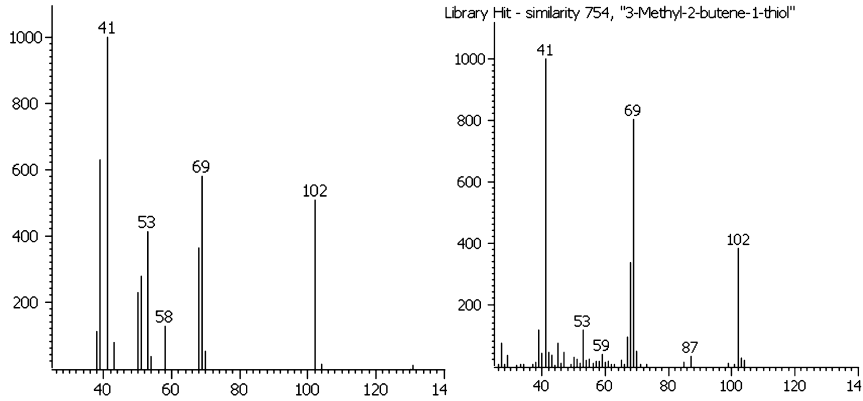

Figure 4. Identification of 3-methyl-2-butene-1-thiol (MBT) in coffee brew using SPME-GC×GC-TOFMS and column setup A; obtained mass spectrum (left) and library reference mass spectrum (right).

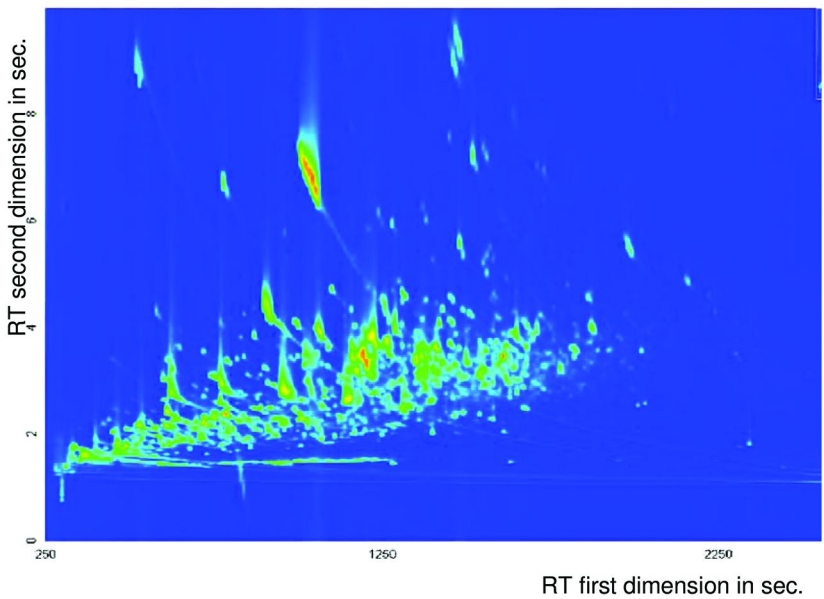

Figure 5. 2D diagram for the column setup B (Equity-1701/DB-FFAP), coffee brew extracted by SPME (40 °C for 10 min.); 4 K/min, 20 K off-set, modulation time 10 s.; carrier gas flow of 1.2 ml/min. (see color insert)

The advantage of the extraction of volatiles by SPME is the rapid aroma isolation (generally from 5 min to 60 min) from a little amount of sample. At the same time, the latter implicates the main drawback as an increase of sample amount is limited. To prove the capacity of the SPME method for the assessment of targeted compounds, it was compared to the more "classical" aroma isolation approach comprising of high vacuum transfer (HVT) distillation with subsequent

solvent extraction. For the aroma isolation by HVT, 200 mL of coffee brew were prepared and distilled. The complete distillate was than submitted to solvent extraction with dichloromethane. After concentration of the extract by distillation, the obtained solvent extract was evaluated by 2D-GC-TOF-MS (column setup A and B) as well as GC-O. Surprisingly, only methional could be positively detected in the solvent extract by 2-dimensional GC-TOF-MS evaluation (Table 1). Despite of the high amount of prepared sample detection of MBT was not achieved; the concentration in the solvent extract is even below the detection level of the TOF-MS. In parallel the solvent aroma extract was assessed by linear GC-O as well. This led to the additional detection of MBT at the sniffing port of the GC-O device. These results clearly demonstrate that sample work-up by solvent extraction and subsequent distillation lead to important degradation of sulfur compounds such as MBT. Despite of the larger sample volume prepared for the solvent extraction, less targeted trace compounds could be detected as compared to SPME isolation. In conclusion, it can be stated that the assets of comprehensive GC×GC-TOF-MS are best used when it is combined with mild isolation techniques.

Quantification of Trace Sulfur Compounds

Methional was shown to be an important shelf life marker in roasted coffee (*9*). Its quantification in a former storage test on R&G coffee (*9*) could only be achieved by a labor-intensive solid phase extraction (SPE) in conjunction with liquid injection and heart-cut GC/GC-MS analysis. Same is valid for MBT that is highly susceptible to oxidative degradation. MBT provides a characteristic fresh odor character and was positively detected in different coffee samples, such as in R&G and brew (*10*). Due to its very low odor threshold and its high volatility it may play an important role particularly in freshly ground coffee samples, but also in coffee brew as an extraction yield of 85% is given in the literature (*10*). Therefore, the development of a more rapid method for the quantification of this sulfur compound in routine analysis is of high interest.

The method developed for the identification of MBT and methional by SPME in combination with 2D-GC×GC-TOF-MS was adapted to quantitative analysis using an isotope dilution assay (IDA) for these sulfur compounds in R&G coffee and coffee brew, respectively. In theory, MBT and methional should be quantified with the column setup A where both compounds were sufficiently separated to obtain clean mass spectra. For the quantitative assessment of a compound in 2D-GC by IDA, it is essential that the peaks of the analyte and its isotopic labeled standard are sufficiently separated in the 2nd dimension. Only then a positive identification of the components is possible by the deconvolution software. Figure 6 illustrates how this condition is fulfilled for MBT, where a slight shift in the retention of only about 0.04 s was enough to be clearly detected by the deconvolution tool as two distinct peaks, i.e. compounds. Both the analyte and labeled standard are almost base-line separated for their quantification masses m/z 102 and m/z 108, respectively. Unfortunately, this requirement was not obtained for methional; m/z 107 of the isotopic labeled standard was not sufficiently separated from m/z 104 to ensure a clear identification. Therefore,

an assay was also tested on the column setup B. As discussed before, MBT can not be detected by this column combination. In the quantification of methional the apexes of m/z 107 and m/z 104 were separated by less than 0.03 s (Figure 7). Nevertheless, automatic detection and thus quantification was feasible. As a conclusion of the quantitative assessment, the targeted sulfur compounds MBT and methional cannot be quantified using the same combination of columns; the polar/ medium-polar configuration was evidenced as suitable for the quantification of MBT, whereas the reversed configuration (setup B) represents the only possibility to quantify methional.

The accuracy and applicability of a new method is generally characterized by detection limits, the level of detection (LoD) and quantification (LoQ), the repeatability as well as the reproducibility of the analysis. For MBT a very low LoD of 0.022 ppb was determined in R&G coffee, and a LoQ of 0.07 ppb resulted thereof (Table 2). The LoD and LoQ of 3-(methylthio)-propionaldehyde were assessed at 1.7 ppb and 5.6 ppb, respectively. These results are fully satisfactory, especially when taking into account that each analyte was split into 2-3 2nd dimension peaks which had to be added up. The low LoD and LoQ levels, particularly for MBT, are only possible by the high resolution capacity of the comprehensive 2-dimensional separation.

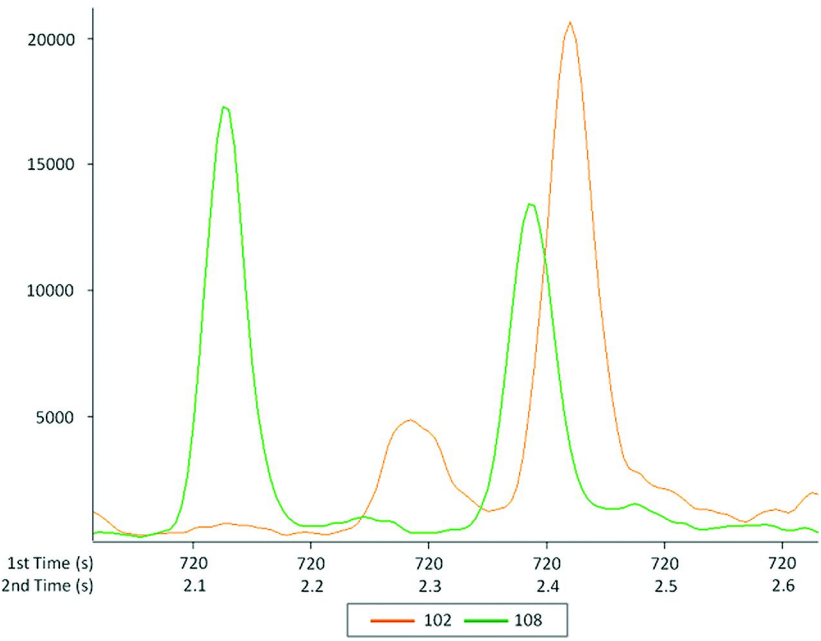

Figure 6. Quantification of 3-methyl-2-butene-1-thiol by IDA; m/z 102 of analyte (orange) and m/z 108 of D_6-labeled standard (green) in R&G coffee; column setup A. (see color insert)

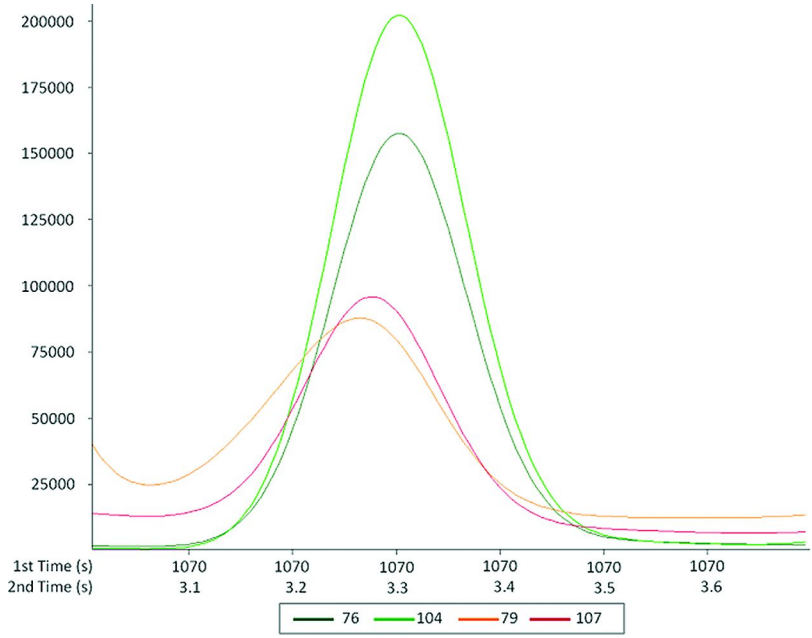

Figure 7. Quantification of 3-(methylthio)propionaldehyde by IDA; molecular mass m/z 104 of analyte (green) and m/z 107 of D_3-labeled standard (red); column setup B. (see color insert)

Table 2. Detection limit (LoD) and quantification limit (LoQ) determined for MBT and methional by SPME GC×GC-TOF-MS in R&G coffee

Compound	*GC×GC-TOFMS*	
	LoD [ppb]	*LoQ [ppb]*
3-methyl-2-butene-1-thiol	0.022	0.07
3-(methylthio)-propionaldehyde	1.7	5.6

For the evaluation of the simple repeatability of the SPME-GC×GC-TOF-MS measurement one R&G coffee as well as one brew sample were quantified by IDA and measured in at least six repetitions. The simple repeatability of the GC×GC-TOF-MS measurements for MBT as well as for methional is given in Table 3. Quantification of methional in both matrices resulted in rather low relative standard deviation of repeatability CV(r). This is easily explained by the conspicuous separation of the analyte in the 2nd dimension, the very low background signal, and thus, the high signal to noise ratio resulting thereof. Due to the considerable lower quantities of MBT in the assessed samples, the CV(r) were determined at higher level of around 14% in the R&G coffee and 12% in the brew, respectively.

Table 3. Simple repeatability for the quantification of MBT and methional in R&G coffee and coffee brew by SPME-2D-GC-TOF-MS (number of measurement repetitions n≥ 6)

	R&G coffee			*Coffee brew*		
Compound	*Range [µg/kg]*	*Mean [µg/kg]*	*CV(r) [%]*	*Range [µg/kg]*	*Mean [µg/kg]*	*CV(r) [%]*
3-methyl-2-butene-1-thiol	31.1 – 39.9	34.8	14.1	0.137 – 0.160	0.145	11.9
3-(methylthio)-propionaldehyde	659.4 – 714.5	678.7	2.93	37.5 – 42.6	39.4	3.74

The concentration of MBT was determined at 31.8 µg/kg in the R&G coffee when measured by SPME in combination with 2D-GC TOF-MS (Table 4) with a relative standard deviation (RSD) of 3.3%. Whereas, the coffee brew revealed a very low amount of only 0.12 µg/kg, with a RSD of 10%. Calculating the concentration in brew normalized to µg per kg R&G coffee, namely 1.60 µg/kg R&G coffee, only 5% of MBT was found to be recovered in the final coffee beverage. In comparison, the reference methodology comprising of HVT for the aroma isolation from the brew and solvent extraction combined with heart-cut GC/GC-MS measurement resulted in a content of 0.18 µg/kg on the same coffee blend (Table 5). The values are in a similar magnitude of concentrations, hence, the method for the quantification of MBT by SPME and 2D HC TOF-MS can be assumed as an accurate and reliable alternative to the conventional approach. However, Mayer et al. (*15*) found a significantly higher value of 0.6 µg/l in a brew by applying solvent liquid-liquid extraction, vacuum distillation, entrapment of the thiols by *p*-hydroxymercury salt with subsequent release, and analysis by dynamic headspace in conjunction with HRGC-MS. Based on the assessed concentration of MBT in R&G coffee of 13 µg/kg, they calculated an extraction yield of 85% when preparing 1 L of brew from 54 g R&G coffee. This value seems to be quite high considering the volatility of MBT as well its rapid degradation behavior. The important analytical differences can simply result from unequal brewing process, drip filter machines or sample treatment afterwards.

Considering the orthonasal detection threshold of 0.3 ppb in water, the concentration of MBT found in coffee brew (0.12 µg/kg) most likely does not contribute to the overall aroma of a filter coffee beverage. However, synergistic effects with other thiols cannot be excluded. In contrast, MBT may play a key role for the aroma of freshly ground coffee considering the very low detection threshold in air. Thiols are supposed to have an important contribution for the fresh coffee aroma; beside other high volatile compounds, the extremely high volatility of MBT may explain the fact that fresh ground coffee powder rapidely loses its desired aroma.

Table 4. Quantification of MBT and methional in R&G coffee and coffee brew; data are means of 3 assays. Extraction yield based on 60 g coffee powder for 1 L brew

Compound	*R&G coffee*			*Coffee brew*			*Extr. yield [%]*
	Range [µg/kg]	*Average [µg/kg]*	*RSD [%]*	*Range [µg/l]*	*Average [µg/l]*	*RSD [%]*	
3-Methyl-2-butene-1-thiol	31.3 - 33	31.8	3.3	0.103 - 0.134	0.12	10.0	5.1
3-(Methylthio)-propionaldehyde	663 - 703	679	3.2	38.5 - 40.8	39.4	3.7	77

Table 5. Comparison of quantitative results to literature data obtained by reference methods (solvent extraction (SE) and GC-MS)

Compound	*R&G coffee [µg/kg]*		*Coffee brew [µg/l]*		
	GC×GC TOF-MS	*SE-GC-MS* [1,2,3,4,5]	*GC×GC TOF-MS*	*HVT-GC-GC-MS* [6]	*SE-GC-MS* [25]
3-Methyl-2-butene-1-thiol	31.8	7 - 17.9	0.12	0.18	0.6
3-(Methylthio)-propionaldehyde	679	213 - 303	39.4	34.1	5.7 - 10

[1] Semmelroch et al. 1995 (*11*), 100% Arabica, Colombia. [2] Semmelroch et al. 1996 (*10*), 100% Arabica, Colombia. [3] Czerny et al. 2000 (*12*), 100% Arabica, Columbia. [4] Mayer et al. 1999 (*13*), 100% Arabica, Columbia. [5] Mayer et al. 2000 (*14*), in 100% Arabica, Colombia. [6] Internal data; 85% Arabica, Colombia, 15%, Robusta, Indonesia.

The quantification of methional in R&G coffee by SPME in combination with 2D-GC TOF-MS resulted in a concentration of 679 µg/kg, with RSD of 3.2 % (Table 4). The brewed beverage from the same coffee revealed a quantity of 39.4 µg/kg of methional with a RSD of 3.7%. The value in the filter coffee corresponds to 525 µg/kg calculated on the base of R&G coffee. When the extracted quantity of the brew is set in relation to the R&G coffee, it becomes obvious that methional is quite efficiently extracted at 77% level during the brewing process. With a detection threshold of 0.43 µg/L in water (*16*) methional must be considered as an aroma relevant odorant in the coffee beverage, but most likely also in R&G coffee. The analysis of methional in the same blend by the reference method resulted in a concentration of about 34 µg/L (Table 5). These results indicate that the SPME-2D-GC-TOF-MS approach represents a reliable alternative for the quantification of methional.

Conclusions

The present study demonstrated the potential of the 2-D GC-TOF-MS technique combined with SPME aroma isolation as a rapid, sensitive, accurate, and ecological method for the quantification of trace sulfur compounds in coffee. Based on its superiority in terms of sensitivity and resolution, this method can be used to quantify sensitive trace odorants like MBT and methional without time consuming clean-up procedures.

As it was shown for MBT and methional, a well defined selection of the column setup for GC×GC as well as optimization of separation parameters, and the modulation conditions, (i.e. modulation time and offset temperature) are essential. However, slowing down of the temperature-programming rate is needed in 2-D GC. Therefore, a practicable solution using the GC×GC approach goes at the expense of the separation time that is slightly longer as compared to 1D-GC analysis. Another and more important draw-back is given by the data treatment of the software that is not designed for routine quantification by IDA. Due to the cutting of peaks, some time-consuming hand operation in data treatment is needed. Therefore, in the future main emphasis has to be put on a more reliable automatic data treatment in the routine quantification of multiple compounds to facilitate the use of this methodology in industrial research.

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Chapter 5

Progress on Volatile Sulfur Compound Analysis in Wine

Peter M. Davis and Michael C. Qian*

Department of Food Science and Technology, Oregon State University, Covallis, Oregon 97330
***E-mail: Michael.qian@oregonstate.edu.**

Volatile sulfur analysis of wine holds great importance from the standpoints of both off-odor and varietal character. For many years the source, impact, and control of sulfur volatiles have been studied in wine using various techniques. The history and current methods of sample preparation, analysis, and detection are explored herein, with emphasis on light and heavy sulfur volatile differentiation.

1. Introduction

1.1. A Word on Sulfur

Sulfur is an abundant and naturally occurring element. Sulfur has the atomic number 16 with four natural isotopes, primarily ^{32}S (approx. 95%) and ^{34}S (approx. 4%), that average to an atomic weight of 32.065(5)amu. Set directly beneath oxygen in the periodic table, sulfur is the second member of the group 16 family of elements known as the chalcogens. In its natural, elemental solid state, sulfur is composed of eight-membered rings stacked upon each other in an ordered fashion, exhibiting a dull yellow color. It often accumulates around volcanic openings, and was known by the ancients as *brimstone*. Polysulfides, involving chains of sulfur-sulfur bonds, are not uncommon, though elemental sulfur defaults most naturally to cyclic S_8 (*1*, *2*).

Sulfur's self-afinity has enormous biological and technological significance. Introduction of the vulcanization of rubber in the 19th century revolutionized industrial machines, relying on the cross-linking of natural rubber polymers with polysulfide bonds to improve cohesion and restriction. Disulfide bonds formed between cysteine residues in biological macromolecules have importance

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in protein stability and irreversible substrate binding (also a notable device in medicinal chemistry), trumping more prevalent hydrogen bonds and intermolecular forces with its covalent strength. Numerous examples of disulfide bonds in biological systems abound, reinforcing the great importance of sulfur in biology, living organisms, and food systems. However, the tendency to form disulfide bonds creates difficulty for chemical analysis, disturbing the natural analyte system during testing.

1.2. Volatile Sulfur Compounds (VSCs)

Volatile sulfur compounds (VSCs), especially at lower molecular weights, are known for giving off strong, offensive odors. Hydrogen sulfide is likely the most well-known VSC, characteristic of rotten eggs. Cabbage patches are a familiar reference for sulfurous odors, as the existence of smaller thiols and sulfides in many cultivars of cabbage is well known (*3*). Other noted sources of VSCs are allium vegetables such as onion, garlic, and chive (*4–7*), asparagus (*8*), broccoli and cauliflower (*9*), tropical fruits like grapefruit, guava, and passion fruit (*10–13*), lychee (*14*), *etc.* in which VSCs occur naturally, and roasted systems having undergone Maillard reaction such as cooked meats (*15–17*), toasted sesame seeds (*18*), and coffee (*19–21*). Many sulfur volatiles have extremely low odor thresholds, many in the parts per trillion (ppt) range (*22*), contributing significantly to overall aromas. In many cases, this is a less-than-desirable effect. Commonly accepted theory of perception suggests that highly volatile small sulfur compounds elicit a strongly negative response to warn consumers of rotten or spoiled foods; many odor-active products are formed through decomposition and putrefaction (*23*, *24*).

However, not all VSCs are necessarily foul-smelling. It has been suggested that very minute amounts of certain sulfur compounds, including the usual low-molecular-weight offenders, can actually enhance and benefit the aroma of certain foods, including wines. Dimethyl sulfide (**1**), for instance, has been shown to bring out fruity character in red wines at low concentrations (*25*, *26*). Some larger structures exhibit some earthy, green, and tropical notes which are of great importance to some varietal character, particularly in Sauvignon blanc (*27*). Generally aromas compared to gooseberry, boxtree, black currant, grapefruit, and other tropical fruits are elicited from such compounds, the three most prominent of which are 4-mercapto-4-methylpentan-2-one (**2**), 3-mercaptohexan-1-ol (**3**), and 3-mercaptohexyl acetate (**4**).

Some of the most noxious sulfur volatiles are small, low-molecular-weight compounds referred to as "light" VSCs. The 'light' indication is not solely based on molecular weight, but on boiling point, which, by definition, falls below 90ºC. These commonly include hydrogen sulfide (H_2S), dimethyl sulfide (**1**), ethyl methyl sulfide(**5**), methanethiol (CH_3SH), ethanethiol (C_2H_5SH), carbon disulfide (**6**), and carbonyl sulfide (**7**). Methyl thioacetate (**8**) is also of note, as, though technically considered 'heavy' as its boiling point is above 90ºC, the margin by which it surpasses the 90ºC threshold is slight (only a few degrees at STP). Heavy volatiles are especially abundant, with a variety of structures and organoleptic properties. Some notable entries include ethyl thioacetate (**9**), dimethyl disulfide

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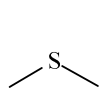
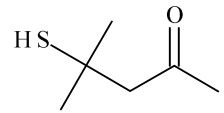
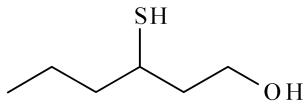
(**10**) and other alkyl disulfides, dimethyl trisulfide (**11**), dimethyl sulfoxide (**12**), 2-mercaptoethanol (**13**) and other mercaptoalcohols, esters of sulfides like 3-methylthiopropyl acetate (**14**), and various heterocyclic species such as 2-methyltetrahydrothiophenone (**15**), and 5-(2-hydroxyethyl)-4-methylthiazole (**16**), to name a few. Sensory thresholds of some of these compounds are given in Table 1.



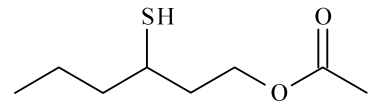
Dimethyl Sulfide
1



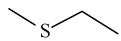
4-Mercapto-4-methyl-pentan-2-one
2



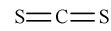
3-Mercaptohexan-1-ol
3



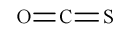
3-Mercaptohexyl Acetate
4



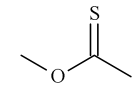
Ethyl Methyl Sulfide
5



Carbon Disulfide
6

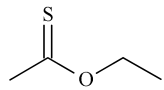


Carbonyl Sulfide
7

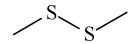


Methyl Thioacetate
8

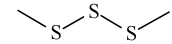
Some ‘light’ sulfur volatiles.



Ethyl Thioacetate
9

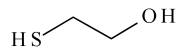


Dimethyl Disulfide
10

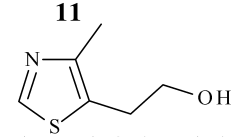


Dimethyl Trisulfide
11

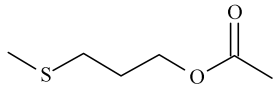
Dimethyl Sulfoxide
12



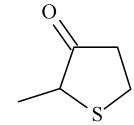
2-Mercaptoethanol
13



5-(2-Hydroxyethyl)-4-methylthiazole
15



3-Methylthiopropyl Acetate
14



2-Methyltetrahydrothiophenone
16

Some ‘heavy’ sulfur volatiles

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Table 1. Sensory Thresholds of Some VSCs found in Wine (*28–30*)

Compound	*Threshold value (ppb)*			*Aroma description*
	Wine		*12% Ethanol (aq)*	
Hydrogen sulfide	0.001-150*		0.8	Rotten egg, decaying seaweed, rubbery
	40-100**			
Methanethiol	1.72-1.82	(red)	0.3	Rotten cabbage, cooked cabbage, burnt rubber, pungent, putrefaction
Ethanethiol	1.1	(white)	0.1	Onion, rubber, fecal, burnt match, earthy, durian
	0.19-0.23	(red)		
Carbon disulfide	30	(white)		Rubber, choking repulsive, cabbage, sulfidy
Dimethyl sulfide	10-160		5-10	Cabbage, asparagus, cooked corn, truffles, vegetal, molasses, black olive
	25	(white)		
	60	(red)		
Diethyl sulfide	0.92-18		6	Garlic, onion, cooked vegetables, rubbery, fecal
	0.92	(white)		
Dimethyl disulfide	20-45		2.5	Cabbage, cook cabbage, onion-like
	29	(white)		
	11.2-23.6	(red)		
Diethyl disulfide	4.3-40		20	Garlic, onion, burnt rubber
	4.3	(white)		
	1.4-2.2	(red)		
Dimethyl trisulfide				Beany
Methyl thioacetate				Sulfurous, rotten vegetables, cheesy, onion, burnt
Ethyl thioacetate				Sulfurous, cheesy, onion, burnt
Methionol	1200-4500			Raw potato, soup-like, meat-like
Methional			50	Onion, meat, mashed potato, soup, bouillon
Benzothiazole	24		50	Rubber
	50-350			

Continued on next page.

Table 1. (Continued). Sensory Thresholds of Some VSCs found in Wine (*28–30*)

Compound	*Threshold value (ppb)*		*Aroma description*
	Wine	*12% Ethanol (aq)*	
2-Mercaptoethanol	130-10000	1000-10000	"Boxer," poultry, farmyard, alliaceous
4-Methylthio-1-butanol	100	80-1000	Chive, garlic, onion, earthy, alliaceous

* Aroma threshold. ** Flavor threshold.

Distinction between light and heavy VSCs will play a key role in analytical methods. Often the same method cannot effectively evaluate content of both light and heavy volatiles. For many analyses, separate steps must be taken to extract, precondition, or derivatize certain compounds in order to ensure their measurability. There is some margin, though, where heavier compounds with relatively low molecular weights can be analyzed in the same assay as lighter compounds. For instance, ethyl thioacetate can be measured simultaneously with dimethyl sulfide, ethanethiol, and methyl thioacetate (*31*). Nevertheless, it is germane to examine analytical methods of these compounds based on their light and heavy character.

2. Light Volatile Sulfur Compounds

2.1. Formation in Wine

The evolution of hydrogen sulfide and dimethyl disulfide has been well-researched (*32–42*). While several factors play an integral role in the production of hydrogen sulfide, ultimately its liberation relies on yeast metabolism of sulfur-containing precursors. For many years when strong, scientific wine research was still relatively nascent, a widely accepted and assumed precursor to hydrogen sulfide was elemental sulfur sprayed onto the grapes during the growing season. Elemental sulfur proves to be a highly effective antimicrobial and antifungal agent, and from the 1960s through 80s many papers were published examining the role of elemental sulfur residue in hydrogen sulfide production. Preliminary results suggested this was a probable cause for high amounts of hydrogen sulfide, as laboratory tests with sulfur-supplemented synthetic must fermentations produced observable hydrogen sulfide. Acree *et al.* (*43*) observed in 1972 that synthetic musts with a 10 mg/L sulfur addition suffered from high hydrogen sulfide production, more so than a similar synthetic must with sulfate addition. They measured hydrogen sulfide production with a cadmium hydroxide trap and methylene blue; a nitrogen stream displaced dissolved hydrogen sulfide into the trap and resulted in a colorimetrically measurable result. Schütz and Kunkee (*32*) measured hydrogen sulfide formation in 1977 using both a lead-acetate-soaked cellulose system borrowed from Rankine (*44*), and

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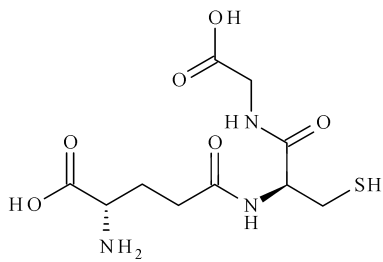
a sulfur-specific ion-selective electrode. The lead acetate method is subject to some criticism (*37*), however, as the method is considered highly inaccurate and qualitative, relying on visual ascertainment of hydrogen sulfide levels based on black color formation in the system. Furthermore, Thomas *et al.* (*45*) examined specific concerns about the amount of sulfur added to the synthetic musts in past experiments. In 1993 they published a method for determining sulfur residue on the grape berries, involving washing the residue off of whole clusters using Tween 20, and analyzing the wash solution for sulfur using vacuum inductively couple plasma (ICP) spectrometry. This led to a realization of relatively low amounts of elemental sulfur residing on grapes within days after dusting in the vineyard. Average values of 1-3μg/g berry weight, which translates to roughly 1.2-3.4 mg/L juice, were found across several vineyards, paling in comparison to the average analyses of 10-100mg/L of several preceding studies. This led to a repeat of Acree's (*43*) experiment utilizing a cadmium hydroxide trap, but with the lower concentrations of elemental sulfur measured on the grapes (0, 1.7, and 3.4 mg/L) (*37*). Conclusions from this study confirmed suspicions of previous reports, ultimately showing the lack of significance of even the largest (3.4mg/L) amount of elemental sulfur found on the grapes. Thus, hydrogen sulfide production was attributed to yeast manipulation of other precursors.

Other factors which may have a hand in hydrogen sulfide production include a pantothenate deficiency (*33*, *46*, *47*), levels of glutathione (**17**) in the yeast (*33*), and reduction of both (or either) sulfate and/or sulfite (*36*, *43*, *47*). Spiropolous *et al.* (*47*) have suggested that the underlying issue in all situations is that of nitrogen levels, specifically of both easily assimilable amino acids and sulfur-containing amino acids. In reviewing yeast metabolism and assimilation of nitrogen sources, levels of cysteine and methionine, which tend to suppress sulfate and sulfite reductases, are in balance with important non-sulfur-containing amino acids, which may or may not (depending on certain conditions) suppress these enzymes. Without delving as deeply into the enzymology and genetics of their research, some influencers of hydrogen sulfide production, namely sulfate and sulfite, are merely extensions of a latent nitrogen-related influencer. Readers are directed toward a great review of these findings in reference (*47*). These factors are highly complex, and even such a complex solution cannot encompass the entirety of chemical behavior among grapes, must, and yeast. A single, unified, comprehensive explanation of hydrogen sulfide formation in wines is unlikely.



Glutathione
17

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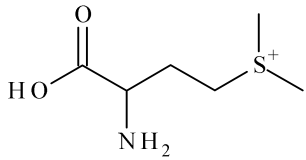
ACS Symposium Series; American Chemical Society: Washington, DC, 2011.



S-Methylmethionine

18

Dimethyl sulfide also receives considerable attention for its presence (in most respects unwanted) in many wines (*25*, *35*, *38*, *42*, *48*, *49*). Believed to also stem from sulfur-containing amino acid precursors, its formation is similarly esoteric. De Mora *et al.* (*50*) performed a radiolabeling experiment with ^{35}S-cysteine, and confirmed a pathway of dimethyl sulfide formation. However, these findings were related to yeast contact with the wine and presence in yeast lees after racking. Commonly dimethyl sulfide off-odors are generated during bottle-aging, without lees contact (*51*, *52*). This led Segurel *et al.* (*49*) to investigate potential precursor compounds, and design a metric for potential dimethyl sulfide (P-DMS). In examining various candidates, they found S-methylmethionine (**18**) to produce reasonable levels of dimethyl sulfide during a heat-alkaline synthetic aging trial. Still, the absolute cause of dimethyl sulfide liberation post-bottling is not clearly understood.

2.2. Analysis of Lighter Sulfur Volatiles

One difficult aspect about light sulfur volatiles is, by definition, their volatility. Precautions must be taken to procure accurate measurements. Past enlightening studies have revolved around analysis as a response to sensorial input; a foul off-odor is noticed in several wines, and the bottles are passed on to an analyst. In many of these studies, the most common compounds are those mentioned above; hydrogen sulfide, dimethyl sulfide, carbon disulfide, and the small thiols including methanethiol and ethanethiol. Among these, disulfides are also present (or subsequently formed) by the oxidation of said thiols. As aforementioned, methyl and ethyl thioacetates are also appropriately studied with other smaller VSCs. Several methods have been used to quantify these compounds, and important submissions will be outlined.

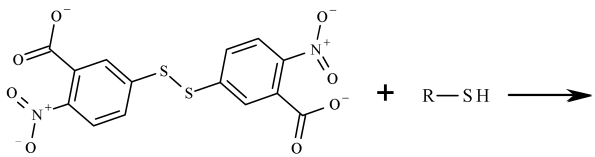
2.2.1. Sample Preparation

A common assay in biological (*53*) and food (*54*, *55*) systems for various smaller thiols involves a chromophore-based compound called 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB, **19**), or Ellman's reagent (*56*). This specific compound relies on a highly electronically withdrawn disulfide bond to react (eq. 1) with smaller, nucleuophilic thiols, leaving a dianion chromophore (2-nitro-5-sulfido benzoate, **20**), and its alkyl-disulfide analogue. The production of said chromophore imparts a yellow color to the solution (the remaining disulfide analogue is colorless), which can be measured spectrophotometrically by UV-VIS

spectrometry. Because wine pigments would convolute UV-VIS readings, however, this method only proves useful for verification of concentrations of prepared standards, and not direct wine analysis (*57*). Measured amounts of a single thiol are placed in a pH 7 solution (using a phosphate buffer) with DTNB and the resultant yellow color is calibrated and measured at 412nm. This method is useful in preparatory stages for accurate measurement of volatile thiols (*57*). However, due to the impartial nature of DTNB, this method offers little aid in simultaneous analysis of multiple thiols.

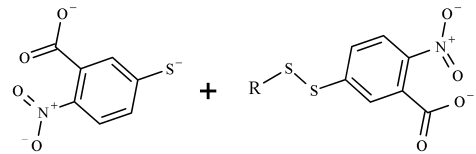
Some small thiols are highly reactive in the presence of certain species. Transition metals, for instance, even in trace amounts, can catalyze oxidation of thiols into disulfides (*38*, *58*). Sampling containers must also be considered. Generally direct gas analyses of sulfur mixtures involved storage in poly(vinyl fluoride) bags to ensure chemical inertness (*59*). Glass vials, commonly used for storage and sampling, contain relatively active hydroxyl groups on the surface. Additional precautions must be taken to deactivate the surfaces of these vials. Common treatment is deactivation using trimethylchlorosilane, dimethyldichlorosilane, methyltrichlorosilane, and hexamethyldisilazane (*60*). Cleaning glassware with a 5% solution in toluene, hexane, or dichloromethane will replace the hydroxyl groups with silyl ether groups.

Volatile sulfur compounds can be separated and analyzed by gas chromatography. Due to the low concentration in wine, futher concentration is necessary. The purge-trap enrichment method can be used to improve the sensitivity of detection. Poly(2,6-diphenyl-*1*-*4*-phenylene oxide) (**21**), also known as Tenax™, was used for its high thermostability required for thermal desorption (*61*). The volatile sulfur compounds can also be cryogenically trapped (*4*, *57*) on a small portion of the capillary column submerged in liquid nitrogen. The frozen material trapped in the column is re-volatilized after the trapping.



5,5'-Dithiobis(2-nitrobenzoic Acid)

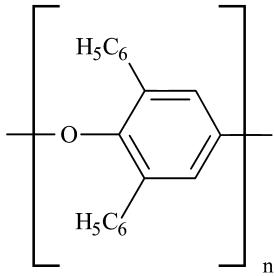
19



2-Nitro-5-sulfido Benzoate

20

(1)



Poly(2,6-diphenyl-*1-4*-phenylene Oxide)
"Tenax"
21

Presently, the most common method of volatile sulfur analysis does not function on removal of unwanted compounds, but rather the selectivity of sulfur compounds for analysis. Mestres *et al.* (*62*) used head space solid phase micro-extraction (HS-SPME) to analyze volatile sulfur compounds in wine. Polyacrylate (PA) and poly(dimethylsiloxane) (PDMS) fibers, as well as a bi-layered activated carbon/PDMS fiber in a later study (*63*), have been evaluated for the extraction efficiency of volatile sulfur compounds. These studies proved highly enlightening. PDMS, which is significantly less polar than polyacrylate and prefers moderate- to non-polar compounds, proved more effective in extracting some VSCs, namely ethylmethyl sulfide, diethyl sulfide, and methylpropyl sulfide, but showed little advantage (or disadvantage) in extracting dimethyl sulfide, methyl and ethyl thioacetates, carbon disulfide, and other alkyl disulfides. However, smaller volatiles like hydrogen sulfide, methanethiol, and ethanethiol were not examined. The activated carbon/PDMS fiber showed a considerable affinity for sulfur compounds, and has since been adopted as the standard for HS-SPME VSC analysis. Recently, reports of triple-phase fibers coated with activated carbon/PDMS/poly(divinylbenzene) (DVB) used for larger, heavy thiol derivatives have surfaced, which will be discussed later.

Increased polarization of the sample liquid *via* addition of sodium chloride effectively increases partition coefficients at the gas-liquid interface, and improves extraction. However, some of the most volatile compounds showed the least absorption by the SPME fiber. This phenomena has been attributed by competitive absorption that larger, less volatile compounds to displace more volatile compounds and consume more space on the fiber (*31*, *59*, *62*, *64*). For this reason, shorter extraction times are generally preferred, at the sacrifice of proper equilibrium. Activated carbon phases somewhat compensate for this problem due to its pore structure, which can detract from displacement by groups too large to fit in smaller crevices. Murray (*59*) has criticized activated carbon/PDMS fibers, citing that other compounds like carbon disulfide can interfere with other molecules' ability to bind to the fiber, even if not by a competitive mechanism. The mere presence of carbon disulfide can reduce the accuracy of other VSCs like dimethyl sulfide. Mestres' group also noted the decomposition and artifact formation with high-temperature extractions, suggesting a 30ºC was optimal.

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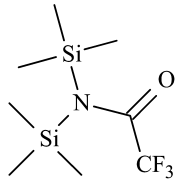

N,N-bis(Trimethylsilyl)-acetamide
22

Artifact formation was further addressed by Fang and Qian (*31*), concurring with low extraction temperatures and suggesting a deactivation step for the injector using N,N-bis(trimethylsilyl)-trifluoroacetamide (**22**) and deactivation of sample vials. It is also common practice to flush all sample vials with nitrogen or argon to avoid any oxidation and disulfide artifact formation (*31*, *65*, *66*). Lastly, a precautionary measure should be taken to eliminate the activity of metal ions present in the system, as such are known to catalyze thiol oxidation as mentioned. Addition of EDTA or other organic acids such as citric acid and malic acid (*31*, *65–67*) will chelate trace metals in solution and prevent catalysis.

Extraction is further improved by agitation, increased headspace, and dilution of ethanol. Still, the matrix effect surmises one of the greatest challenges of wine sulfur analysis, due to the great variability of wine (and wine-based products like Cognacs and brandies), and remains as a major challenge (*58*, *62*, *63*, *68–72*).

2.2.2. Separation

Originally researchers used dimethylpolysiloxane columns for separation *via* GC, which vary in composition and thickness. Some have utilized non-polar PDMS (DB-1) (*39*, *58*), or PDMS fluid (HP-101) (*36*) columns. Slighter higher polarities are reached through partially-substituted PDMS with phenyl (DB-35) (*73*), or cyanopropyl groups (DB-1701) (*74*). Specific sulfur columns (SPB-1, Figure 1) have also been used successfully (*63*, *72*), which rely on a a very thick film (4 μ) of PDMS to retain highly volatile compounds (*60*). Others have used packed columns (*48*, *75*), but the most common used for sulfur analysis are polar poly(ethyleneglycol)-based (wax) columns (*29*, *31*). Good separation is achieved by Qian's group using a 2-nitroterephthalic acid-substituted wax column (FFAP, Figure 2)(Qian, unpublished chromatogram). Siebert *et al.* (*76*) have also shown effective separation with a dual-column approach (Figure 3), using serially connected wax and (5%phenyl)-PDMS (VB-5) columns with a 2m retention gap.

Temperature programs are often used, generally ramping from 35-60ºC to 150-300ºC in anywhere from 5 to 30 minutes (*29*). A common nuisance in wine matrices is the large peak from sulfur dioxide, a compound utilized at multiple stages in winemaking (*31*). The large, wide peak eclipses more pertinent, odor-active thiols; thus, its removal is critical. Simple addition of acetaldehyde can solve this issue (*31*) (Figure 4), as a known affinity between the two compounds exists (*77*). Other aldehydes can also be used to eliminate the interference of SO_2 in wine.

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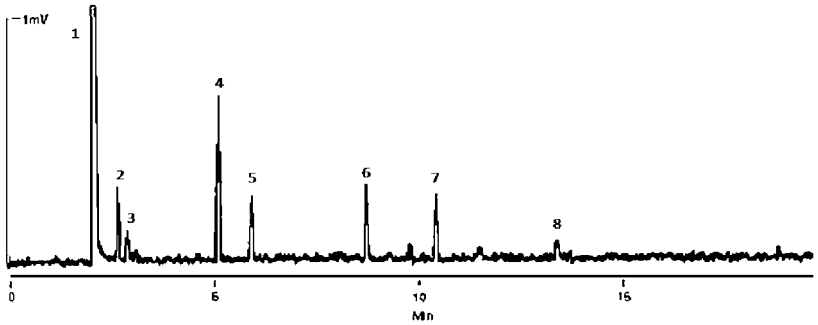


Figure 1. Chromatogram of sulfur analysis of sour natural gas using SPB-1 Column. 1. H_2S, 2. COS, 3. SO_2, 4. DMS, 5. MeSH, 6. EtSH, 7. Isopropanethiol, 8. Sec-Butanethiol. (Courtesy of Supelco, Bellafonte, PA)

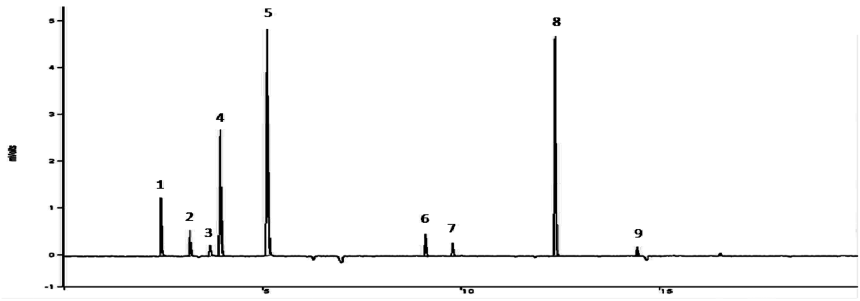


Figure 2. Chromatogram of sulfur analysis of Chardonnay using DB-FFAP column. 1. H_2S, 2. MeSH, 3. CS_2, 4. DMS, 5. EMS (IS), 6. MeSOAc, 7. EtSOAc, 8. DIDS (IS), 9. DMTS. (Qian, unpublished chromatogram)

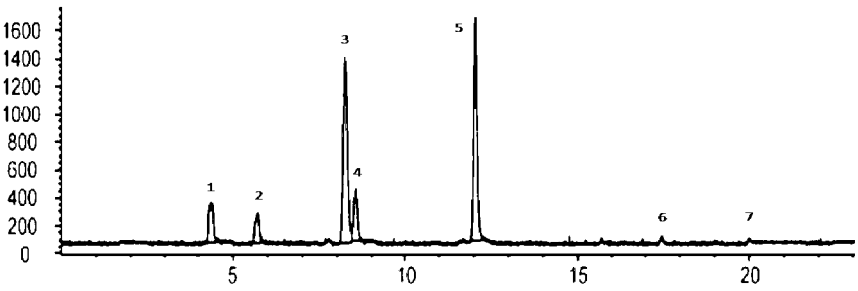


Figure 3. Chromatogram of sulfur analysis of white wine using VFWAXms to VB-5 dual column. 1. H_2S, 2. MeSH, 3. DMS, 4. CS_2, 5. EMS (IS), 6. MeSOAc, 7. EtSOAc. (reproduced with permission from reference (76), copyright 2010).

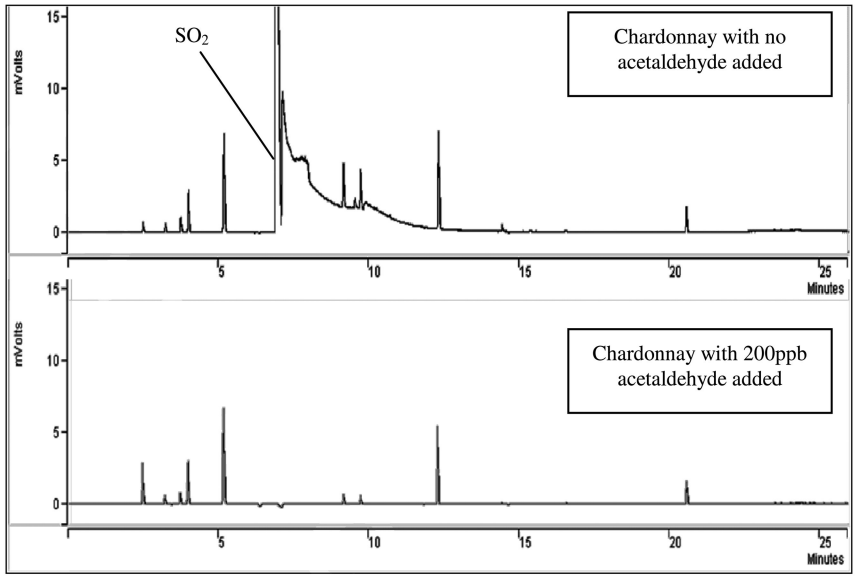


Figure 4. Chromatograms showing the effects of acetaldehyde on SO_2. (Unpublished chromatogram from Qian's Laboratory).

2.2.3. Sulfur Detectors

Both flame ionization detection (FID) and mass spectrometry (MS) have been used to quantify VSCs in wine. However, its detection limits are generally too poor to analyze sulfur in wine samples. Both FID and MS have been overshadowed by sulfur-specific detection methods.

2.2.3.1. Sulfur Chemiluminescence Detector

Sulfur chemiluminescence detectors (SCDs) are popularly used for volatile sulfur detection in wines due to their high sensitivity and equimolar response functionality. The principle behind the system involves decomposition of sulfur molecules (equation 2) into sulfur dioxide, which then reacts with hydrogen to produce a sulfur chemiluminescent species, notated here as ~SCS, the details of which are somewhat unclear (*78*). However, it is accepted that this chemiluminescent species reacts with ozone to produce an excited state of sulfur dioxide, SO_2*, which relaxes to yield a spectrum focused around 380nm. The response functionality is dependent on the concentrations of ozone and ~SCS, meaning a constant overabundance of ozone would create a first-order, linear relationship between response and concentration: response=kC (*79*). SCDs also often incorporate integrated FIDs, though this has been known to cause problems involving the transfer line temperature between the two detectors. Some other

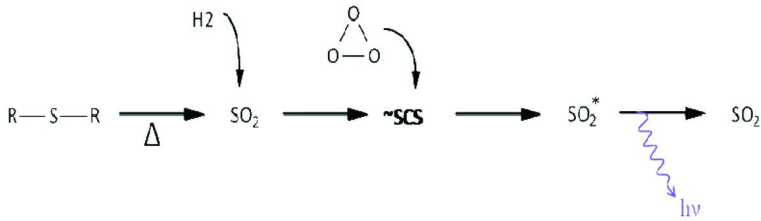
downsides of the SCD are the cost and maintenance as certain maladies like probe alignment can result in a finicky system (*29*).

2.2.3.2. Atomic Emission Detector

Much like those of trace metal analysis, atomic emission detectors can be used to analyze sulfur compounds based on specific sulfur-atom emission spectra. The technique, though still in use, was never quite as popular for wine analysis as some others. Atomic emission is a universal detection mechanism, keying in on specific emission spectra unique to each atom's excitation-relaxation cycle. Samples are volatilzed and atomized *via* heat source, and individual atoms are excited (S*), releasing photons in relaxation. The method is sulfur-specific by measuring emission of wavelength 132nm or 181nm (*80*, *81*)

2.2.3.3. Flame Photometric Detector

Flame photometric detectors (FPDs, Figure 5) are similar to FIDs, but rely on an excitation-relaxation photon emission stimulated in lieu of ionization. Within the flame, sulfur species become oxidized and react (equation 3) to form the excited sulfur dimer species, S_2*, which relaxes to S_2, emitting a photon at 394nm. The photon is then absorbed by a photomultiplier in the detector, offering exceedingly low detection limits (*82*). FPD was the most popular detector for some time (*24*, *55*, *64*), though some limitations were well-noted. Specifically, introduction of hydrocarbon species co-eluting with sulfur species is known to cause quenching (equation 4) of the response (*83*). As hydrocarbons are burned by the flame, carbon monoxide is produced which chemically interacts with the stimulated S_2* units and lessens the observed signal. Responses are based on the number of sulfur atoms present in each compound, with quadratic functionality: response=kC^b. Generally the b value ranges from 1.5 to 2. Calibrations thus involve logarithms of analyte/internal standard ratios, comparing peak areas and peak heights (*24*, *62*).



(2)

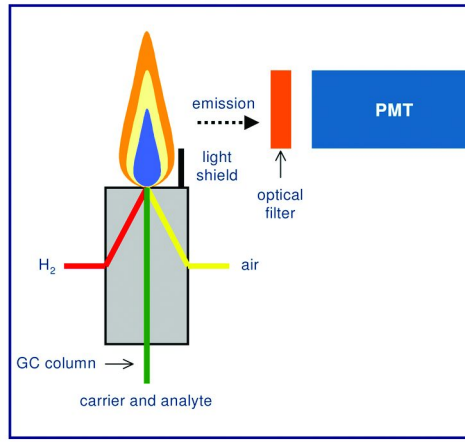
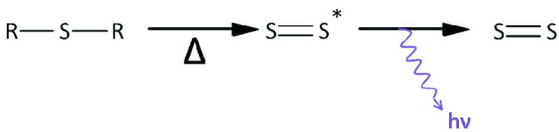
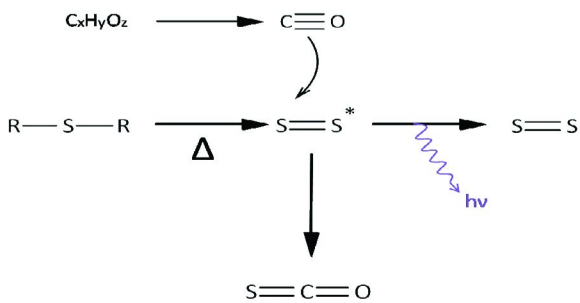


Figure 5. Schematic of flame photometric detector. (Courtesy of Hewlett-Packard Co., Analytical Customer Training, Atlanta, GA)



(3)



(4)

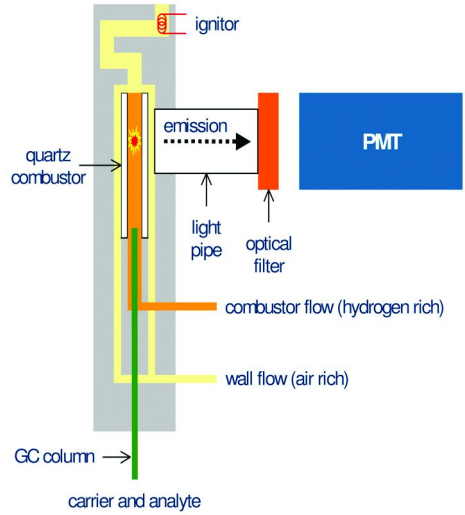


Figure 6. Schematic of pulsed flame photometric detector. (Courtesy of Varian Inc., Palo Alto, CA).

2.2.3.4. Pulsed-Flame Photometric Detector

In an effort to overcome issues with sensitivity in traditional FPDs, the pulsed-flame photometric detector (PFPD, Figure 6) forgoes a constant, steady flame in favor of a punctuated mechanism. Effectively, hydrocarbons, carbon monoxide, carbon dioxide, and sulfur dioxide have different relaxation patterns based on time. The former three relax much more quickly (2-3ms) than sulfur dioxide (5ms), and this emission lag is utilized to focus strictly on sulfur species. The PFPD works just like a FPD, allowing a buffer time amidst the pauses between flame pulses to ignore early emissions from C and other atoms and greatly increase sensitivity (*83*, *84*). This detector has been popularized due to its high selectivity and reproducibility, if slightly less sensitive than SCD (*31*, *69*).

3. Heavy Volatile Sulfur Compounds

3.1. Formation in Wine

Some larger, heavy volatiles, as discussed, can often impart beneficial flavor to wines. 3-Mercaptohexan-1-ol and its acetate ester are known to impart tropical, passion fruit, grapefruit, and guava aromas to wine (*13*, *29*). Such heavy volatiles are essential to varietal aromas of white wines, notably Sauvignon Blanc (*27*, *39*, *85*) and Muscat (*34*, *86*). Several prize-winning rosé wines from Provence were found to contain both aforementioned mercaptohexyl compounds at concentrations above their thresholds, attributing as well to varietal aroma (*87*). Origins of some heavy thiols involve reactions of amino acid methionine and ethanol. Well-known VSC methionol (3-methylthiopropan-1-ol, **23**) and

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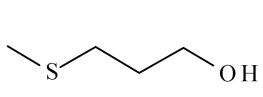
other C_3 sulfur compounds are believed to originate in this manner. Other larger forms are found as conjugate species with amino acid cysteine, which are cleaved enzymatically during fermentation. A β-lyase enzyme present in the yeast liberates bound thiols and contributes to the fermentation bouquet (*88*). This explains why such sulfur compounds, despite their low thresholds, are not immediately detectable in the grapes. However, anecdotal reports for many years have documented the 'Sauvignon-blanc-like' aftertaste that arises 30 seconds after consumption of the grapes. This is believed to be a retro-olfactory phenomenon caused by compounds hewn from their precursors by mouth enzymes (*89*). Within the grape, Peyrot des Gachons *et al.* (*90*) have shown that, while 4-mercapto-4-methylpentan-2-ol (**24**) and its ketone analogue are equivalent in the skins and juice, 3-mercaptohexan-1-ol is considerably more present in skins. Thus more extraction can be achieved by extended maceration. However, other factors will affect its retainment in red wines; oxygen and phenolic compounds greatly reduce the presence of 3-mercaptohexan-1-ol over time, though sulfur dioxide, and to a lesser extent anthocyanins, can offer protection. Dozens of other heavy volatiles exist in wines (*29*). Because of their lower volatility and concentrations, heavy VSCs have generally been analyzed differently than lighter forms.

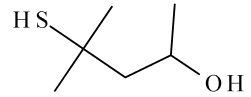
3.2. Analysis of Heavy Sulfur Volatiles

3.2.1. Sample Preparation

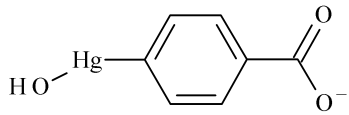
The standard method for analyzing heavy VSCs was pioneered by Tominaga *et al.* in 1998. (*19*, *27*, *90–94*). Preparation begins with a solvent extraction at neutral pH, generally in dichloromethane, ethyl acetate, Freon 11, or any combination thereof. The organic phase is centrifuged and separated, then further extracted with *p*-(hydroxymercuri)benzoate (**25**). This derivatization reagent preferentially binds to sulfur species, and allows the bound conjugates to adhere to a strong anionic exchange column for concentration and washing of unwanted material. To liberate the thiols from the column, an abundance of larger thiol compounds like cysteine or glutathione can replace the analytes. The heavy-thiol-rich eluate is then extracted again with dichloromethane before analysis by GC.



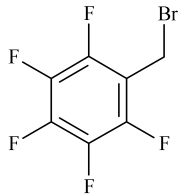
Methionol
23



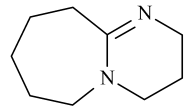
4-Mercapto-4-methylpentan-2-ol
24



p-(Hydroxymercuri)benzoate
25



Pentafluorobenzyl Bromide
26



1,8-Diazabicyclo[5.4.0]undec-7-ene
27

Recently, Rodríguez-Bencomo *et al.* analyzed larger thiols through the use of SPME and alternate derivatization (*70*). In their method, wines are extracted and similarly loaded onto solid phase extraction cartridges containing styrene divinylbenzene phases. The compounds are derivatized with pentafluorobenzyl bromide (**26**) with the aid of strong alkaline agent 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU, **27**), and washed with mercaptoglycerol. For the SPME fiber, a triple-phase activated carbon/DVB/PDMS coating was used to improve extraction specifically of the fluorobenzyl conjugates. SPME analysis of the derivatized forms proved promising (*70*). This method was adapted from a previous work by Mateo-Vivaracho *et al.* (*95*), who used the derivatized species for direct injection into GC-MS.

3.2.2. Sample Analysis

For the most part, the difference in heavy and light volatile sulfur analysis is comprised of the preparatory measures necessary for proper extraction. The basics of separation (GC) and detection (most often MS) have been adequately discussed, and are applicable for heavier volatiles. Mass Spectrometry seems to gain greater preference for heavier compounds; however, pretreatment of samples must be done to concentrate the VSCs to meet the systems' detectability.

Conclusions

Sulfur is very interesting in its behavior and properties, and important in the wine and food industry. Though most well-known for the foul odors associated with them, VSCs play an integral role in complexity and depth. Because of their high volatility, propensity toward oxidization, and existence only in minutiae, the

analysis of VSCs has proven difficult in complex matrices. But with the prevalence of natural sulfur sources and emerging science, the understanding and efficacy of analytical techniques will progress.

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Chapter 6

Recent Advances in Volatile Sulfur Compounds in Cheese: Thiols and Thioesters

A. M. Sourabié,[a,b] H.-E. Spinnler,[a] A. Saint-Eve,[a] P. Bonnarme,[a] and S. Landaud[*,a]

[a]AgroParisTech/INRA, UMR 782 GMPA, Rue Lucien Brétignières, 78850 Thiverval-Grignon, France
[b]SAF-ISIS, Zone Artisanale, 40140 Soustons, France
[*]E-mail: landaud@grignon.inra.fr.

Among the numerous compounds involved in cheese aroma, sulfur compounds are of particular interest because of their very powerful odors and low perception thresholds. Nevertheless, little attention has been focused until now on the possible presence of polyfunctional thiols in cheese, even if these compounds have been found to be associated with the flavor of different foods, including fermented ones. The difficulty in isolating volatile thiols in cheeses is probably due to the complexity of this matrix and to the very low concentrations of these compounds that make them undetectable using classic means. Consequently, different methods of sample preparation followed by extractions with *p*-hydroxymercuribenzoate were used to investigate the possible occurrence of thiol compounds in ripened cheeses. The analysis of cheese extracts by GC coupled with pulse flame photometry, MS and olfactometry detections made it possible to identify ethyl 2-mercaptopropionate (ET2MP) and ethyl 3-mercaptopropionate (ET3MP) in smear and mold-ripened cheeses for the first time. The presence of ET3MP in cheeses at concentrations of around 3 µg/kg, significantly higher than its perception threshold in cream (723 ppt), suggests that this thiol may significantly contribute to the aroma of these cheeses.

Concerning other potent odors that contribute to the aroma of numerous cheeses, we also investigated the ability of *Brevibacteria strains* to produce *S*-methyl thioesters in

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the presence of (i) methanethiol, (ii) fatty acids, or (iii) branched-chain amino acids as precursors. All the strains studied were able to yield the corresponding *S*-methyl thioesters (i.e., *S*-methyl thioisovalerate from L-leucine) from precursors. It was interesting to note that the data also showed that other *S*-methyl thioesters e.g., *S*-methyl thioacetate or *S*-methyl thioisobutyrate, were also produced following the addition of an individual precursor (e.g., L-leucine). Enzymatic and tracing experiments allowed us to propose the catabolic pathways used by the strains to produce *S*-methyl thioesters.

Keywords: smear soft cheese; polyfunctional thiols; *S*-methyl thioesters; *Brevibacteria*; fatty acids; branched chain amino acids

Introduction

Cheese ripening is a complex phenomenon in which a wide variety of microorganisms (e.g., yeasts, bacteria) are involved. Their action results in the synthesis of a variety of aroma compounds, especially volatile sulfur compounds (VSC), which give specificity to a variety of ripened cheeses. Even if the VSC found in cheeses belong to a large number of chemical families (*1*), we chose to focus on thiols and thioesters because little is still known about their nature (thiols) and their biosynthesis (thioesters).

Little attention has been given to the possible occurrence of thiols in cheese until now. Only methanethiol (MTL) and H_2S, which are common precursors for a variety of other VSC, have been frequently reported in cheeses (Table I).

MTL, found in high amounts in Camembert cheese, gives a characteristic "cooked cabbage" flavor note. It has also been reported in vintage Cheddar, Parmesan, Pecorino, Grana Padano and blue cheese. Among those cheeses, MTL was perceived as a "strong aroma" in Cheddar and blue cheese, and as a "very strong" aroma in Parmesan (*3*). MTL is the first product of methionine degradation.

Moreover, H_2S has the unpleasant odor of "rotten eggs" and has been reported in Limburger cheese (Table I) but only in traces, probably because of its high reactivity and its difficulty to be quantified. H_2S is considered as the primary degradation product of cysteine.

Few other thiols have been reported in cheeses to date. 4-mercapto-4-methyl-pentan-2-one has been reported in Gouda cheese as a catty flavor compound (*4*). More recently, Kleinhenz carried out studies that highlighted the hypothetical presence of several thiols in Cheddar cheese (*5*). However, they used a phosphine reagent, TCEP (tris(2-carboxyethyl) phosphine) to prevent the oxidation of thiols and to enable their recovery from Cheddar cheese oil. Given that TCEP is a very powerful reducer, the hypothetical thiols that they found could have been formed by the reduction of the polysulfur molecules. Moreover, these authors were not able to unequivocally characterize the identified thiols.

Table I. Examples of thiols found in cheese (*2*)

Thiol compounds	*Flavor note*	*Odor threshold (ppb)*	*Probable precursor*	*Cheeses in which they occur*
Hydrogen sulfide	Rotten eggs	0.18[a]	Cysteine	Limburger Cheddar
Methanethiol	Cooked cabbage; fermented cabbage	0.06[b]	Methionine	Camembert Cheddar blue cheese Parmesan Grana Padano Pecorino
4-mercapto-4-methyl-pentan-2-one	catty	nd [c]	nd	Gouda

[a] In air. [b] In sunflower oil. [c] nd: not determined.

Nevertheless, thiols possess a wide variety of odors ranging from cheese to blackcurrant, depending on their chemical structures (*6*) and their concentrations, and have been found to be associated with the flavor of different foods, including fermented ones (*7–10*). Because of the multiple sulfur descriptors usually used to characterize smear soft cheeses like Munster, it is reasonable to assume that thiols may be involved in their characteristic flavors. The difficulty in isolating volatile thiols in cheese is due to the complexity of the cheese matrix and to the very low concentrations of these compounds, which make them undetectable using classic means.

In the first part of this chapter, we present the adaptation of a thiol extraction method to the cheese matrix, allowing us to detect and identify two new thiols in cheeses. The hypothesis concerning their biosynthesis is also developed.

Once formed, certain thiols such as MTL can be oxidized to form other VSC such as sulfides and thioesters. *S*-methyl thioesters have been reported in several cheeses and extensively studied with respect to their detection thresholds—which ranged from 1 to 3 ppb—and flavor notes (Table II).

The most common descriptors cited for thioesters were “cabbage”, “garlic” and “cheesy” (*11*). For example, *S*-methyl thioacetate (MTA) was detected in Vacherin, Pont-l’Evêque, Langres and Epoisses; *S*-methyl thiopropionate (MTP) was found in Vacherin and Pont-l’Evêque (Table II).

The production of *S*-methyl thioesters, particularly those with a carbon chain length of 2-6 carbons, has been extensively studied over the years. Hence, it was shown that strains of *Geotrichum candidum* were able to produce *S*-methyl thioesters in a liquid cheese medium (*12*, *13*) and that *Micrococcus* (*14*) and *Brevibacterium* strains were able to produce *S*-methyl thioacetate (*15*). The production of these volatiles was achieved with cell-free extracts of *G. candidum* and their synthesis was shown to be essentially spontaneous (*16*). Furthermore,

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Lamberet *et al.* (*17*) succeeded in generating various *S*-methyl thioesters by resting cells of coryneform bacteria including *Brevibacterium* strains.

S-methyl thioesters are believed to originate from MTL and acyl-CoAs (*14*, *18*) that could be generated by numerous metabolic pathways such as those of sugar, fatty acids and branched-chain amino acids. During cheese ripening, *Brevibacterium linens*, a well-known cheese-ripening bacterium, is able to produce methanethiol from methionine degradation (*19*) during cheese ripening via a methionine γ-lyase (*20*, *21*), and acyl-CoAs probably intermediates in catabolic pathways involving FAs and BCAAs. After examination of the genome sequence of *B. linens*, it was in fact shown that this species may have the ability to synthesize FAs and branched-chain FAs from BCAAs (*22*) and could therefore generate acyl-CoAs in a series of reactions initiated by aminotransferases (ATases). These enzymes, which were previously reported in *B. linens* (*23*, *24*), catalyze the first step of amino acids catabolism, converting them into cheese flavor compounds. In addition, based on the same genomic data, it has been reported that this species possesses all the genes required for the degradation and biosynthesis of FAs (*22*). However, despite these findings, the mechanisms and biosynthetic routes for the production of *S*-methyl thioesters in cheese have not yet been conclusively established.

Table II. Examples of thioesters found in cheese (*2*)

Thioesters	*Flavor note*	*Odor threshold (ppb)*[a]	*Probable precursor*	*Some cheeses in which they occur*
S-methyl thioacetate	Cabbage, cheesy, crab	3	MTL and acetyl-CoA	Pont-l'Evêque Langres Epoisses Vacherin Limburger
S-methyl thiopropionate	Cabbage, cheesy, garlic, crab	2	MTL and propionyl-CoA	Vacherin Pont-l'Evêque
S-methyl thiobutyrate	Cabbage, cheesy, rancid, garlic	3	MTL and butyryl-CoA	
S-methyl thioisovalerate	Cheesy, garlic, cabbage	1.2	MTL and isovaleryl-CoA	
S-methyl thioisobutyrate	Garlic, cheesy, cabbage	2.6	MTL and isobutyryl-CoA	

[a] In water.

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Consequently, in the second part of this chapter, we demonstrate the ability of *Brevibacterium* strains to produce *S*-methyl thioesters from FAs and BCAAs and to elucidate the metabolic pathways associated with their synthesis.

Polyfunctional Thiols Are Involved in Cheese Aroma

Identification of Two New Thiols in Cheese

A triangular sensory test was firstly performed to evaluate the possible impact of thiols on overall aroma of cheese. To do this, smear-ripened cheese (Munster), alone or mixed with *p*-hydroxymercuribenzoate (*p*HMB), was tested using the well-known property of thiols to react with mercury. This chemical bond normally leads to the loss of the sensory properties of thiol.

Due to the fact that the sensory difference between the two samples, with or without *p*HMB, was clearly significant and that the flavor descriptors used for samples with *p*HMB were solvent, ammonia and white cheese, whereas those of the controls were fruity, sulfured and cheese, we assumed that thiols were key compounds responsible for aroma in smear-ripened cheese. These results may have been due to thiols such as hydrogen sulfide or methanethiol, which have been reported to contribute to cheese flavor. However, given that these two thiols are very reactive and that cheese flavor is a complex mixture of a wide range of compounds, other thiols was purchased.

Consequently, we attempted to isolate these compounds in cheese using a method adapted from that of Tominaga (*25*, *26*). The preparation of samples was considerably modified to overcome the problems associated with the fat content and heterogeneity of cheese. Consequently, three methods were evaluated (*27*) and parameters such as length and the number of the steps were taken into consideration in the overall extraction efficiency. Only the protocol that considers the thiol content of the cheese surface layers made it possible to eliminate the fat content drawback. This procedure gave the best results for isolating the compound and was therefore selected (see Figure 1 for experimental details). Given that flavor formation in cheese often begins at the surface where microbial activity is the highest before diffusion into the core, this result is not surprising. Moreover the layers were wide enough to include a small part of the cheese core, which probably improved extraction efficiency.

Thiols could be identified in 40% of the cheeses analyzed, including the smear and mold-ripened ones. Using two specific and sensitive detectors (a Pulsed Flame Photometric Detector (PFPD) and Mass Spectrometry (MS) coupled with Gas Chromatography (GC)), thiol identification was based on their retention times, PFPD indices (*27*) and mass spectra in comparison with commercial references.

One of them did not match any compound in the GC-MS library (NIST Mass spectral database), perhaps because this detector is not sensitive enough and undoubtedly because the concentration of the compound was too low for detection. Nevertheless, it has been tentatively identified as ethyl 2-mercaptopropionate (ET2MP) by comparison to its PFPD retention time, PFPD index and those of the commercial reference. This volatile is known to be found in apple juice and strawberries (*28*, *29*).

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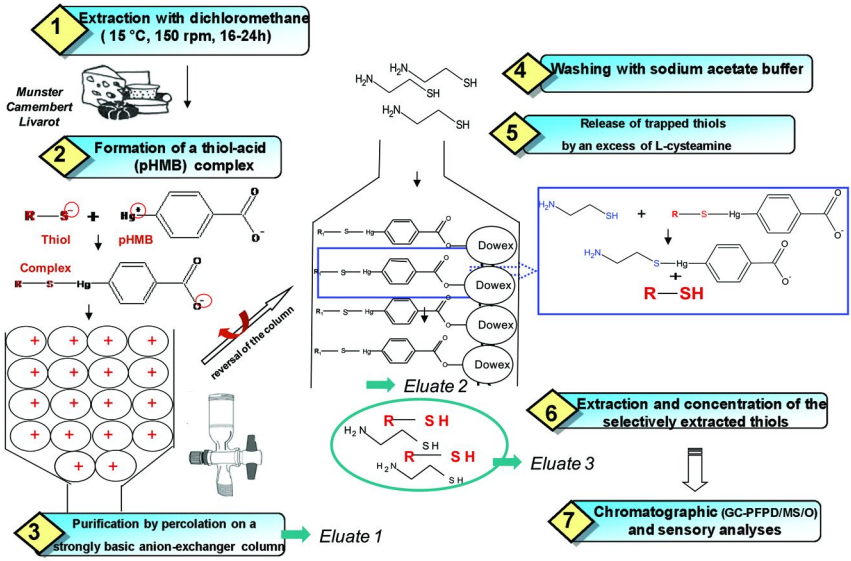

Figure 1. Protocol for thiol extraction from the cheese matrix. The cheese surface was cut into pieces before dichloromethane extraction.

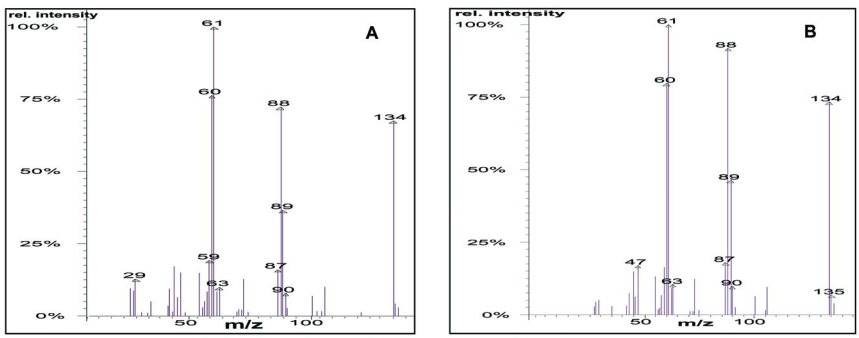

Figure 2. Mass spectra (MS/EI) of Ethyl 3-mercaptopropionate detected in a final L-cysteamine extract of Munster cheese (A) compared to that of its commercial analogue (B).

A second sulfur compound was identified as ethyl 3-mercaptopropionate. The mass spectrum of this compound (Figure 2A) closely matched that of the commercial reference (Figure 2B). The calculated linear retention index of ET3MP was also identical to that of the reference (on a DBXLB column). In addition, ET3MP identification was confirmed in SIM mode by overlapping selected ions, *m*/*z* 61, 88, and 134, at the linear retention index of the reference compound. Moreover, an additional attribute for ET3MP identification was represented by the

odor quality of this compound (assessed by Gas Chromatography-Olfactometry), which was identical to that of the commercial reference at the same concentration. Ethyl 3-mercaptopropionate was previously reported in various foods such as wine (*30*) or Concord grape (*31*). To our knowledge, however, this is the first time that it has been found in cheese.

Evidence for an Unequivocal Impact of ET3MP in Cheese Aroma

To evaluate the impact of ethyl 3-mercaptopropionate on the overall aroma of cheese, the concentration of this compound was quantified using the standard addition procedure. The concentrations for ET3MP were approximately 2 µg/kg and 4 µg/kg for a mold-ripened cheese and a smear-ripened cheese, respectively (standard deviation ± 0.2 µg/kg). Furthermore, the perception threshold of this compound was measured in fresh cream using triangle tests with ascending flavor concentrations. The group perception threshold for detection of ET3MP in fresh cream, as detected by panelists (geometric mean of the individual thresholds), was 723 ppt, evidence of its very powerful odor (Figure 3). The presence of ET3MP in cheese at concentrations higher than its perception threshold in cream suggests that this thiol may play a significant role in the aroma of these cheeses.

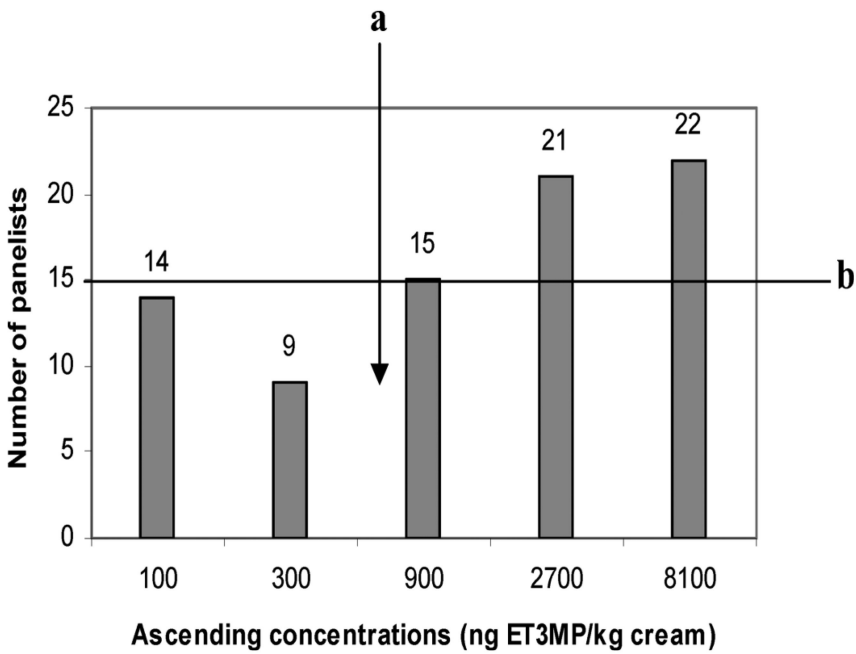

Figure 3. Number of panelists with a correct answer to triangle tests with ascending concentrations of ET3MP in fresh cream. Key: a, group BET was the geometric mean of the individual BET; b, critical number (minimum) of correct answers required for significance at the stated significance level (5%).

Hypothetical Metabolic Pathways of These New Thiols in Cheese

The metabolic routes leading to the formation of both ET2MP and ET3MP in cheese have not yet been elucidated and may be relatively complex. However, it can be assumed that they are quite similar to those described for other thiol compounds in wine and beer. ET2MP and ET3MP synthesis could therefore be related to the microbial catabolism of amino acids, mainly alanine, cysteine and methionine, which are present in caseins. Regardless of the pathway, it is likely that 2- and 3-mercaptopropionate were formed before subsequent esterification with ethanol. Alanine could first be converted by an ammonia lyase to synthesize prop-2-enoic acid (acrylate). Acrylate could then react with hydrogen sulfur (H_2S) derived from cysteine to yield either ET2MP or ET3MP in a Markovnikov-type reaction (Figure 4). Moreover, alanine could undergo a dehydrogenation catalyzed by an alanine dehydrogenase (EC 1.4.1.1) to produce pyruvate, which would finally yield 2-mercaptopropionate by a nucleophilic substitution with H_2S (Figure 4). Alternatively, pyruvate could be formed by glycolysis during cheese ripening.

Furthermore, since methionine can produce homocysteine by a methyl transfer, an additional possible route for the formation of ET3MP could be the Ehrlich degradation of this amino acid, which is known to occur in fermented beverages and cheese.

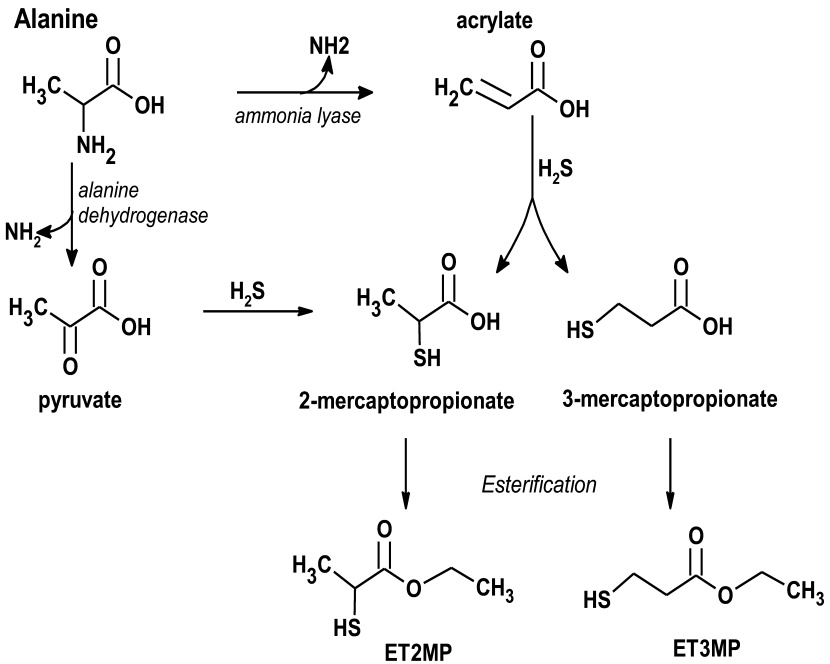

Figure 4. Hypothetical formation pathways of ethyl 2- and ethyl 3-mercaptopropionate.

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Conclusion: Could Hydrophobic Thiols Be Efficiently Extracted from the Cheese Matrix?

To conclude, the promising results presented here are a first step towards a better understanding of thiol contribution to cheese flavor.

Nevertheless, the gas-liquid partition coefficient (measured using the Phase Ratio Variation (*32*)) of ET3MP was 1.75 10^{-3} in water and four times less in cream (4.87 10^{-4}), showing that this volatile is only moderately retained in fresh cream. This result is not surprising since the molecule is slightly hydrophobic (log P = 1.40).

Consequently, identification of more hydrophobic thiols in cheese will require improvements of the extraction method. For example, the use of more apolar solvents (first step of extraction) or the use of "AFFi-Gel" (PhHgloaded Agarose gel) for purification and enrichment of volatile thiols could be tested.

Finally, the fact that ET3MP was only found in certain types of cheeses, and not in all of the tested samples strongly suggests that the microbial flora in the ripening chamber must be involved in its synthesis. Hence, differences between cheese-making environments must be taken into account when assessing cheese samples from the same variety.

Involvement of Branched Chain Amino Acid (BCAA) and Fatty Acid (FA) Catabolism in the Biosynthesis of *S*-methyl Thioesters

Requirement of an Enzymatic Step in the Synthesis of *S*-methyl Thioesters

To determine the involvement of microorganisms in the pathways leading to *S*-methyl thioesters, we performed preliminary experiments using different precursors without microbial cells: acyl-CoAs, methanethiol (MTL), dimethyldisulfide (DMDS), FAs or BCAAs. The results obtained showed that in the absence of cells, thioesters were formed only in the mixtures containing acyl-CoAs and MTL, and that their synthesis was not significantly enhanced in the presence of resting cells. Consequently, *S*-methyl thioesters are formed by a spontaneous reaction between acyl-CoAs and MTL. Considering the chemistry of thiols, it is assumed that a very reactive compound such as MTL replaces the large thiol molecule, coenzyme A, via a nucleophilic substitution reaction. In contrast, since FAs and BCAAs only generate thioesters in the presence of cells, we suspect that their activation to thioester precursors might occur via an enzymatic reaction to yield acyl-CoAs.

Consequently, we studied two species of *Brevibacteriaceae* (*B. antiquum* and *B. aurantiacum)* as major actors in cheese ripening, particularly via their ability to produce volatile sulfur compounds (VSCs). Although significant differences in thioester concentrations were observed, both species exhibited very similar thioester production patterns.

Involvement of BCAA and FA Catabolism in *S*-Methyl Thioester Synthesis

As shown in Figure 5, the addition of a specific BCAA (leucine or valine) or a specific FA (propionate, butyrate or isovalerate) led to a significant increase in the corresponding thioester: (i) *S*-methyl thioisovalerate (MTiV) for leucine or isovalerate; (ii) *S*-methyl thiopropionate (MTP) for propionate; (iii) *S*-methyl thiobutyrate (MTB) for butyrate; and (iv) *S*-methyl thiosiobutyrate (MTiB) for valine.

Utilization of labeled precursor compounds (the precursor was totally substituted with the labeled precursor for tracing experiments) coupled with GS-MS for thioester analyses enabled us to confirm the strong connection between the precursor added and the formation of the corresponding thioesters.

Concerning the addition of a specific FA, labeled propionate, butyrate or isovalerate generated the corresponding labeled *S*-methyl thioester. The labeling efficiency (calculated according to the method of Arfi *et al.* (*33*)) was always higher than 85%, regardless of the thioester. The small proportion of unlabeled compounds may have been derived from intracellular pools of FAs, amino acids or sugars, as suggested by the controls during thioester biosynthesis assays. Depending on the structure of the labeled precursor (Table III), different types of labeling were observed by GC-MS.

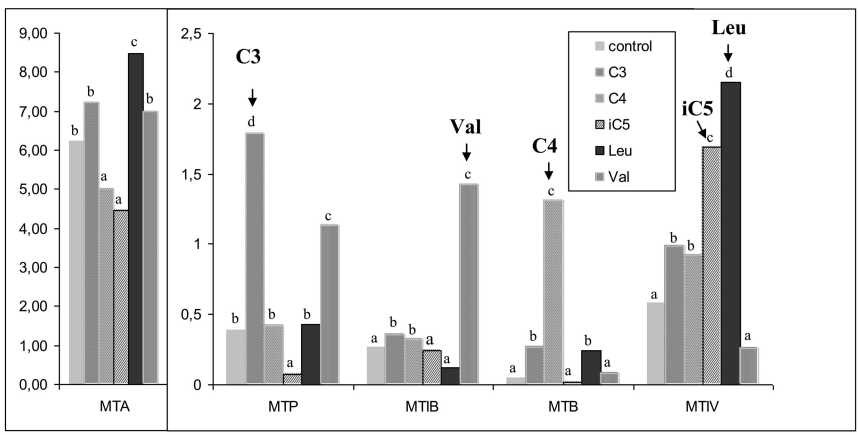

Figure 5. Production of S-methyl thioesters (in mMole) from propionate (C3), butyrate (C4), isovalerate (iC5), L-leucine (Leu), L-valine (Val) and without the addition of precursors (control: cells in plain buffer) by whole cells of B. antiquum incubated for 5 h with methanethiol. MTA (S-methyl thioacetate); MTP (S-methyl thiopropionate); MTB (S-methyl thiobutyrate); MTiB (S-methyl thioisobutyrate); MTiV (S-methyl thioisovalerate). Distinct letters (e.g., a, b, c and d) were assigned to significantly different groups (one-way analysis of variance and Newman-Keuls ($P < 0.05$) tests).

For example, the mass spectra of labeled MTiV produced from [1-^{13}C] isovalerate showed a molecular ion at *m/z*=133 (Figure 6-B) that corresponded to a mass increase of M+1 compared to the unlabeled molecule (molecular ion

at *m/z*= 132) (Figure 6-A). Since the labeled precursor had one ^{13}C in the first position, the labeled fragment in MTiV may have been derived from this part of the [1-^{13}C] isovalerate. This M+1 increase was also observed for labeled MTB while a 2-mass unit increase was observed for labeled MTP, corresponding to the two deuterium atoms of its labeled precursor, [D2] propionate.

Table III. Structures of labeled compounds used in the study of thioester biosynthesis

Labeled molecule	*Structure*
[D2] propionate	CH_3CD_2COOH
[1-^{13}C] butyrate	$CH_3CH_2CH_2{}^{13}COOH$
[1-^{13}C] isovalerate	$(CH_3)_2CHCH_2{}^{13}COOH$
[D10] L-leucine	$(CD_3)_2CDCD_2CDNH_2COOH$
[D8] L-valine	$(CD_3)_2CDCDNH_2COOH$

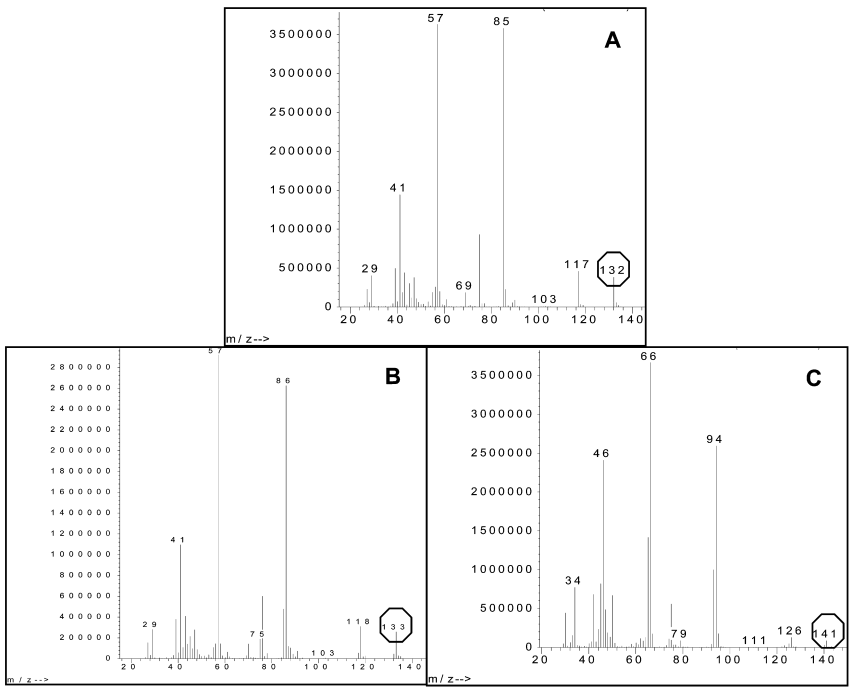

Figure 6. GC-MS spectra of unlabeled S-methyl thioisovalerate (MTiV) (A) and labeled MTiV derived from [1-^{13}C] isovalerate (B) and [D10] L-leucine (C). The labeled MTiV molecules exhibit a molecular mass increase of m+1 and m+9, respectively, compared to the unlabeled molecule (A; molecular mass at m/z=132).

In light of these results, it is possible to suppose that when added to the mixtures, label-free FAs are activated by an acyl-CoA synthase to form acyl-CoAs before reacting with MTL to synthesize thioesters. The mechanism of this reaction using ATP and free CoA could be schematized as follows:

$$RCOOH + ATP + CoA\text{-}SH \rightarrow RCO\text{-}S\text{-}CoA + AMP + PPi$$

The trans-acylation reaction is known to be the first step in FA catabolism. This reaction is catalyzed by acyl-CoA synthases. These enzymes are members of the so-called acyl-adenylate/thioester-forming family.

Concerning thioesters derived from BCAAs, spiking of labeled L-leucine and L-valine led to the generation of the corresponding labeled thioesters (MTiV and MTiB for L-leucine and L-valine, respectively), with a labeling efficiency close to 90%. The mass spectra of labeled MTiV (Figure 6-C) and MTiB (data not shown) showed molecular ion mass increases of M+9 and M+7, respectively, compared to the unlabeled molecules (molecular ions at *m/z*=132 and *m/z*=118, respectively). The analysis of the mass spectra of labeled compounds indicated that they could be derived from labeled isovaleryl-CoA and isobutyryl-CoA, respectively. The type of labeling observed with MTiV perfectly matched the following series of reactions: labeled [D10] L-leucine is assumed to lose one deuterium atom when it is transaminated to α-ketoisocaproic acid (KIC); labeled KIC is subsequently oxidatively decarboxylated to isovaleryl-CoA before yielding MTiV with an M+9 increase, compared to the unlabeled molecule. This observation supports the hypothesis that the studied strains are able to perform transamination of amino acids to α-keto acids.

Consequently, we can hypothesize that the conversion of L-leucine and L-valine to *S*-methyl thioesters involved enzymatic steps and was initiated by aminotransferases (ATases). Once they were formed, the α-keto acids were subsequently converted to acyl-CoAs by a KADHase before reacting with MTL to generate thioesters. Hence, the strains studied exhibit ATase and KADHase activities, as shown by our enzymatic assays (data not shown). The drastic reduction in the synthesis of thioesters caused by the addition of sodium arsenite, a known inhibitor of the α-keto acid/pyruvate dehydrogenase complex, reinforces this view. The branched-chain α-keto acid dehydrogenase is a multienzyme complex that catalyzes the oxidative decarboxylation of branched-chain keto acids with the formation of branched-chain acyl-CoAs and the reduction of NAD^+ to NADH. The KADHase complex, which exhibits a broad specificity for BCAAs, was already reported in *Bacillus subtilis* (*34*) and *Oenococcus oeni* (*35*). It may convert the corresponding keto acids formed by BCAA catabolism to acyl-CoAs. Moreover, this complex was previously shown to be responsible for the production of isovaleryl-CoA from L-leucine in *S. cerevisiae* (*36*).

Since the concentrations of MTiV and MTiB were significantly enhanced ($P < 0.05$) in both strains with L-leucine and L-valine utilization, these observations confirm that the strains were capable of producing KIC and KIV before activating them to isovaleryl-CoA and isobutyryl-CoA, which react with MTL to synthesize thioesters. Taken together, these findings and the results of labeling experiments

with [D10] L-leucine allow us to propose the hypothetical pathway for L-leucine presented in Figure 7.

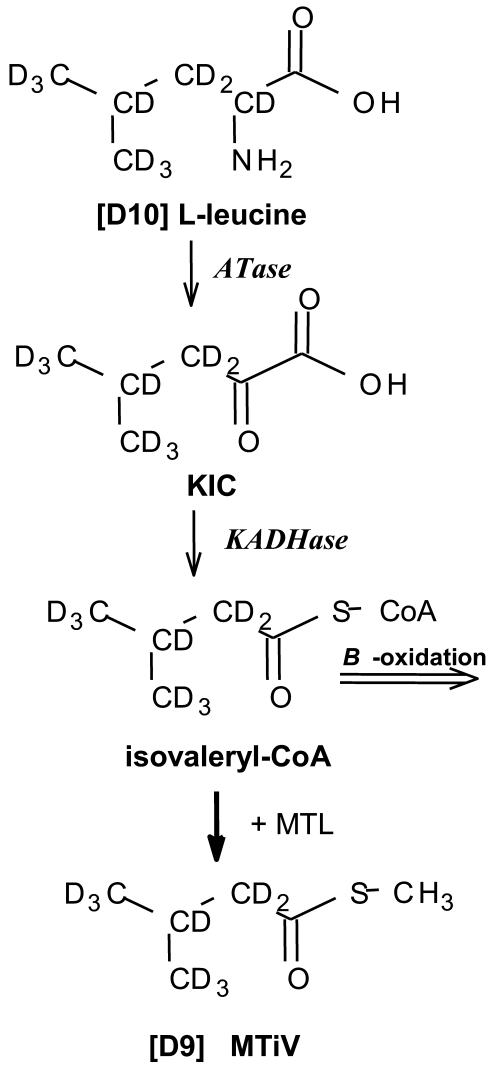

Figure 7. Hypothetical pathways of S-methyl thioisovalerate (MTiV) formation from [D10] L-leucine using whole cells of Brevibacterium strains.

Is the Great Variety of Thioesters Due to the Combination of FA and BCAA Catabolism and a Concomitant β-Oxydation of acyl-CoAs?

As shown in Figure 5, the addition of a specific FA or BCAA significantly increases the production of unexpected thioesters. We therefore investigated the formation of these unexpected compounds (e.g., MTA, MTP or MTB).

First, these thioesters were also labeled following supplementation with labeled precursors, showing the link between these volatiles and putative precursors. Three types of labeled MTAs exhibiting molecular mass increases of m+1, m+2 and m+3 compared to the unlabeled molecule were identified in reaction mixtures with [D10] L-leucine. The overall labeling efficiency for the three types of labeled MTA molecules was greater than 45%. Among them, the MTA molecule exhibiting a mass increase of m+3 represents two-thirds of the labeled forms of this thioester.

Regarding L-valine catabolism, a [D7] MTiB (labeling efficiency of 90%) and a [D3] MTP (labeling efficiency of 15%) were identified in the samples spiked with [D8] L-valine. This observation provides evidence that L-valine could sequentially be converted into α-ketoisovaleric acid (KIV) and isobutyryl-CoA ,which either generates MTiB or is alternatively degraded to yield propionyl-CoA, the precursor of MTP.

FA and BCAA degradations have a common step, which is to generate acyl-CoAs, leading to the corresponding thioesters (e.g., MTiV with isovalerate or L-leucine). Indeed, in addition to reacting with MTL, acyl-CoAs are also substrates for β-oxidation (Figure 7). Consequently, they could undergo series of reactions that ultimately generate molecules of acetyl-CoA and/or propionyl-CoA. The latter compounds could, in turn, be responsible for the generation of some of the other thioesters (i.e., MTA, MTP and MTB) in reaction mixtures supplemented with a single substrate alone.

The general mechanism could proceed as follows. First acyl-CoAs are oxidized to enoyl-CoAs that are, in turn, hydrated to hydroxyacyl-CoAs. The latter compounds are subsequently oxidized in the presence of a free molecule of coenzyme A to ketoacyl-CoAs that undergo a scission catalyzed by a thiolase to release an acetyl-CoA. The remaining acyl-CoAs (shorter by two carbon units) undergo additional cycles of β-oxidation until they are completely transformed to acetyl-CoA, the precursor of MTA.

In the case of L-leucine, this molecule is first converted to KIC, which is further activated to yield isovaleryl-CoA, a substrate of the β-oxidation enzymes. These enzymes e.g., acyl-CoA dehydrogenase, enoyl-CoA carboxylase and hydratase, convert isovaleryl-CoA to generate 3-hydroxy-3-methylglutaconyl-CoA (HMG). HMG is subsequently cleaved by the action of an unidentified lyase either into acetyl-CoA or acetoacetate. Acetoacetate is then activated by an acetyl-CoA C-acetyltransferase to acetoacetyl-CoA that, in turn, undergoes the action of an unidentified lyase to generate two molecules of acetyl-CoAs with different types of labeling. These compounds finally yield two labeled MTA molecules with mass increases of *m*+2 and *m*+3 compared to the unlabeled molecule. Furthermore, due to the ketoenolic equilibrium, acetoacetyl-CoA derived from L-leucine degradation could undergo

dehydration to form butanoyl-CoA that subsequently reacts with MTL to form MTB. The identification of a labeled MTB molecule with a mass of *m+4* supports this hypothesis. Examination of the genome sequence of *B. linens* ATCC 9174 showed that this organism has all the genes required to produce and degrade FAs as previously reported (*22*).

Moreover, the proposed L-leucine degradation pathway could be expanded to L-valine based on the same genomic and genetic data. Thus, L-valine could first be converted to KIV by an ATase and then to isobutyryl-CoA, which subsequently generates MTiB. Isobutyryl-CoA could, in turn, yield 3-hydroxyisobutyryl-CoA via the action of two enzymes of β-oxidation including the previously reported butanoyl-CoA dehydrogenase (EC 1.3.99.2). After the release of coenzyme A, it is assumed that 3-hydroxyisobutyrate is converted into methylmalonate semialdehyde, which finally yields propionyl-CoA and carbon dioxide.

All these hypothetical pathways are under investigation in our laboratory using techniques that allow the identification of non-volatile intermediary compounds such as acyl-CoAs.

Towards the Improvement of the Aromatic Quality of Cheeses

This study raises the question of the physiological role of *S*-methyl thioester synthesis. As far as we know, the physiological role of thioester synthesis in *Brevibacterium* is unknown. Their role can be compared to that of esters in beer and sake yeasts. It has been suggested that ester synthesis could be a useful way for cells to regenerate free CoA in these organisms without releasing high concentrations of free acetic and medium chain FAs that could be toxic (*37*).

Our results provide new insights into the understanding of the formation of these important volatiles that contribute to the aroma of numerous cheeses. We have demonstrated that the catabolism of FAs and BCAAs, the β-oxidation of acyl-CoAs and the catabolism of methionine have to be considered when assessing the pathways leading to the generation of VSCs. The impact of the transport phenomenon for substrates and products as well as the expression and regulation of the genes involved in FA and BCAA catabolism need to be investigated before implementing the production of cheeses with the desired types or balances of *S*-methyl thioesters.

General Conclusion and Prospects

These recent advances concerning key aromatic sulfur compounds will significantly contribute to the better control of their microbial production and to the support of high organoleptic quality of cheeses.

Cheese-making results in the combined action of an ecosystem made of yeasts and bacteria, which implies the concept of co-metabolism. The understanding and/or control of the mechanisms of VSC synthesis would therefore remain partial without taking the simultaneity and/or the succession of biochemical reactions generated by the microbial consortium into account. The functional analysis of

the ecosystem and its interaction with the cheese matrix is therefore essential for the improved control of the full ripening process.

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Chapter 7

Contribution of Volatile Sulfur Compounds to the Characteristic Aroma of Roasted Garlic

Keith Cadwallader,* David Potts, Laura Brisske-BeVier, and Samira Mirarefi

University of Illinois, Department of Food Science and Human Nutrition, 1302 W. Pennsylvania Ave., Urbana, IL 61801
***E-mail: cadwlldr@illinois.edu.**

Characteristic aroma components of roasted garlic were identified by combined sensory-instrumental techniques. Sensory descriptive analysis revealed the predominance of *raw garlic*, *pungent*, *fatty/brothy* and *caramelized/hydrolyzed protein* aroma notes in roasted garlic (177 °C for 1.5 h). Relative potency of individual odorants was determined by gas chromatography-olfactometry of decreasing static headspace samples (GCO-H) and by aroma extract dilution analysis (AEDA) of solvent extracts. Predominant odorants were mainly sulfur compounds (allyl methyl trisulfide, diallyl trisulfide, 2-vinyl-*4H*-1,3-dithiin, dimethyl trisulfide and diallyl disulfide). Additional characterizing compounds included acetaldehyde, guaiacol, *p*-vinylguaiacol, eugenol, (*Z*)-and (*E*)-isoeugenol, 4-hydroxy-2,5-dimethyl-3(*2H*)-furanone and vanillin. Based on these findings sulfur-containing compounds and thermally-derived nonsulfur-containing compounds are important contributors to the characteristic aroma of roasted garlic.

Garlic (*Allium sativum* L.) is extensively cultivated and consumed throughout the world (*1–3*). Garlic is recognized as both a medicinal and nutritious food, and as a valuable herb/spice used in everyday cooking (*2–4*).

The characteristic sulfurous odor of garlic is attributed to various sulfur-containing compounds, such as diallyl sulfide, diallyl disulfide and diallyl

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trisulfide (*3*, *5*, *6*). The major volatile sulfur compounds of cut or minced raw garlic are formed by the spontaneous degradation of allicin (ally 2-propenethiosulfinate) which is formed from alliin (S-ally cysteine sulfoxide) via the enzyme alliinase (*3*, *7*). This enzyme also coverts S-methyl cysteine sulfoxide, S-(*E*)-1-propenyl cysteine sulfoxide and γ-glutamylalk(en)yl cysteine to minor amounts of the corresponding alk(en)yl thiosulfinates (*3*). These thiosulfinates are very unstable and readily decompose or rearrange to form numerous volatile sulfur compounds, including thiols, sulfides and vinyl dithiins, among other compounds (*3*).

The method used to prepare culinary forms of garlic has a great effect on its flavor (*3*). Roasted garlic is a popular culinary form of garlic. Garlic roasted in olive oil (400 °F for 45 min) was reported to have a sweet, mellow, nutty and caramel flavor (*3*), which differs greatly from the typical intense and pungent flavor of cut or minced raw garlic. To the best of our knowledge, the aroma components of whole roasted garlic bulbs have not been reported; however, several studies have examined the effects of various types of heat processes on flavor generation in garlic (*8–15*).

Boiling (blanching) of intact garlic (bulb or clove) causes deactivation of alliinase and minimizes the formation of pungent and volatile sulfur-containing compounds (*9–16*). Garlic cloves blanched in boiling water (20 min) were sliced and then fried (180 °C for 22 min in soybean oil) or baked (180 °C for 25 min) (*9*). Less volatile compounds were detected in the blanched cloves than in blanched-fried or blanched-baked garlic. The authors suggested that volatile compounds in heated garlic should be grouped into one of four categories: (1) those generated from thermal degradation of nonvolatile flavor precursors of garlic; (2) those generated from thermal interactions of sugars and nonvolatile flavor precursors of garlic; (3) those generated from thermal interactions of lipids and nonvolatile precursors of garlic; and (4) those generated from thermal interactions of sugars, lipids, and nonvolatile flavor precursors of garlic (*9*, *14*).

In a related study, stir-fried garlic was extracted by supercritical CO_2 and the volatile components isolated by either purge-and-trap (P&T) or simultaneous distillation-solvent extraction (SDE) (*13*). Major volatiles detected by the P&T method were dimethyl sulfide, allyl alcohol, diallyl sulfide, methyl allyl disulfide and diallyl disulfide. Meanwhile, diallyl disulfide, diallyl trisulfide and dithiins were the major volatiles isolated by the SDE method. A similar SDE method was employed by *Yu and coworkers* (*8*) for the analysis of the volatile constituents of deep-oil fried, microwave heated and oven-baked garlic slices. Diallyl disulfide and diallyl trisulfide were the predominant volatile compounds in the baked or microwave heated garlic; whereas, major volatile compounds in fried, oil-cooked or microwave-fried garlic were diallyl disulfide, allyl methyl disulfide and vinyl dithiins. Numerous nitrogen-containing compounds, generated from the interactions of reducing sugars and flavor precursors of garlic, were found in both baked and oil-heated garlic samples. Subsequent studies confirmed the thermal interaction of glucose and alliin or deoxyalliin (*11*). Thiazoles were reported to contribute roasted, savory flavors in the glucose/alliin model system. Meanwhile, the combined lack of thiazoles and the presence of appreciable levels of allylthio-compounds in deoxyalliin/glucose model systems were responsible for more typical garlic/pungent flavors. Pyrazines were identified in both model systems,

even in the absence of glucose. Pyrazines were later reported as products of the interaction of inosine-5′-monophosphate and alliin or deoxyalliin (*10*).

Gas chromatography-olfactometry (GCO) combined with GC-MS has been extensively used for the identification of potent odorants in foods (*17*). A popular procedure based on GCO is aroma extract dilution analysis (AEDA), in which serial dilutions of an aroma extract are evaluated by GCO to obtain a flavor dilution (FD)-factor for each odorant in the extract (*17*, *18*). The FD factor for an odorant is equal to its concentration in the initial aroma extract divided by its concentration in the highest dilution in which it is detectable by GCO (*18*). FD factors provide estimates of relative odor potencies of odorants in the aroma extract. Another GCO method based on the dilution concept is GCO of decreasing headspace samples (GCO-H). This method compliments AEDA in that it provides odor potency estimates for highly volatile compounds, which might not be adequately recovered during the preparation of the aroma extract (*19*). In GCO-H, a decreasing series of sample headspace volumes is evaluated by GCO. Odorants detected in the lowest headspace volume have the highest FD-factors and, thus, are considered the greatest odor impact in the headspace of the food.

The objective of the present study was to characterize the aroma compounds present in roasted garlic through the combined use of sensory evaluation and instrumental-sensory analysis technques.

Experimental

Materials

Whole garlic (bulbs) and other reference food materials used in sensory evaluation were purchased from a local market (Urbana, IL) and stored at room temperature (~20 °C) until needed. Chemicals, high purity solvents and authentic flavor standards listed in Tables II and III were obtained from Sigma-Aldrich (St. Louis, MO), except for no. **8** which was synthesized using a previously published procedure (*20*). The surface of all glassware used in this project was deactivated by treatment with Sylon CT™ (dimethyl-dichlorosilane) (Supelco, Bellefonte, PA). After deactivation, the glassware was rinsed successively with toluene, methanol and then hot water. Glassware was baked at 190 °C for 24 h prior to use.

Preparation of Blanched and Roasted Garlic

Garlic bulbs were tightly wrapped in aluminum foil and baked at 350 °F (177 °C) for 0.5 h (blanched) or 1.5 h (roasted). The heads were removed from the oven and allowed to cool for 1 h at room temperature. Each clove was squeezed by hand to produce a puree, which was either used immediately for analysis or stored at -60 °C.

Sensory Aroma Profiling

Aroma profiles were determined by descriptive sensory analysis using a published procedure (*21*). The panel was composed of university students and

staff (4 males and 8 females), between the ages 21 and 50. Panelists were trained for 9-10 h to identify and define descriptive terms for blanched and roasted garlic aromas and to determine appropriate aroma references. Samples consisted of garlic puree (1 g) in 125-mL Nalgene Teflon PTFE wash bottles (Nalge Company, Rochester, NY). Bottles were labeled with random 3-digit codes and were covered with aluminum foil to prevent any visual bias. Samples were presented at room temperature (~23 °C). Panelists evaluated each sample by gently squeezing the bottle and sniffing the air released from the nozzle. Reference materials (standards) for "pungent", "fatty/brothy", "caramelized/hydrolyzed protein" and "raw garlic" were presented along with the samples at room temperature (Table I). Aroma intensities were marked on 15-cm universal scales anchored on the left with "none" and on the right with"extreme," which corresponded to intensity ratings of 0 and 15, respectively (*22*). The assigned intensity ratings of the standards were used as references for rating the intensities of the garlic samples. Individual rating results were revealed at the end of each sensory analysis session, and final aroma profiles of the samples were reported on the basis of discussion and consensus ratings by the panel.

Gas Chromatography-Olfactometry of Decreasing Static Headspace Samples (GCO-H)

Roasted garlic puree (5 g) was placed in a 50-mL vial which was sealed with a PTFE-faced septum. The flask was incubated at 35 °C for 25 minutes and then a headspace volume (5, 1, 0.2 or 0.04 mL) was withdrawn by means of a heated (45 °C) gastight syringe and then injected into a CIS-4 cooled injection system (Gerstel) operating in the solvent vent mode [vent pressure, 6 psi; vent flow, 10.0 mL/min for 0.1; splitless time, 1.1 min; initial temperature -120°C (0.1); ramp rate, 12°C/s; final temperature, 240°C (3 min hold)]. A fresh sample was used for each headspace volume.

Table I. Sensory Descriptive Terms and References

Term	*Description*	*reference material*
pungent	aroma associated with raw onion	2.6 g of raw onion (minced)
fatty/brothy	aroma associated with chicken broth	1 g of Swanson's chicken broth
caramelized/ hydrolyzed protein	aroma associated with soy sauce	2 g of LaChoy lite soy sauce
raw garlic	aroma associated with raw garlic	1 g of chopped raw garlic

GCO-H was conducted Agilent 6890 GC (Agilent Technologies, Inc.; Palo Alto, CA) equipped with an flame ionization detector (FID) and olfactory detector port (ODP2, Gerstel), and an RTX-Wax or RTX-5 column (15 m x 0.54 mm; 1.0 μm film thickness; Restek, Bellefonte, PA). Column effluent was split between FID and olfactory detection port using deactivated fused silica tubing (1 m x 0.25 mm i.d.; Restek). FID and olfactory transfer line temperatures were maintained at 250 °C. The GC oven temperature was programmed from 35 to 225 °C at a rate of 10 °C/min (RTX-Wax) or 6 °C/min (RTX-5) with initial and final hold times of 5 and 30 min, respectively. Helium was used as the carrier gas at a constant flow rate of 5 mL/min. Other details have been previously described (*21*).

Preparation of Aroma Extracts

Direct Solvent Extraction (DSE)

Fifty grams of roasted garlic puree was mixed well with 50 g of NaCl and 75 mL of odor-free water in a 250-ml PTFE screw-capped bottle (Nalge Company). Diethyl ether (50 mL) was added and the bottle was shaken (20 min at 200 rpm) on an orbital shaker (VWR Scientific Product, West Chester, PA) and then centrifuged (3000xg for 4 minutes on an IEC HC-SII centrifuge; Damon/IEC Division, Ramsey, MN) and the ether layer recovered. The above extraction procedure was repeated two more times using 40 and 30 ml of ether for the second and third extractions, respectively. The pooled solvent extract was frozen overnight at -60 °C to remove bulk water as ice crystals.

Solvent-Assisted Flavor Evaporation (SAFE)/Fractionation

SAFE was conducted on the above solvent extract in order to isolate the volatile constituents from nonvolatile components present in the extract. This was an important step since on-column injection was used for GC analysis. Solvent extracts were subjected to SAFE and fractionated into neutral (N), basic (B) and acidic (A) components as earlier described (*23*). Final fractions were dried over 1 g of anhydrous sodium sulfate and then further concentrated under a gentle stream of nitrogen to 1 mL. Extracts were stored in 2-mL glass vials at -60 °C until analysis.

Aroma Extract Dilution Analysis

Each aroma extract fraction was stepwise diluted (1:3) with diethyl ether according to the aroma extract dilution analysis technique (*18*). Dilutions were stored at -60 °C prior to analysis.

Gas Chromatography-Olfactometry (GCO)

The GCO system consisted of a 6890 GC (Agilent Technologies Inc.) equipped with an FID, an on-column injector and an olfactory detection port

(DATU Technology Transfer, Geneva, NY). Each extract was injected by cool on-column mode (+3 °C oven tracking mode) into a polar (RTX-Wax) or nonpolar (RTX-5SILMS) column (15 m x 0.32 mm; 0.5 µm film thickness; Restek). Column effluent was split between FID and olfactory detection port using deactivated fused silica tubing (1 m x 0.25 mm i.d.; Restek). FID and olfactory block temperatures were maintained at 250 °C. The GC oven temperature was programmed from 35 to 225 °C at a rate of 10 °C/min (Stabilwax) or 6 °C/min (Rtx-5MS) with initial and final hold times of 5 and 30 min, respectively. Helium was used as the carrier gas at a constant flow rate of 2.2 mL/min. Other details have been earlier described (*21*).

Compound Identification

Gas Chromatography-Mass Spectrometry (GC-MS)

Each aroma extract (1 µL) was injected by cool on-column mode (+3 °C temperature tracking mode) into a 6890 GC/5973N MSD (Agilent Technologies Inc.). Separations were performed using either a polar (Stabilwax; Restek) or non-polar (SAC-5; Supelco) column (30 m x 0.25 mm i.d.; 0.5 µm film). The oven temperature was programmed from 35 °C to 225 °C at a rate of 4 °C/min with initial and final hold times of 5 and 30 min, respectively. Helium was used as carrier gas at a constant rate of 1.0 mL/min. The MSD conditions were as follows: capillary direct interface temperature, 280 °C; ionization energy, 70 eV; mass range, 35 to 300 amu; electron multiplier voltage (Autotune + 200 V); scan rate, 5.27 scans/s.

Positive identifications of aroma compounds were made by matching GC retention indices (RI, determined using *n*-alkanes analyzed on both polar and non-polar columns), mass spectra and aroma properties of unknowns with those of authentic reference standards analyzed under identical conditions. Tentative identifications were based on MS library data (NIST 08; Agilent Technologies, Inc.) or based on matching RI values with those of authentic reference compounds or published literature.

Results and Discussion

Sensory Aroma Profiles of Blanched and Roasted Garlic

Four main aroma characteristics (plus overall aroma intensity) were rated by a sensory descriptive analysis panel. Overall, *pungent* and *raw garlic* aroma intensities were notably lower in roasted garlic than in blanched garlic (Figure 1). The decreases in *pungent* and *garlic* notes were accompanied by increases in caramelized and *fatty/brothy* aroma notes. Thus, the characteristic sensory aroma profile of roasted garlic is characterized by suppressed *pungent, garlic* and *sulfurous* notes and enhanced *caramel* and *savory* notes.

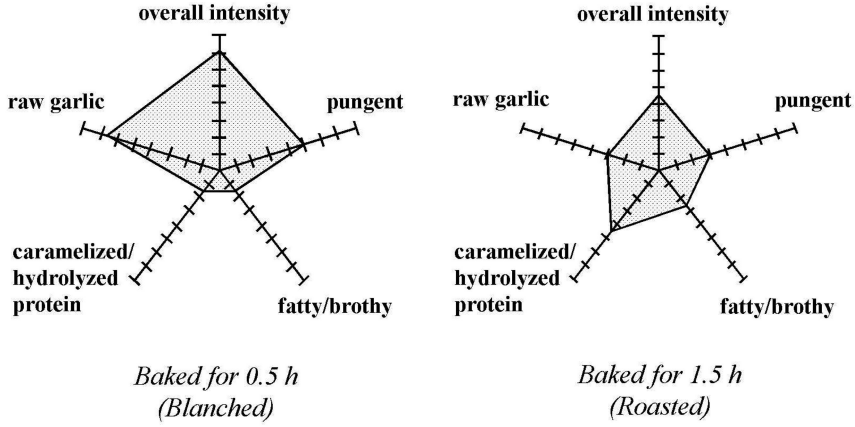

Figure 1. Sensory aroma profile comparison of blanched and roasted garlic.

Predominant Odorants in Roasted Garlic

In the present study two complimentary GCO dilution methods were used to indicate potent odorants of widely varying volatilities. The GCO-H method was suitable for the identification of the potent headspace odorants of roasted garlic.

Typically, these are highly volatile compounds, but sometimes less volatile compounds which have sufficiently high odor-activity are also detectable by this technique. In addition to GCO-H, aroma extract dilution analysis (AEDA) was employed to assess the potent odorants of intermediate and low volatility.

Potent Headspace Odorants

A total of 14 odorants were detected by GCO-H in the static headspace of roasted garlic (Table II). Among these, acetaldehyde (no. **1**; *pungent, sweet, yogurt*), allyl methyl trisulfide (no. **12**; *sulfurous, garlic*) and diallyl trisulfide (no. **13**; *sulfurous, heated garlic*) had the highest FD-factors on both GC stationary phases used for GCO-H. Most of the odorants detected by GCO-H had *sulfurous, garlic-like* odor characteristic (e.g., nos. **2**-**5**, **7**, **9**, **12**-**14**, **21** and **23**). In addition to compound no. **1**, two other non-sulfur containing compounds were detected which had *smoky* (no. **15;** guaiacol) and *smoky/clove-like* (no. **19**; (*E*)-isoeugenol) aroma notes. One limitation of GCO is caused by selective and often strong solute-stationary phase interactions (*17*), which can make it difficult to detect some compounds on certain GC stationary phases. This phenomenon explains why some compounds were detected on only one of the two columns. For example, compounds **5**, **15**, **19** were only detected on the polar (WAX) column; whereas, compounds **21** and **23** were only detected on the nonpolar column. The above two tetrasulfides (nos. **21** and **23**) also were not detected by AEDA on the polar stationary phase (Table III).

Table II. Odorants Detected by GCO of Decreasing Headspace Volumes of Roasted Garlic

no. [a]	*compound*	*odor description* [b]	*retention index* [c]		*FD-factor* [d]	
			WAX	*RTX5*	*WAX*	*RTX5*
1	acetaldehyde [e]	pungent, sweet, yogurt	600	<500	125	125
2	dimethyl sulfide [e]	sulfurous, fresh corn	717	522	1	1
3	allyl mercaptan [e]	sulfurous, meaty, garlic	887	635	1	5
4	allyl methyl sulfide [f]	sulfurous, meaty, garlic	952	691	1	5
5	diallyl sulfide [e]	sulfurous, garlic	1185	- - [g]	25	n.d. [h]
7	allyl methyl disulfide [f]	sulfurous, garlic	1284	894	1	1
9	dimethyl trisulfide [e]	sulfurous, cabbage	1374	972	25	5
12	allyl methyl trisulfide [f]	sulfurous, garlic	1591	1140	125	25
13	diallyl trisulfide [f]	sulfurous, heated garlic	1782	1306	125	25
14	2-vinyl-*4H*-1,3-dithiin [f]	sulfurous, pungent, garlic	1839	1218	5	5
15	guaiacol [e]	smoky	1849	- -	125	n.d.
19	(*E*)-isoeugenol [e]	smoky, cloves	2245	- -	25	n.d.
21	allyl methyl tetrasulfide [f]	sulfurous, garlic	- -	1390	n.d.	5
23	diallyl tetrasulfide [f]	sulfurous, cabbage, garlic	- -	1556	n.d.	5

[a] Numbers correspond to those in Table III and Figure 2. [b] Odor quality as perceived during GCO. [c] Retention index calculated from GCO data. [d] Flavor dilution (FD) factor = highest headspace volume tested (10 mL) divided by the lowest headspace volume required for detection of a compound (i.e., 5, 1, 0.2 or 0.04 mL). [e] Compound positively identified. [f] Compound tentatively identified. [g] - - = not available. [h] n.d. = not detected.

It is well known that the volatile sulfur components of garlic are prone to degradation and/or rearrangements during extraction and GC analysis (*6*, *24*). In the present study, components of intermediate and low volatility were carefully isolated from roasted garlic by direct solvent extraction followed by a mild high vacuum distillation cleanup step (SAFE) to remove nonvolatile material from the extracts. To further reduce artifact formation, aroma extracts were analyzed by cool on-column injection-GCO.

Nineteen odorants with FD factors ≥ 3 (on either polar or nonpolar GC columns) were detected by GCO and AEDA (Table III). Results of AEDA were in good general agreement with the sensory evaluation profile and the GCO-H results for roasted garlic. Ten of the 19 compounds detected by AEDA were also detected by GCO-H. The major odorants identified by AEDA included those with *sulfurous* notes (nos. **3**, **4**, **6**, **9**, **11**-**14**, **21** and **23**), *roasted* notes (nos. **8** and **10**), *smoky, clove-like* notes (nos. **15**, **17**-**20**), and *burnt sugar* and *vanilla* notes (nos. **16** and **22,** respectively).

Based on the results of AEDA, the sulfur-containing compounds with *sulfurous, garlic-like* aroma notes were the predominant odorants of roasted garlic. Among these, allyl methyl trisulfide (no. **12**), diallyl trisulfide (no. **13**) and 2-vinyl-*4H*-1,3-dithiin (no. **14**, *sulfurous, pungent, garlic*) had the highest FD-factors (= 2187). Compounds nos. **12** and **13** were also indicated as potent headspace odorants by GCO-H (Table II); however, compound no. **14** was detectable at low FD-factors by GCO-H. This is in agreement with a previous study which showed that vinyldithiins were poorly isolated by headspace (purge and trap) analysis (*13*). Additional sulfur-containing odorants with moderately high FD-factors (≥ 27) included allyl mercaptan (no. **3**, *sulfurous, meaty, garlic*), allyl methyl sulfide (no. 4; *sulfurous, meaty, garlic*), allyl (*E*)-1-propenyl sulfide (no. **6**; *sulfurous, meaty, garlic*), dimethyl trisulfide (no. **9**; *sulfurous, cabbage*), allyl methyl tetrasulfide (no. **21**) and diallyl tetrasulfide (no. **23**; *sulfurous, cabbage, garlic*). With the exception of nos. **6** and **11**, all of the above-mentioned sulfur compounds were also detected by GCO-H.

Nine non-sulfur containing compounds were also detected by AEDA. Six phenolic compounds with smoky and clove-like aroma notes had high FD factors (≥ 243). These included guaiacol (no. **15**), eugenol (no. **17**; *smoky, cloves*), (*Z*)- and (*E*)-isoeugenol (nos. **19** and **20**, respectively) and vanillin (no. **22**; *vanilla*). Among these, nos. **15** and **19** were also detected by GCO-H. The remaining three odorants in this group were only detected by AEDA. These included 4-hydroxy-2,5-dimethyl-3(*2H*)-furanone (HDMF; no. **16**; *burnt sugar*) with an FD-factor of 81; and 2-acetyl-1-pyrroline (no. **8**; *popcorn, roasty*) and 3-ethyl-2,5-dimethylpyrazine (no. **10**; *potato, earthy*), both with low FD-factors of 9.

Table III. Potent Odorants Detected by Aroma Extract Dilution Analysis of Roasted Garlic

no. [a]	*compound*	*Odor description* [b]	*Fr* [c]	*RI* [d]		*FD-factor* [e]	
				WAX	*RTX5*	*WAX*	*RTX5*
3	allyl mercaptan [f]	sulfurous, meaty, garlic	N	870	636	27	9
4	allyl methyl sulfide [f]	sulfurous, meaty, garlic	N	- - [h]	691	n.d.	81
6	allyl (*E*)-1-propenyl sulfide [g]	sulfurous, meaty, garlic	N	1198	892	27	<3
8	2-acetyl-1-pyrroline [f]	popcorn, roasty	B	1335	925	9	<3
9	dimethyl trisulfide [f]	sulfurous, cabbage	N	1379	970	27	27
10	3-ethyl-2,5-dimethylpyrazine [f]	potato, earthy	B	1434	1055	9	<3
11	diallyl disulfide [f]	garlic, meaty	N	1484	1082	9	9
12	allyl methyl trisulfide [g]	sulfurous, garlic	N	1588	1140	2187	729
13	diallyl trisulfide [g]	sulfurous, heated garlic	N	1787	1305	2187	729
14	2-vinyl-*4H*-1,3-dithiin [g]	sulfurous, pungent, garlic	N	1837	1215	2187	2187
15	guaiacol [f]	smoky	A	1855	1091	729	<3
16	4-hydroxy-2,5-dimethyl- 3(*2H*)-furanone [f]	burnt sugar	A	2015	1056	81	<3
17	eugenol [f]	smoky, cloves	A	2156	1362	243	9
18	*p*-vinyl guaiacol [f]	smoky, cloves	A	2182	1317	243	<3
19	(*Z*)-isoeugenol [f]	smoky, cloves	A	2245	1415	729	<3.
20	(*E*)-isoeugenol [f]	smoky, cloves	A	2332	1456	729	<3
21	allyl methyl tetrasulfide [g]	sulfurous, garlic	N	- -	1388	n.d. [i]	81

no. [a]	*compound*	*Odor description* [b]	*Fr* [c]	*RI* [d]		*FD-factor* [e]	
				WAX	*RTX5*	*WAX*	*RTX5*
22	vanillin [f]	vanilla	A	2536	1402	729	3
23	diallyl tetrasulfide [g]	sulfurous, cabbage, garlic	N	- -	1549	n.d.	27

[a] Numbers correspond to those in Table II and Figure 2 [b] Odor quality as perceived during GCO. [c] Fr., fraction in which odorant was detected [d] Retention index calculated from GCO data. [e] Flavor dilution (FD) factor. [f] Compound positively identified. [g] Compound tentatively identified. [h] - - = not available. [i] n.d. = not detected.

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Structures for 16 potent odorants identified in roasted garlic by GCO-H and AEDA are shown in Figure 2. The present findings indicate that potent odorants in roasted garlic can be subdivided into three general categories based on their possible origins.

Sulfur Compounds

Volatile sulfur compounds make the greatest contribution to the characteristic aroma of roasted garlic. In particular, allyl methyl trisulfide (no. **12**), dially trisulfide (**13**) and 2-vinyl-*4H*-1,3-dithiin (no. **14**) seem to make the greatest overall contribution to roasted garlic aroma. These compounds have been previously identified in heated garlic. For example, the major volatile components of heated garlic oil were identified as diallyl trisulfide, diallyl disulfide, methyl allyl trisulfide, methyl allyl disulfide, diallyl sulfide, allyl methyl sulfide, dimethyl sulfide, 2-vinyl-*4H*-1,3-dithiin and 3-vinyl-*4H*-1,2-dithiin (*25*). Similarly, diallyl disulfide, diallyl trisulfide, diallyl sulfide, diallyl trisulfide, methyl allyl trisulfide, 2-vinyl-*4H*-1,30dithiin and 3-vinyl-*4H*-1,2-dithiin were the predominant volatile sulfur components of oven-baked garlic slices (*8*). In the present study diallyl tetrasulfide (no. **23**) was the highest molecular weight diallyl polysulfide detected by GCO and GC-MS. The diallyl sulfides and polysulfides can be formed from thermal decomposition of allicin (*3*) or by alliinase action on a mixture of alliin and cystine (*26*). The former reaction is the most likely source of diallyl sulfides in roasted garlic. Diallyl polysulfides containing more than four sulfur atoms have been identified in heated garlic (*3*), but these compounds do to impact the aroma since they are essentially nonvolatile compounds and are present in relatively low abundance.

2-Vinyl-*4H*-1,3-dithiin (no. **14**) has been shown to be a Diels-Alder dimer of thioacrolein (*27*, *28*). The roasting conditions used in the present study are adequate to form sufficient amounts of thioacrolein from the abundant diallyl disulfide (*29*). It has been postulated that thermal degradation of dithiins could lead to the formation of additional volatile compounds, but these reactions are not well-characterized (*24*).

Thermally-Derived, Non-Sulfur Containing Compounds

The Maillard reaction is an important source of some potent odorants in roasted garlic. These include 2-acetyl-1-pyrroline (no. **8**) and 3-ethyl-2,5-dimethyl-pyrazine (no. **10**) and 4-hydroxy-2,5-dimethyl-3(*2H*)-furanone (HDMF; no. **16**). Among these three compounds, only no. **10** has been previously reported in heat processed garlic or as a degradation product of nonvolatile garlic flavor precursors (*11*). Nitrogen-containing volatile compounds, and in particular pyrazines, have been previously identified in heated garlic (*8*, *9*). Yu and coworkers postulated that the volatile nitrogen-containing compounds

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could be generated from thermal interactions between reducing sugars and the nonvolatile flavor precursors of garlic, such as γ-glutamylalk(en)ylcystein and alk(en)yl-cystein S-oxides (*8–10*). HDMF can be formed by degradation of sugars, either directly or in the presence of amines or amino acids (*30*). In addition to the above compounds, acetaldehyde (no. **1**) has been previously reported as a product of the thermal degradation of alliin or deoxyalliin (*12*) or formed via the interaction of glucose and alliin or deoxyalliin (*11*). Acetaldehyde has been identified as a volatile component of heat-treated garlic (*9*, *13*).

Phenolic Compounds

In addition to the above compounds, several phenolic compounds with smoky, clovy and vanilla-like aroma notes were identified as potent odorants in roasted garlic. These included guaiacol (no. **15**), *p*-vinylguaiacol (no. **18**), eugenol (no. 17), (*Z*)- and (*E*)-isoeugenol (nos. **19** and **20**, respectively) and vanillin (no. **22**). None of these compounds have been previously identified in heat-treated garlic; however, results of the present study indicate that each compound notably influences the overall aroma of roasted garlic. These compounds might originate from thermal degradation of ferulic acid during the roasting process (*31*).

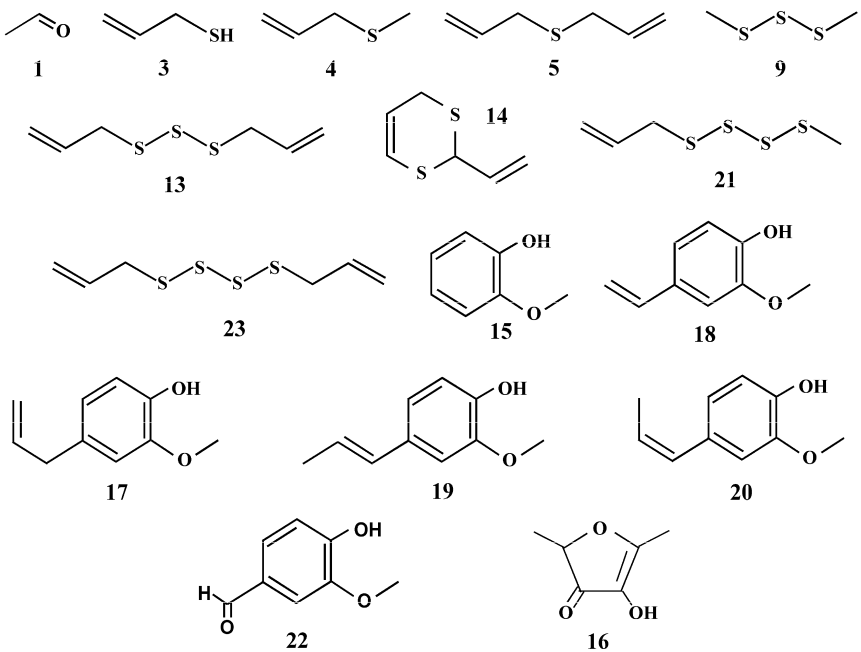

Figure 2. Structures of selected potent odorants in roasted garlic. (Numbers correspond to those in Tables II and III.)

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Conclusions

Results of this study demonstrate the complexity of the aroma profile of roasted garlic. While sulfur-containing compounds are the predominant odorants and contribute characteristic pungent and garlic-like aroma notes, numerous non-sulfur containing compounds impart important *nutty, roasted, caramelized* and *smoky, clovy, vanilla* aroma notes. Together, these potent odorants produce the characteristic aroma profile of roasted garlic.

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Chapter 8

Analysis of Volatile Sulfur Compounds in Swiss Cheese Using Selected Ion Flow Tube Mass Spectrometry (SIFT-MS)

W. James Harper,*[,1] Nurdan A. Kocaoglu-Vurma,[1] Cheryl Wick,[1] Karen Elekes,[1] and Vaughan Langford[2]

[1]The Ohio State University, Department of Food Science and Technology, Columbus, Ohio 43210, USA
[2]Syft Technologies, Christchurch, New Zealand
***E-mail: harper.9@osu.edu.**

The selected ion flow tube mass spectrometer (SIFT-MS) Syft Voice100 has the capability to identify and measure some, but not all, of the high impact sulfur compounds in Swiss cheese at ppb concentrations in the cheese headspace without sample preparation. This requires the selection of those compounds that can be identified and quantitatively measured without conflict with other compounds in the cheese sample. Careful method development is required to achieve this goal. A significant finding of this study was that the formation of propionic acid during warm room curing coincided with the formation of some of the high impact sulfur compounds in the cheese. It was observed in 30 day old cheeses from one manufacturer using the same production methods and milk supply, that dimethyl disulfide and methyl mercaptan concentrations in the cheese increased as the propionic to acetic acid ratio increased.

Introduction

Volatile composition has a very important role in flavor and quality perception of cheese. The flavor of cheese is very complex, with over 600 (*1*) volatile compounds reported. Cheese flavor results from a mixture of volatiles formed as a consequence of proteolysis, lipolysis, and lactose, lactate, and citrate metabolism by microorganisms during ripening. (*2–4*). Cheese flavor is composed of a unique

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balance of key volatile components rather than a single, unique character-impact compound. Therefore, obtaining the flavor fingerprint of the cheese is of great interest to maintain and monitor quality. Compounds with a flavor impact at very low threshold levels are of particular significance in the flavor of cheese, but are also amongst the most difficult to analyze. The sulfur compounds are in this category.

A number of analytical methods have been used to study the aroma of dairy products. Gas chromatography-mass spectrometry (GC-MS) is the standard approach for the analysis of volatile compounds in cheese. Volatile compounds that have low threshold values, in the ppb to ppt range, generally cannot be detected by conventional headspace analysis techniques without some form of extraction and/or concentration of the compounds from the cheese matrix prior to analysis. Pre-separation techniques in aroma analysis were thoroughly reviewed by Qian et al. (*5*). In order to accurately evaluate the composition of volatiles in the sample, the volatile components of the food matrix should not be lost, nor should new compounds be artificially created, during sample preparation, volatile extraction, and chromatographic or analytical separation. The various methods of sampling include static headspace (S-HS), dynamic headspace purge and trap extraction (D-HS), solid-phase micro extraction (SPME), stir bar sorptive extraction (SBSE), and solid phase dynamic extraction (SPDE) (*6*).

All sample preparation techniques have biases and can introduce artifacts. Static headspace analysis involves sampling air equilibrated above a food sample and injection into a GC-MS for separation, identification, and quantitation. This method allows for detection of the most abundant volatile compounds in the headspace. Dynamic headspace analysis uses a carrier gas in the sampling of volatiles above a food sample to purge-and-trap the volatiles for concentration prior to GC-MS analysis. Static headspace SPME provides high extraction speeds and stability and depending on the fiber of choice, has compound selectivity and low concentration capability. For many products, headspace SPME is the sample collection method of choice. Reproducibility of this method depends on sample equilibration, headspace collection time, temperature, sample size and fiber condition (*7*). SBSE has significant concentration capacity, however longer extraction times and fewer choices of polymer coating limit the use of this technique. SPDE is an inside-needle technique that is considered to be a compromise between SPME and SBSE, where a fixed volume of the headspace of the sample under investigation is concentrated by accumulation in the polymer that coats the needle wall (*6*, *8*).

The chemical analysis of flavor compounds has several limitations. The flavor compounds with high odor-impacts may not be extracted by the sample preparation technique. The character-impact compounds may be extracted with 100% recovery but are present at levels too low to be detected by GC-MS. Some chemicals have very low odor thresholds and impact the flavor with their concentrations below the GC-MS detection level (*9*). The odor active chemicals may be too volatile, or not volatile enough, to be trapped on the trapping medium used in D-HS analysis. Thermally labile chemicals might decompose in hot GC injectors. This problem has been reported for some types of sulfur compounds.

Table I. Sulfur compounds found in Swiss cheese and from *Propionibacterium freudenreichii* fermentation

Source	*Sulfur compounds*	*Reference*
Emmenthal	Methional	(*15*)
Emmenthal	Methional, dimethyl trisulfide	(*16*)
Emmenthal	Dimethyl disulfide	(*17*)
Emmenthal	Methional	(*18*)
Gruyere	Methanethiol, dimethyl sulfide, dimethyl disulfide, dimethyl trisulfide, methional	(*19*)
Swiss type	Methyl, ethyl disulfide + 8 other unnamed sulfur compounds	(*20*)
Swiss cheese	Dimethyl sulfide	(*21*)
Swiss cheese	Dimethyl disulfide, dimethyl trisulfide	(*22*)
Swiss cheese and propionibacteria	Hydrogen sulfide, methanethiol, dimethyl disulfide, dimethyl trisulfide	(*23*)
Lactobacillus helveticus fermentation	Dimethyl disulfide	(*24*)
Starter cultures	Methional, methanethiol	(*25*)

Volatile sulfur compounds (VSC) are considered essential for aroma of many food products and were found to be significant contributors to cheese flavor (*10*, *11*). Reviews that include information on the distribution of sulfur compounds in cheeses include: Landaud et al. (*10*); Molimard and Spinnler (*12*); Rattray and Fox (*13*), Sablé and Cottenceau (*14*).

Sulfur compounds, which have very low threshold values in the range of ppb to ppt concentrations, are recognized as being important to the flavor of many cheese varieties. These include hard and semi-hard cheeses such as Cheddar, Swiss, Blue, Romano, Provolone, Parmesan types, Gouda and Edam, and soft mold and smear ripened soft cheeses such as Camembert, Limburger, Brie, Trappist, Maroilles, Livarot, Pont-l'Eveque, Langres, Epoisses, and Vacherin.

Table I lists the sulfur compounds reported in Swiss type cheeses or produced by starter cultures used in cheese manufacture.

There is a lack of consistency in the number of compounds reported for Swiss cheese by the 11 different investigators cited in Table I. These differences can be related to differences in the methodologies used, especially the method of sample preparation. However, dimethyl disulfide (*7*), dimethyl trisulfide (*4*) methanethiol (*4*), and methional (*4*) are the most common sulfur compounds reported. Methanethiol, dimethyl disulfide and methional are considered to have the highest flavor impact of the sulfur compounds.

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Sulfur compounds derived from methionine and commonly associated with a wide range of both hard and soft ripened cheese include: methanethiol, dimethyl sulfide, dimethyl disulfide, dimethyl trisulfide, dimethyl tetrasulfide and methional.

In addition other sulfur compounds expected to be present especially in soft cheeses, include thioesters, thioethers, polyfunctional thiols, and thiazoles.

Formation of Volatile Sulfur Compounds – Microbial Metabolism

The volatile sulfur compounds found in cheese are primarily formed from methionine and cysteine by the action of microorganisms and their enzymes.

Methanethiol and hydrogen sulfide are considered as the primary degradation product of methionine and cysteine, respectively. These thiols are highly reactive, relatively difficult to quantify, and can be oxidized to form other volatile sulfur compounds such as sulfides and thioesters (*10*, *26*).

A recent review of bacterial volatiles by Schulz and Dickschat (*27*) indicated the presence of 30 sulfur compounds among over 300 compounds released from various bacteria. Several bacterial volatiles remain unidentified due to the lack of available reference data in current mass spectrometer (MS) libraries (*28*). The presence of several different microorganisms (bacteria, yeasts, and molds) in consortia in the cheese matrix point toward the potential formation and presence of several highly volatile, low threshold sulfur compounds.

Analytical Techniques for Volatile Sulfur Compound Analysis

The majority of the conventional extraction and/or concentration techniques are not suitable for VSC analysis, and generally only a few sulfur compounds are reported in cheese flavor studies. In Gouda-type cheeses, three sulfur compounds (dimethyl disulfide, dimethyl trisulfide and methional) were detected (in a total of 63 compounds) using simultaneous steam distillation-extraction (SDE) and GC-MS and the sulfur compounds were found to have good correlation with the flavor intensity (*29*). Using SDE-GC-MS, Poveda et al. (*30*) identified approximately 50 volatile compounds in semi-hard goat cheese; however 3-methylthiopropanal was the only sulfur compound detected. Condurso et al. (*31*) have not detected any sulfur compounds during 21 days shelf-life of fresh goat cheese using SPME-GC-MS with a divinylbenzene-carboxen-polydimethylsiloxane fiber, while they were able to identify 47 other volatile compounds. Using the same fiber, Abilleira et al. (*32*) detected two sulfur compounds (dimethyl sulfide and carbon disulfide) in farmhouse cheese (Idiazabal) made in winter and spring. Tornambé et al. (*33*) detected four sulfur compounds (3-methylthio-propanal, carbon disulfide, dimethyl disulfide, and dimethyl sulfone) in 5 month old experimental Cantal-type cheese using dynamic headspace GC-MS. Using the same method along with GC-olfactometry (GC-O), Cornu et al. (*34*) detected dimethyl disulfide and 3-methylthio-propanal in Cantal-type cheese. Odor active volatile compounds in Parmigiano-Reggiano cheese were identified by Qian and Reineccius (*35*) using GC-O/MS after solvent-assisted high vacuum distillation and fractionation.

Dimethyl trisulfide and methional were found to be odor-active (*35*). Dimethyl disulfide, dimethyl trisulfide, dimethyl tetrasulfide, tetramethyl thiourea, and benzothiazole were the sulfur compounds detected in Parmesan cheese using SDE extracts and GC-MS (*36*).

Using SPME-GC-MS with carboxen-polydimethylsiloxane fiber, Hayaloglu (*37*) detected 7 sulfur compounds (methanethiol, carbon disulfide, dimethyl sulfide, dimethyl disulfide, dimethyl trisulfide, dimethyl sulfone, and S-methyl ethanethioate) in mature Kashar cheeses. Burbank and Qian (*11*) were able to detect up to 8 sulfur compounds in Cheddar cheese, using SPME with carboxen-polydimethylsiloxane (CAR-PDMS) fiber coupled with gas chromatography-pulsed flame photometric detection (GC-PFPD). The sulfur compounds detected were carbonyl sulfide, hydrogen sulfide, methanethiol, dimethyl sulfide, dimethyl disulfide, dimethyl trisulfide, dimethyl sulfoxide, and dimethyl sulfone. Because of the different selectivity of the SPME fibers towards various sulfur compounds, a standard calibration curve is necessary for reliable quantification of each compound (*38*). One of the limitations of SPME is the difficulty in calibration due to matrix effects and analyte competition and displacement during adsorption on the fiber (*32*, *39*). In another study, Burbank and Qian (*40*) quantified hydrogen sulfide, methanethiol, dimethyl sulfide, dimethyl disulfide, and dimethyltrisulfide to follow development of volatile sulfur compounds in cheese using the SPME-GC-PFPD method.

Gkatzionis et al. (*41*) utilized solvent-extraction GC-MS, SPME GC-MS and atmospheric pressure chemical ionization-mass spectrometry (APCI-MS) for volatile profiling of Stilton cheeses. No sulfur compounds were detected using solvent extraction GC-MS whereas dimethyl disulfide and methanethiol were detected using SPME GC-MS. APCI-MS allowed for direct headspace analysis in MS, with a soft ionization resulting in protonated molecular ions. In certain cases, some compounds such as alcohols are reported to dehydrate. Ion masses give limited information for compound identification but appear to enable rapid sample profiling.

Other analytical techniques employed in the analysis of cheese flavor include neutral desorption extractive electrospray ionization mass spectrometry (*42*) and proton transfer reaction mass spectrometry (*43*).

Over the past two decades polyfunctional sulfur compounds have been reported in many foods that have both a thiol group and another functional group, such as an acid, alcohol, ketone, aldehyde or ester (*44*, *45*). Sourabié et. al. (*46*), have recently confirmed the presence of ethyl-3-mercapto propionate in Munster and Camembert cheeses.

Kleinheinz et al. (*47*) reported the possible presence of higher molecular weight poly functional thiol compounds in Cheddar cheese over 6 months of age. One compound, 4-mercapto-4-methylpentan-2-one, identified by matching retention times and Kovats retention index of aged Cheddar cheeses with that of a pure sample of this compound, was found in 10 of the 11 Cheddar cheeses. Badings (*48*) reported that catty flavor in Gouda cheese was related to 2 methyl pent-2-en-4-one. However, Vermeulen et al. (*45*) have shown that a number of compounds can cause catty flavors.

Other compounds tentatively identified by Kovats indices in a majority of the cheeses included 4-mercapto-3-methylpentan-2-one and 3-mercapto-3-methylbutanal. Polyfunctional thiols tentatively identified only one or two times included 4-mercapto-2-pentanol, 4-mercapto-3-methylpentan-2-ol, 5-methyl-4-mercaptohexan-2-one, 5-methyl-4-mercaptohexan-2-ol, and 3-mercaptooctanal. Standard samples were not available for any of the compounds, other than 4-mercapto-4-methyl-pentan-2-one, so that their identity could not be confirmed. Sourabié et al. (*46*) suggested that these compounds could have been artifacts due to the use of a strong reducing agent and the length of time the compounds were maintained in a reducing environment causing the interaction of reduced sulfides during the reaction process. This remains to be resolved.

Selected ion flow tube mass spectrometry (SIFT-MS) utilizes three soft ionizing reagent ions (H_3O^+, NO^+ and O_2^+) to permit the analysis of the volatile compounds in the headspace at concentrations of ppb in real time without the necessity of pre-concentrating the volatile compounds. The ability to identify and quantitate the compounds in the sample allows for determination of the volatile flavor compounds at or above their flavor threshold values.

SIFT-MS has been used in a number of studies on the analysis of volatile compounds including bacterial metabolites (*49*) real-time release of volatiles in cut onion, crushed garlic and ripe banana, (*50*) olive oil oxidation (*51*) effect of temperature on lipid related volatile production in tomato puree (*52*) and the formation of alkylpyrazines and other volatiles in cocoa liquor by SIFT-MS (*53*).

Since SIFT-MS can potentially analyze most of the several hundred flavor compounds in cheese, the significant challenge is how to achieve consistent and reliable results for compounds with the same molecular weight and therefore potential conflicts in compound identification and quantification.

SIFT-MS

SIFT-MS is a powerful analytical technique that uses chemical ionization reactions coupled with mass spectrometric detection to rapidly quantify targeted volatile organic compounds (VOCs). The VOCs are identified and quantified in real time from whole-gas samples based on the known rate coefficients for reaction of the chemically ionizing species (reagent ions) with the target compounds.

A schematic diagram of the analytical process used in a SIFT-MS instrument, such as the Syft Technologies Voice100™, is shown in Figure 1. Reagent ions are generated using a microwave discharge source and selected using the quadrupole mass filter in the "upstream" chamber. H_3O^+, NO^+ and O_2^+ are commonly selected because they do not react with bulk components of air. The mass selected reagent ions are then passed into the flow tube where they are reacted with sample under very well defined conditions. The products of the chemical ionization reactions, together with unreacted reagent ions, enter the downstream chamber and are filtered by a second quadrupole mass filter. A particle multiplier detects the ions at the selected mass and the count-rate is passed to the instrument computer for processing. The concentration is readily obtained in real time because it is proportional to the count of product ions divided by the count of reagent ions.

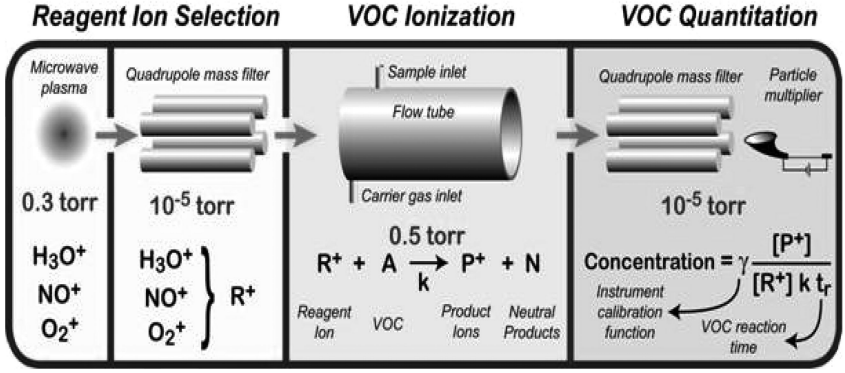

Figure 1. Schematic diagram of the analytical process used in a SIFT-MS instrument. Used with permission from Syft Technologies, Inc. Christchurch, New Zealand.

Generally the soft chemical ionization used in SIFT-MS yields a smaller range of product ions than is common in electron impact mass spectrometry, as used by GC-MS. Hence, the need for gas chromatographic separation of the sample is circumvented, speeding sample throughput and providing instantaneous quantification of VOCs. The use of several reagent ions to independently quantify target analytes also greatly reduces interferences and increases the specificity of SIFT-MS versus other whole-gas analysis technologies.

The Syft Technologies Voice100™ SIFT-MS instrument can be operated in two modes as follows:

Full mass scans: Mass scans aid identification of unknown compounds but also allow concentrations to be derived. Full mass scans were obtained using each of the three standard SIFT-MS reagent ions (H_3O^+, NO^+ and O_2^+) over the mass range 15 to 200 Daltons.

Selected ion mode (SIM): SIM targets specific compounds for sensitive quantitative analysis. Concentrations are reported in parts-per-billion by volume (ppbv).

SIM provides better limits of quantification and better precision than mass scans because it targets compounds at their specific product masses, allowing a longer counting time than with a mass scan.

Different compounds have different reaction rate coefficients which have a marked impact on calculation of concentrations and must be known for any given compound. Product ions formed through the reaction with the three reagent ions do not always occur at a single mass. However, concentrations are calculated individually, often helping to minimize conflicts. The signal at a single mass is not always the product of a single analyte and may arise from more than one compound.

In SIFT-MS five different types of reactions can occur between the analyte and the reagent ion, depending on both the reagent ion and the chemical nature of the analyte. Therefore it is necessary to characterize the product ions for each reactant that is suspected to be in the sample via mass scan. The Syft library currently has

data for more than 500 volatile organic compounds (VOCs) that provide product masses for each reagent. The most recent Syft library includes 34 sulfur containing VOCs (*54*).

Common reactions mechanisms include:
Proton transfer (H+)
$H_3O^+ + Analyte \rightarrow Analyte.H^+ + H_2O$

Charge transfer (reagent ion accepts an electron from an analyte)
$O_2^+ + Analyte \rightarrow Analyte^+ + O_2$
$NO^+ + Analyte \rightarrow Analyte^+ + NO$

Dissociative charge transfer (Charge transfer and fragments)
$O_2^+ + Analyte \rightarrow Fragment^+ + Neutral\ fragments + O_2$
Also occurs occasionally with NO^+ for compounds with low ionization energy

Association (three body collision – reagent ion, analyte, carrier gas or nitrogen or oxygen. Common for NO^+ and occasionally with H_3O^+)
$NO^+ + Analyte + M \rightarrow Analyte.NO^+ + M$
$H_3O^+ + Analyte + M \rightarrow Analyte.\ H_3O^+ + M$

Hydride extraction (an H^- ion is removed by the reagent ion)
$NO^+ + Analyte \rightarrow [Analyte\text{-}H]^+ + HNO$

This chapter concentrates primarily on the sulfur compounds and other compounds known to be important to Swiss cheese flavor. Understanding the very complex changes that occur during manufacture and ripening is essential to gaining a full understanding of how to control the quality of Swiss cheese.

Methodology

SIFT-MS Method Development

Compound Selection, Product Masses Utilized in the Method and Reaction Constants Used for Quantification

The current Syft compound library contains over 500 compounds that could be used to develop SIM methods for compound quantitation. Various SIM methods that target the quantification of the volatile compounds reported to be most important for cheese flavor were developed. The method development software (LabSyft and Voice100) (*54*) indicates available reagent channels and masses that can be used to quantify each compound. Concentrations for the compounds are calculated using known reaction rates and branching ratios for each reagent channel (H_3O^+, NO^+, O_2^+) as described by Spanel and Smith (1999)

(*50*). For each compound selected, there may be multiple m/z that can be used to quantitate the compound. When all channels used for a compound concentration calculation do not report the same concentration value, the software reports the lowest concentration calculated, because higher concentrations may include known or unknown conflicting compounds. When several masses return the same concentration within a user-determined percentage (termed tolerance) of the lowest value, the average concentration from those masses is reported. The masses that would cause potential conflicts within the method are indicated by the current software. Clearly, the presence of potential conflicting compounds is not strictly limited to the compounds in the method but rather to the compounds in the sample headspace.

Initially, a method was developed using 50 compounds considered to be important for Swiss cheese. The compounds included acids, alcohols, aldehydes, ketones, esters, lactones, pyrazines and sulfur compounds. Only the following 12 compounds were without any potential conflicts from the other compounds included in the method: butyl methyl ketone, capric aldehyde, dimethyl disulfide, dimethyl trisulfide, ethanol, ethyl mercaptan, ethyl octanoate, furaneol, hydrogen sulfide, methyl amyl ketone, and tetramethylpyrazine. Fifteen additional compounds had no conflict in at least one of the product ion channels. These are propionic acid, acetic acid, acetone, butyric acid, ethyl hexanoate, formaldehyde, lactic acid, methional, methionol, methyl heptyl ketone, 1-octen-3-one, 2-phenethyl acetate, pyrazine, and tetramethylpyrazine. The other compounds had conflicts but were included in the method because of the potential to calculate their concentration by subtraction, using concentrations of conflicting compounds calculated from non-conflicted channels and masses.

The most common cause of conflicts is related to the differences in fermentation products formed in the same type of cheese, even when it is made from the same milk supply, using the same method and the same starter organisms. This can result in the formation of a product of the same m/z for two or more different compounds present in some, but not all of the samples.

For evaluation of the sulfur compounds, a simpler method was used to include selected sulfur compounds of interest as well as propionic acid, acetic acid, isovaleric acid, butyric acid, ethanol, pyrazine, and furaneol (Table II).

The full mass scans provide data on product masses for each reagent ion. Collection of full mass scan data is crucial in proper method development as it helps identify the masses that are present in the sample headspace. Mass scans are essential in helping to resolve reaction products for a compound in the sample that is not in the method. Mass scans also can be used for differentiation of a particular characteristic of the cheese without complete knowledge of the full identification of all the compounds in the mass scan. In addition, by using multivariate analysis techniques the masses that are most influential in discrimination and classification of samples can be identified. Using the Syft library database, target compounds that are known to be associated with that specific mass can be identified allowing for further evaluation in future studies.

When evaluating mass scan data it should be taken into consideration that the detection of less abundant masses might be compromised because of the greater number of masses to be detected, within a given time frame, compared to a SIM method.

Table II. Reagents, rate coefficients, and product ions used to quantify target aroma compounds for the sulfur cheese method

Ref.	*Compound*	*Mass*	*Reagent*	*k (cm³/s)*	*m/z*	*Potential Interferences*
(*55*)	acetic acid	60	H_3O^+	2.6E-09	61, 79[a], 97[a]	
			NO^+	9.0E-10	**90**, 108[a]	
(*55*)	propionic acid	74	H^3O^+	2.7E-09	75, 93[a], 111[a]	
			NO^+	1.5E-09	57, **104**, 122[a]	methional
			O_2^+	2.2E-09	56	
(*55*)	n-butyric acid	88	H_3O^+	2.9E-09	**89, 107**, 125[a]	methionol
			NO^+	1.9E-09	118	
			O_2^+	2.1E-09	88	
(*54*)	isovaleric acid	102	H_3O^+	3.0E-09	103, 121[a], 139[a]	
			NO^+	2.5E-09	85, 132	
(*56*)	hydrogen sulfide	34	H_3O^+	1.6E-09	35	
(*56*)	methyl mercaptan	48	H_3O^+	1.8E-09	49	
			O_2^+	2.2E-09	48	
(*56*)	n-propyl mercaptan	76	NO^+	1.9E-09	76	
(*57*)	dimethyl sulfide	62	H_3O^+	2.5E-09	**63**	
			NO^+	2.2E-09	**62**	
			O_2^+	2.2E-09	62,**47**,46	ethanol
(*57*)	dimethyl disulfide	94	NO^+	2.4E-09	94	
(*58*)	dimethyl trisulfide	126	H_3O^+	2.8E-09	127, 145[a]	
			NO^+	1.9E-09	126	

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Table II. (Continued). Reagents, rate coefficients, and product ions used to quantify target aroma compounds for the sulfur cheese method

Ref.	*Compound*	*Mass*	*Reagent*	*k (cm³/s)*	*m/z*	*Potential Interferences*
(*54*)	dipropyl thioether	118	O_2^+	2.4E-09	72, 99, 114	
(*54*)	methional	104	H_3O^+	3.0E-09	105	
			O_2^+	2.5E-09	**76**, 104	methionol
(*54*)	methionol	106	H_3O^+	3.0E-09	**107**	n-butyric acid
			NO^+	2.5E-09	106	
			O_2^+	2.5E-09	89, 106	
(*59*)	ethanol	46	H_3O^+	2.7E-09	47, 65[a], 83[a]	
			NO^+	1.2E-09	45, 63[a], 81[a]	
(*54*)	pyrazine	80	H_3O^+	3.4E-09	81, 99[a]	
			NO^+	2.8E-09	80	
			O_2^+	2.7E-09	**80**	dimethyl trisulfide
(*54*)	furaneol	128	H_3O^+	4.00E-09	129, 147[a]	
			NO^+	2.50E-09	128, 158	

[a] Indicates water clusters. Mass to charge ratios in bold indicate conflict with potential interferent that could be present in some cheese samples, but not others.

Selection of Conditions for Analysis

Sample Preparation

Cheese samples were grated, vacuum packed and stored in a frozen state until analysis. Prior to analysis, grated samples were placed in 500 ml Schott bottles and sealed with cap and/or septa. The bottles were placed in a water bath to allow for headspace equilibration for a selected time and temperature. Headspace sampling was accomplished by using a passivated sampling needle connected to the sampling arm of the instrument. In cases where the bottle was capped with a septum, a second "by-pass" needle was utilized to minimize pressure variations during sampling. A standard cheese sample, prepared in the same way, was run each time the other cheese samples were run to provide a reference control.

Sample Size

The effect of sample size was determined by evaluating the VOCs in 1, 5, and 10 g samples using full mass scans and a SIM method. Full mass scans indicated that the counts per second (cps) for the m/z's of interest generally increased when the sample size increased (data not shown). Small increases or no change was observed when the sample size increased from 5 g to 10 g. Based on the results, the sample size of 5 g was selected for further studies. The use of larger samples sizes did not increase the concentration of most of the VOCs, whereas a 1 g sample did give smaller concentrations of most VOCs (Figures 2, 3, and 4).

As shown in Figure 2, the concentration of propionic and acetic acids were effectively unchanged by increasing the sample size from one to ten grams. Similarly, there was not an effect of sample size for the other organic acids in the sample.

Figure 3 shows the effect of sample size on the concentration of key sulfur compounds. Increasing the sample size from one to 5 grams showed a slight increase in the concentration of dimethyl disulfide, dimethyl trisulfide, and methyl mercaptan. There was no significant increase in the concentration of these samples when evaluated at 5 and 10 grams. This is attributed to the formation of an oil film over the melted cheese, so that only the compounds at the oil/air interface were being evaluated.

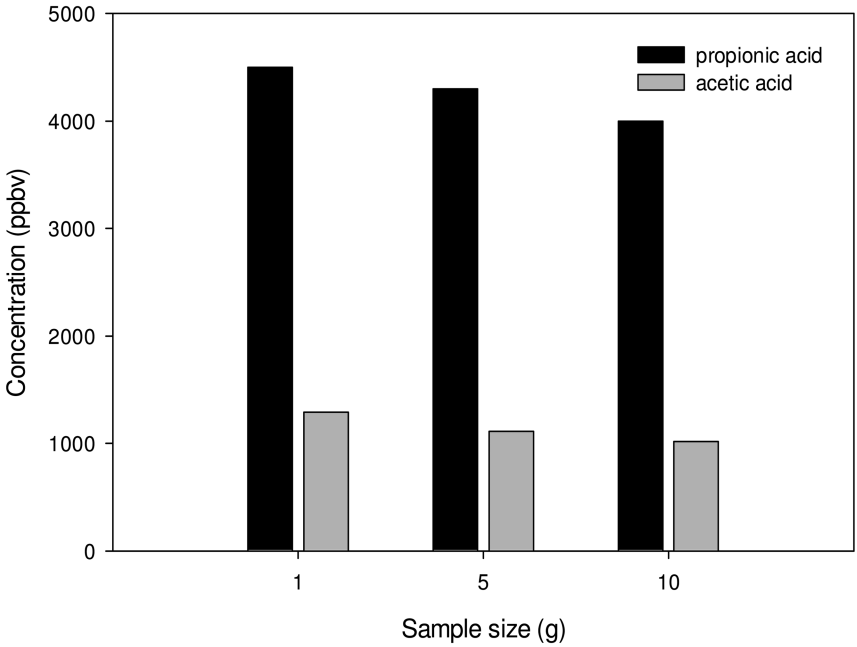

Figure 2. Effect of sample size on the concentration reported for propionic acid and acetic acid.

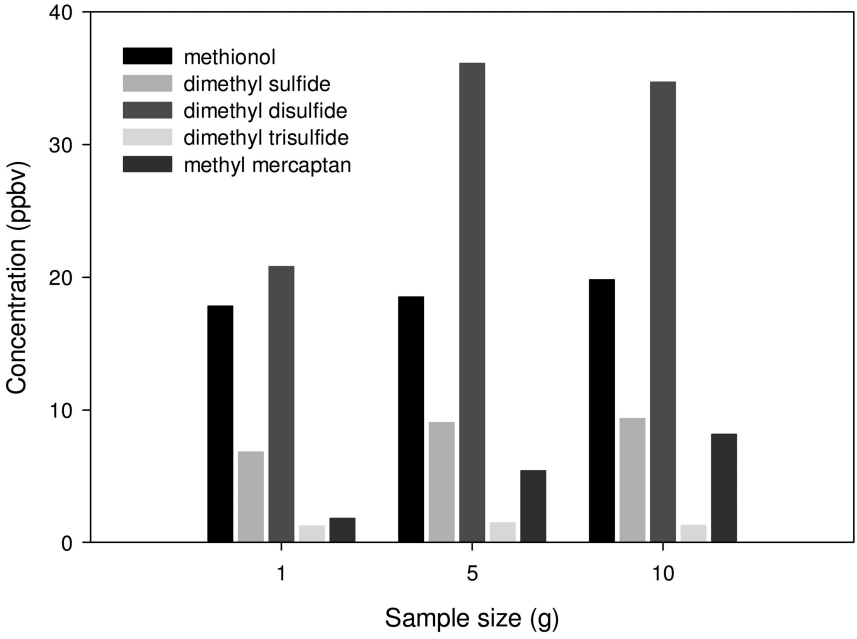

Figure 3. Effect of sample size on the concentration reported for selected sulfur compounds.

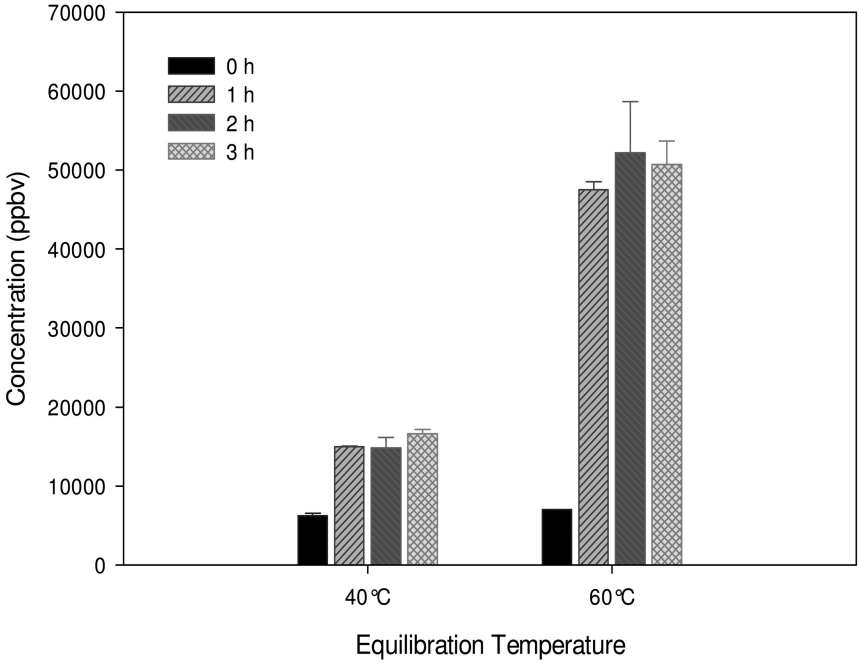

Figure 4. Effect of equilibration time and temperature on the concentration reported for propionic acid.

Equilibration Time and Temperature

The SIM method containing 50 compounds known to be important in Swiss cheese was used to evaluate the effect of equilibration time and temperature on the reported compound concentrations of 5 g shredded samples of Swiss cheese. The samples were equilibrated at 40 and 60°C for 0, 1, 2 and 3 hours.

It should be mentioned that for all compounds evaluated, the standard error was greater at 60°C that at 40°C. During the run there was a small, but continual decay in the signal due to a reduction in the pressure in the sample bottle. However, because this decay rate was consistent from sample to sample, it was not necessary to correct for the average values from run to run for a given sample.

The majority of the compounds (*37*) showed no statistically significant differences for all temperatures and all times. These include all the acids, alcohols and aldehydes. Figures 4 and 5 are representative figures for two of these compounds – propionic acid and dimethyl disulfide.

Neither dimethyl disulfide nor dimethyl trisulfide (not shown) showed a significant change in concentration over equilibration time and temperature.

Of the sulfur compounds included in the method, only methional showed an effect of time and or temperature. Methional showed an increase over time when equilibrated at 60°C, whereas there was no change at 40°C as shown in Figure 6.

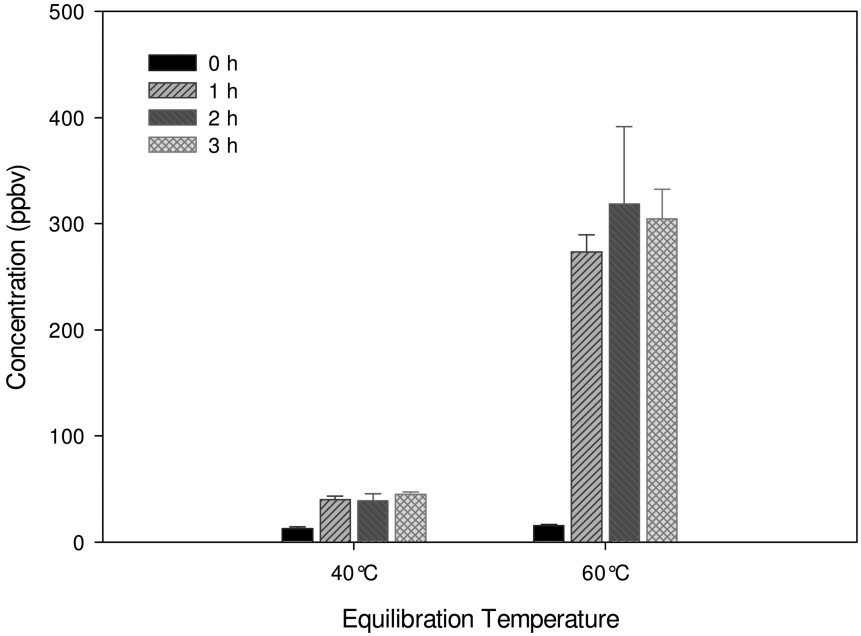

Figure 5. Effect of equilibration time and temperature on the concentration reported for dimethyl disulfide.

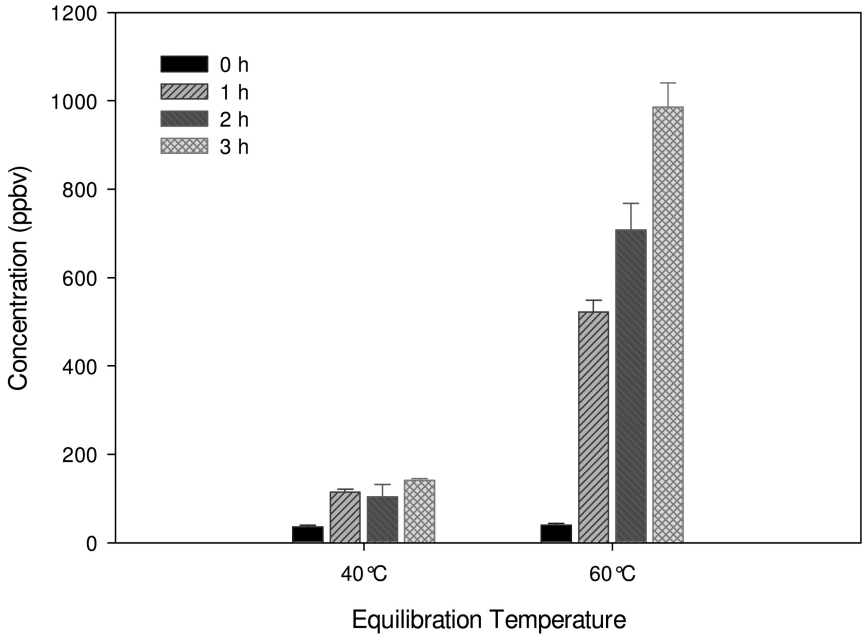

Figure 6. Effect of time and temperature on the concentration reported for methional.

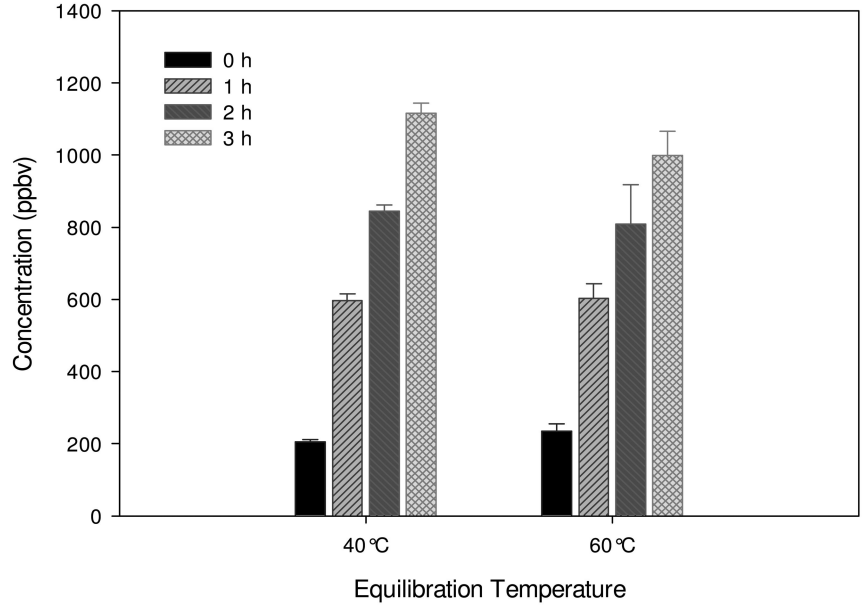

Figure 7. Effect of time and temperature on the concentration reported for ethyl acetate.

Other compounds that showed an effect of different equilibration times and temperatures included ethyl acetate, (Figure 7), acetyl methyl ketone, ethyl butyl ketone and diacetyl. The temperature and time effect on the ketones and diacetyl were not statistically significant. The reported ppb concentration for ethyl acetate increased at both 40 and 60°C.

Of all the compounds that might be in mass conflict with ethyl acetate, only diacetyl showed any tendency to increase with time at both temperatures. Of the 6 other potentially conflicting compounds, there was no change in reported concentrations at either 40 or 60 °C.

Selection of Cheese Samples

Analysis was made on different sets of cheese samples: (a) ten Swiss cheeses of different ages, and (b) 30 day old Swiss cheese just out of warm room with different ratios of propionic and acetic acids.

Swiss Cheese of Different Ages

10 cheese samples were selected as follows:
1 week of age prior to warm room treatment – 2 samples
2 months of age after warm room and ready for packaging – 6 samples
4 months of age –1 sample
14 months of age 1 sample

The designation of the cheeses, their manufacturer and age are shown in Table III. All but the 14 month old cheese were made in the same factory using the same milk supply and starter cultures.

The headspace of 5 g samples were analyzed with a Syft Voice100 SIFT-MS using three soft ionizing reagent ions in both full scan mode and SIM mode. Identification and quantification of the VOCs were determined based on the knowledge of the known ion products and reaction rate coefficients for each compound in a method that contained 18 compounds including 4 volatile fatty acids, 11 sulfur compounds, 1 alcohol, 1 pyrazine, and furaneol.

Results

Analysis of Swiss Cheese of Different Ages

A method was developed that included all the compounds for assignment of product masses for calculation, but only used those compounds that included sulfur compounds and high impact marker non-sulfur compounds (propionic acid and acetic acid) for which there were no mass conflicts or for which the mass conflicts could be resolved. A SIM method targeting selected sulfur compounds along with propionic and acetic acids for Swiss cheese is shown in Table II. The compounds selected, their molecular weight, reaction rate coefficient, mass to charge ratio and potential conflicting compounds are listed.

In Volatile Sulfur Compounds in Food; Qian, M., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2011.

Compounds selected for analysis were made on the basis of those compounds known to be most important to the flavor of Swiss cheese and for which Syft had library data for the mass (m/z) for products generated by the 3 reagent ions, the branching ratios and the reaction rate. The sulfur compounds of greatest interest, for which conflicts did not exist for more than one other compound, included: hydrogen sulfide, methyl mercaptan, dimethyl disulfide, dimethyl trisulfide, dipropyl thioether, methional, and methionol (Table II).

Representative Mass Scans

Figures 8, 9 and 10 provide the mass scans for representative 2 month old cheeses with a low (<1:1) and high (>2:1) propionic acid to acetic acid ratio using reactions from reagent ions H_3O^+, NO^+ and O_2^+ respectively. For each of the reagent channels, the data reports the relative intensities of the counts per second (cps). For each reagent ion, the intensity of the product masses (m/z) was higher for the cheese with high propionic acid to acetic acid ratios. This was true for masses that could be associated with sulfur compounds as well as the masses associated with non-sulfur compounds. NO^+ gave less fragmentation than did H_3O^+ or O_2^+. The O_2^+, as expected, gave the most fragmentation.

Table III. Designation of the cheeses by age and manufacturer

Manufacturer	*Cheese Code*	*Cheese Age (days)*
1	A	7
1	B	7
1	C	55
1	D	61
1	E	48
1	F	49
1	G	50
1	H	61
1	I	131
2	J	420

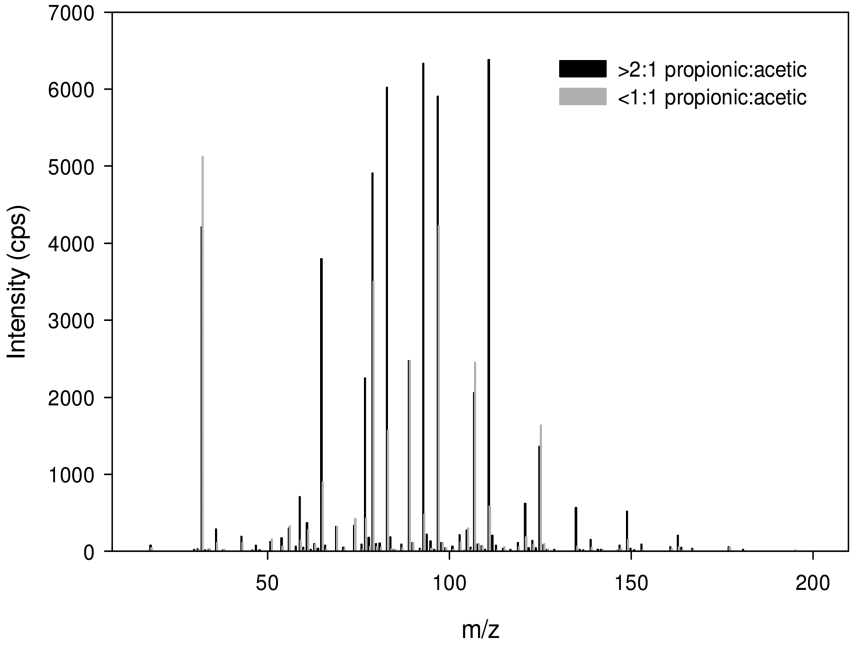

Figure 8. Representative H_3O^+ mass scan of 2 month old Swiss cheese with low and high propionic to acetic acid ratio.

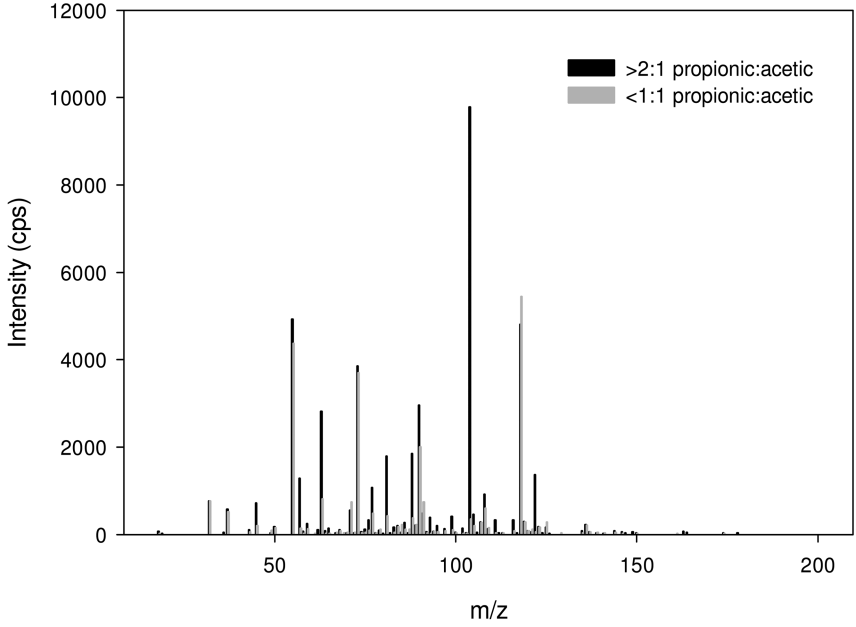

Figure 9. NO^+ mass scan of 2 month old Swiss cheese with low and high propionic to acetic acid ratio.

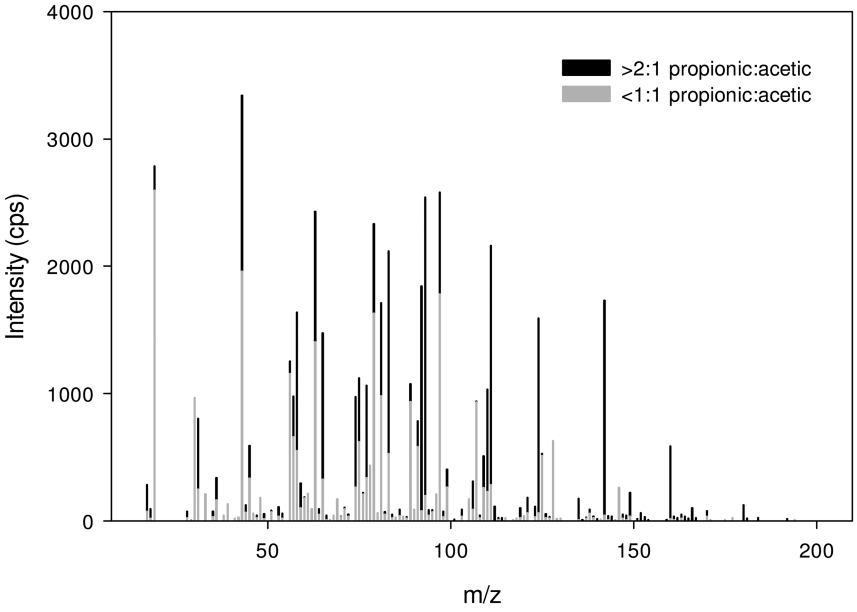

Figure 10. O_2^+ mass scan mass scan of 2 month old Swiss cheese with low and high propionic to acetic acid ratio.

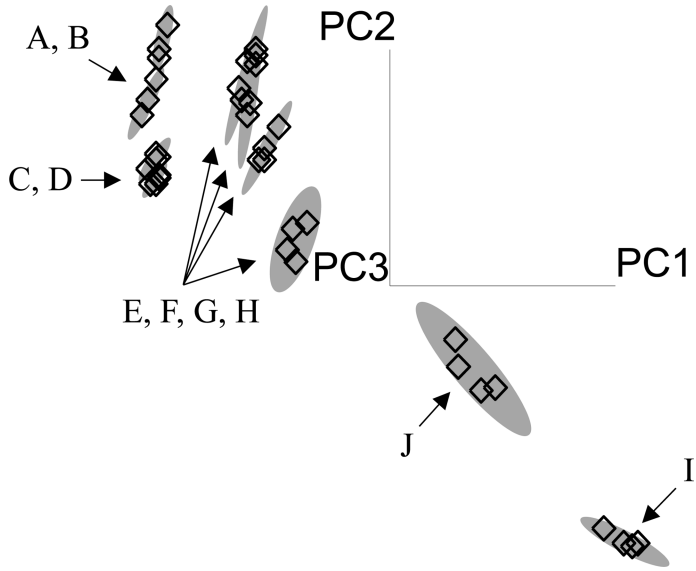

Figure 11. Soft independent modeling of class analogy (SIMCA) projection plot for full mass scan classification of 10 samples using autoscaled and normalized data from NO^+ reagent ion reactions. (low p/a = low propionic to acetic acid ratio; all other two month old samples have p/a ratio close to or > 2:1).

Soft independent modeling of class analogy (SIMCA) analysis (Pirouette v. 4.0 rev. 2, Infometrix, Inc. Bothell, WA) for full mass scan classification of 10 samples for each of the reagent ions used, indicated good separation and classification of each of the samples. The two 7 day old samples clustered together, and the two samples of 2 month old samples with low propionic acid to acetic acid ratios clustered together and were closer to 7 day old samples than the other 2 month old samples with higher propionic acid to acetic acid ratio. The two aged cheese samples manufactured by 2 different cheese manufacturers also formed well isolated clusters distant from the other samples (Figure 11).

Multivariate analysis is a powerful technique for understanding how a set of chemicals may impact flavor. Traditional univariate methods could be more restrictive for meaningful interpretation of the data as important relationships exist between combinations of variables. Multivariate analysis methods examine many variables simultaneously and attempt to reduce the number of factors (linear combinations of independent variables). This enables the classification of samples and quantitative prediction of flavor score and shelf life (*60*) to be used to interpret the simultaneous variations of many compounds.

Figure 11 demonstrates the potential of using unit mass scan data from cheese headspace for rapid, high-throughput classification of cheese samples for quality control purposes, based on the head space volatile organic compound composition without extensive sample preparation.

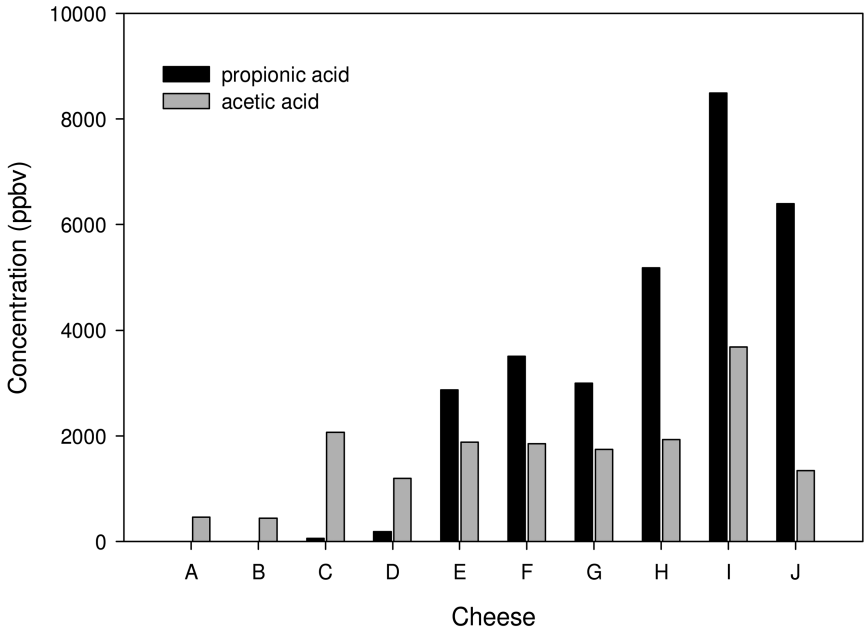

Figure 12. Concentration of propionic acid and acetic acid in cheese of different ages.

The propionic to acetic acid ratio is used by the industry to indicate control of the propionic acid bacteria during fermentation in the warm room. A theoretical ratio of propionic to acetic acid is 2:1, although a slightly lower theoretical ratio is to be expected in the headspace due to the relatively higher volatility of acetic acid compared to propionic acid. Low production of propionic acid during the warm room fermentation has been associated with a reduction in Swiss cheese flavor.

The ratios of the propionic to acetic acid levels in the head space are shown in Figure 12. Propionic acid levels in the two one week old cheeses are low, as would be expected, since propionic acid production only occurs during the warm room fermentation. However, after two months, the ratios would be expected to approximate 2:1. Cheeses C and D have propionic to acetic acid ratio of <1. The relationship between the propionic/acetic acid ratios in the cheese and the concentrations of the various common sulfur compounds in cheese are shown in Figures 12 and 13. The propionic acid fermentation during the warm room may have a relation to the formation of some of the sulfur compounds.

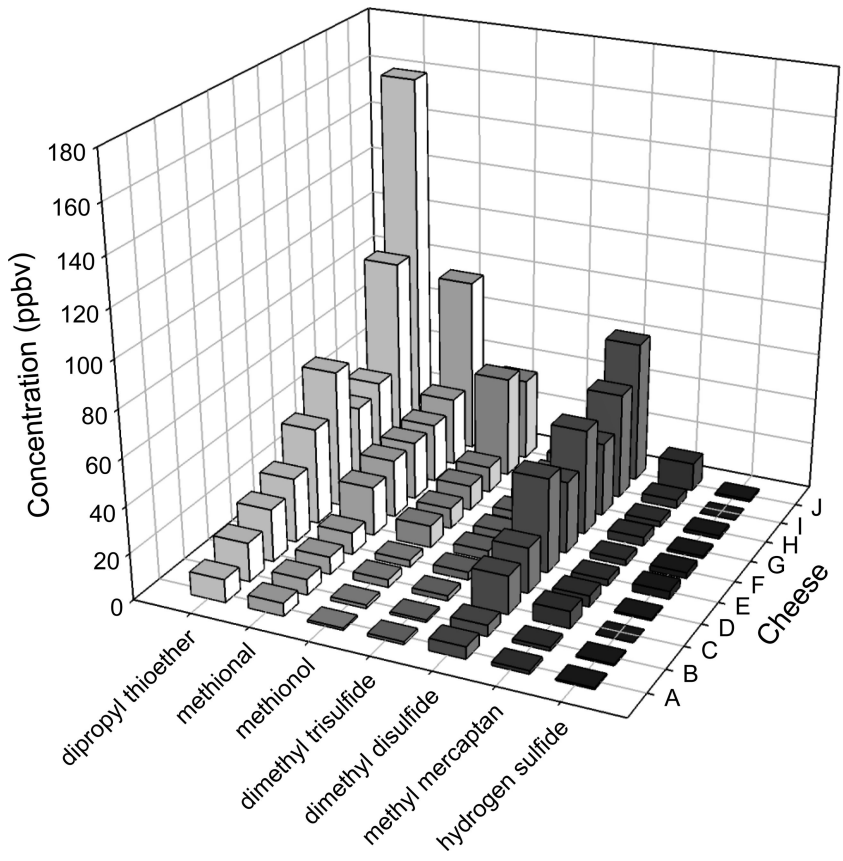

Figure 13. Concentration of sulfur compounds in cheese of different ages.

Propionic Acid to Acetic Acid Ratio Relationship to Volatile Compounds in 30 Day Old out of Warm Room Swiss Cheese from a Single Factory

To obtain more information on the relationship between the propionic to acetic acid ratio and the formation of sulfur compounds, eight Swiss cheese, just removed from the warm room (30 days of age), were obtained from the same factory that produced the cheeses reported in the first section of the study. In these samples the same relationship was found between the propionic acid concentration and the sulfur compound concentrations. As propionic acid concentration increased, the key sulfur compounds also increased. One day old samples were also collected, but the results are not reported. The Swiss cheese SIM method that contained 50 compounds was used to quantify compound concentrations.

Figure 14 shows the propionic to acetic acid concentrations and their ratios for these eight 30 day old cheeses. The propionic to acetic acid ratios, ranged from 0.1 to 2.4 in the eight cheeses of the same age. Five of these cheeses had ratios of less than 1.0 and three had ranges greater than 1.0. Pairwise correlation analysis indicated that the propionic acid to acetic acid ratio was highly correlated with propionic acid concentration (0.95) at a significance level of $p<0.000$. Acetic acid on the other hand had a lower correlation (0.34) with the propionic to acetic acid ratio ($p=0.048$).

Figure 15 shows the relationship of differences in the propionic to acetic acid ratio to the selected sulfur compounds. The cheeses are arranged (from front to back) in order of increasing propionic to acetic ratios. Cheeses with a propionic to acetic acid ratio approximating 2:1 theoretical ratio had significantly higher ($p < 0.0001$) concentrations of dimethyl disulfide and methyl mercaptan compared to cheeses with very low propionic to acetic acid ratio.

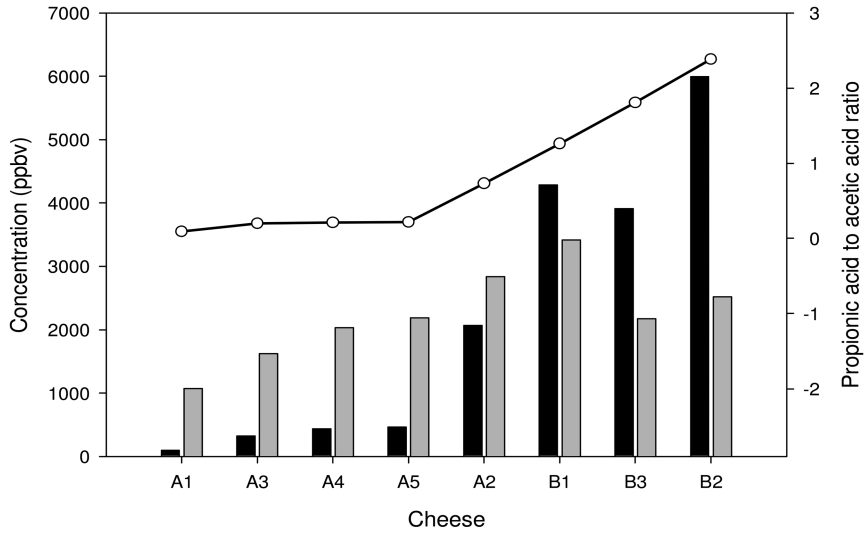

Figure 14. Propionic acid to acetic acid ratios and concentrations of propionic acid (black bar) and acetic acid (gray bar) in the headspace of 30 day old (out of warm room) Swiss cheese.

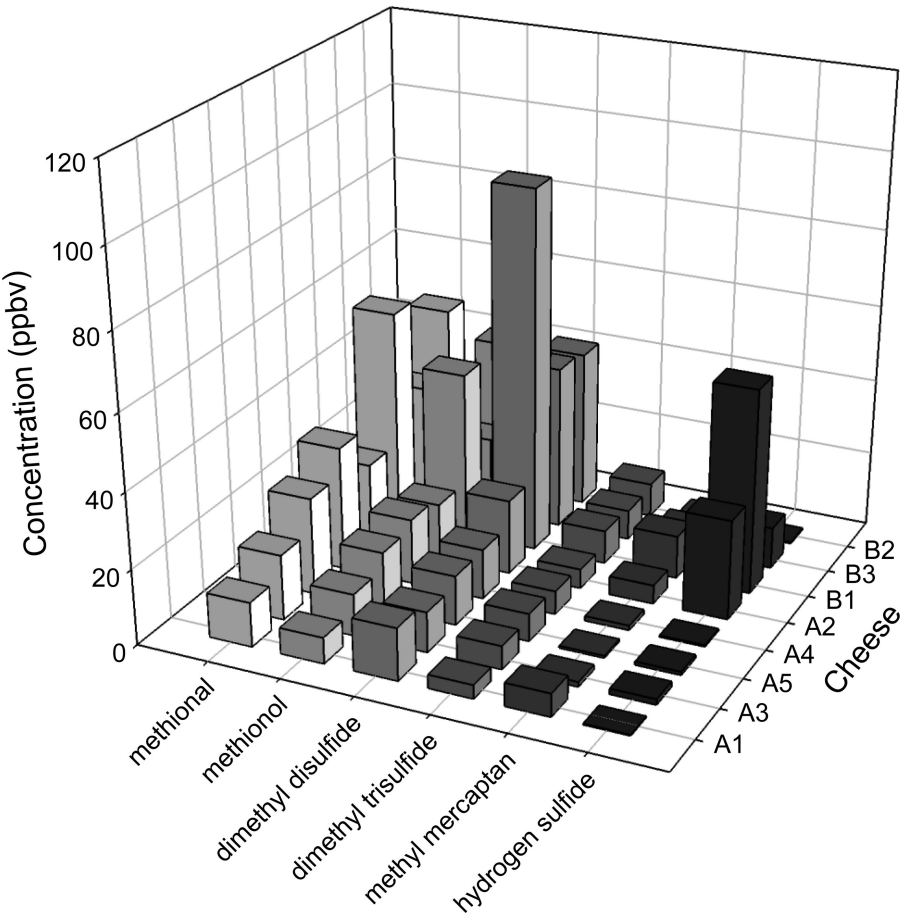

Figure 15. Concentration of sulfur compounds in 30 day old cheese with varying propionic to acetic acid ratio.

A SIMCA plot based on concentrations of seven sulfur compounds (methionol, dimethyl trisulfide, hydrogen sulfide, dimethyl sulfide, dimethyl disulfide, methional, and methyl mercaptan) obtained using the method consisting of 50 compounds showed good discrimination of the samples based on propionic acid to acetic acid ratio (Figure 16).

Additionally, samples were classified based on propionic acid to acetic acid ratio using unit mass scan data from each of the three reagent ions (H_3O^+, NO^+, and O_2^+). All masses (m/z) associated with propionic acid and acetic acid along with masses associated with the reagent ions were excluded from classification data. Figure 17 shows a representative SIMCA plot obtained using mass scan results using the H_3O^+ reagent ion. SIMCA analysis allowed for classification of the samples based on propionic to acetic acid ratio and the discriminating power plot indicated the masses (m/z) that contributed greatest to the classification of the samples. The m/z values influencing differentiation with the H_3O^+ reagent ions included 35, 163, 103, 89, 161, 123, 119, 65, and 59. The masses highly

influencing differentiation using NO^+ were 43, 102, 118, 132, 88, 105, 71, 62, 81, and 85. Relative to the number of masses scanned for H_3O^+ and NO^+ reagent ions, fewer numbers of masses (*18*) were selected for scanning and calculating the compound concentrations using O_2^+. The majority of the m/z's were associated with sulfur compounds. Of the masses selected, m/z's 72, 126, 106, 48, 44, 122, 76, 62, 94, and 105 were highly important for classification. Masses associated with sulfur compounds, such as hydrogen sulfide, dimethyl sulfide contributed highly to discrimination of samples with different levels of propionic to acetic acid ratios. Furthermore, masses associated with other important compounds for Swiss cheese flavor such as isovaleric acid, butyric acid, 3-methyl butyric acid, and trimethylpyrazine were found to contribute to classification of the samples. This suggests the propionic acid fermentation may have an influence on the formation of other VOCs, including the sulfur compounds.

Discussion

Selected ion flow tube mass spectrometry (SIFT-MS) has significant value as method that could potentially identify and quantitatively measure the volatile compounds in ppb concentration in the headspace of a food sample without sample preparation. The method may provide a basis for differentiating the factors influencing the development of flavor and provide a chemical basis for understanding that differentiation

Many methods can differentiate the factors influencing flavor without actually determining the role of the exact chemical compounds in the differentiation. This includes the electronic nose and Fourier transform infrared spectroscopy (FTIR). However, these methods do not have the ability to associate that differentiation with specific chemical compounds. SIFT-MS generally does provide additional chemical information of the basis of differentiation by chemical species present.

SIFT-MS analysis permits the identification and quantification of more compounds in Swiss cheese than other direct methods that have been used. It may also give a more complete picture of the actual compounds present and provide their relative concentration in the headspace. Although there may be an association between the levels of some of the sulfur compounds and the formation of propionic acid during warm room treatment. Proof of this will require further study.

The Syft Voice100 selected ion flow tube mass spectrometer permits the detection of key flavor compounds in the headspace of a cheese, or other food sample at ppb concentrations without the need for sample preparation or concentration. This sensitivity of the method permits direct detection of volatile organic flavor compounds not attainable by GS/MS without first increasing the concentration of the compounds introduced into the instrument. Frequently, sample preparation for GC/MS requires sample concentration and may also result in a different ratio of compounds in the sample injected into the GC than the ratio of the compounds in the original sample.

The use of the three different soft ionizing reagent ions (H_3O^+, NO^+ and O_2^+) allows for improved identification of the compounds by forming different m/z

reaction products. The reaction rate constants permit direct calculation of product concentrations.

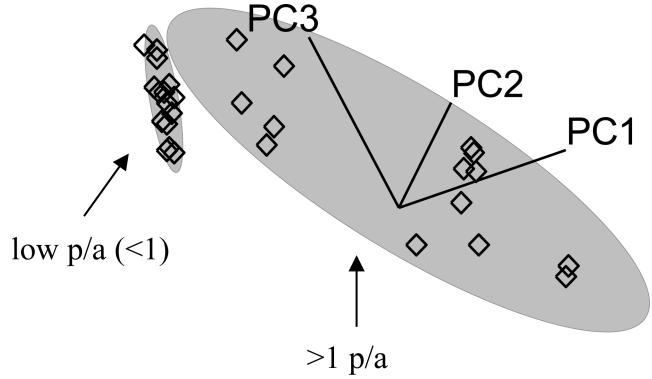

Figure 16. SIMCA class projection plot based on concentrations of seven sulfur compounds (methionol, dimethyl trisulfide, hydrogen sulfide, dimethyl sulfide, dimethyldisulfide, methional, and methyl mercaptan) showing discrimination based on propionic acid to acetic acid ratio (p/a).

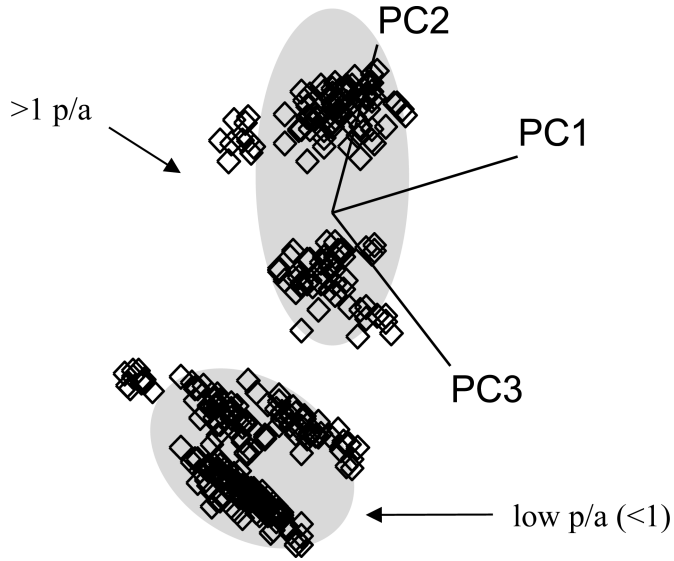

Figure 17. SIMCA class projection plot based on sulfur compound concentrations of samples with < 1 and > 1 propionic acid to acetic acid ratio (p/a).

The use of soft ionizing reagents results in a less complex mixture of reaction products, making it easier to identify the source of the compound. For H_3O^+, the most common reaction product is the molecular weight +1 and for NO^+, the most common reaction product is the molecular weight. The O_2^+ reagent generally gives much more complex mixtures of reaction products.

Due to the complexity of volatile compounds in most food products, the headspace of many foods, including cheeses, contains more than 200 different volatile compounds at concentrations greater than one ppb. Therefore, it is impossible to avoid potential conflicts between compounds, especially those with the same molecular weight.

When developing a method for a product like cheese, it is important to obtain information for the reaction products m/z for each compound significant to the flavor of the product. It is imperative to determine where the m/z values for a specific compound are free from conflicts. The inability to avoid all conflicts in a matrix as complex as cheese is a limitation of SIFT-MS methodology.

In this study, which focused on sulfur compounds in Swiss cheese, the m/z values for compounds of interest were limited by mass conflicts. Of the 50 compounds in the original method, only the following sulfur compounds could be quantitatively determined by the SIFT-MS method, either without conflict, or where the conflict could be resolved by subtraction: hydrogen sulfide, methyl mercaptan, dimethyl disulfide, dimethyl trisulfide, methional and methionol. Even so, the method did provide a chemical basis of differentiation of some of the key factors affecting the flavor characteristics of Swiss cheese not possible with other methods.

Using both SIM scans and mass scans it was possible to differentiate Swiss cheese on the basis of age and on the basis of the differences in the ratio of propionic to acetic acid ratios. The latter is especially important since the industry has long known that a high propionic to acetic acid ratio in cheese coming out of the warm room is associated with a higher quality cheese.

An attempt to add additional sulfur compounds, often reported in mold and yeast ripened cheese proved unsuccessful. Thiolactic acid, 2-methylthioacetic acid and 2-mercapto-1-propanol all have the same molecular weight which results in an unresolvable conflict. It was possible using subtraction technique to quantify 3-mercapto-1 propanol.

Conclusions

The selected ion flow tube mass spectrometer (SIFT-MS) Syft Voice100 has the capability to identify and measure some, but not all, of the high impact sulfur compounds in Swiss cheese at ppb concentrations in the cheese headspace without any sample preparation. Careful method development is necessary to identify and quantify the un-conflicted compounds to achieve reliable results.

It cannot be expected that SIFT-MS can identify and quantify all compounds present in a cheese sample. This is due to the complexity of the number of compounds that may be present and the inability to avoid and resolve

mass conflicts. Nevertheless, it does provide a means for achieving a better understanding of the chemical basis of factors affecting the flavor of Swiss cheese.

A significant finding of this study was that formation of propionic acid during warm room curing coincided with the formation of some of the high impact sulfur compounds in the cheese. It was observed in 30 day old cheeses from one manufacturer using the same production methods and milk supply, that dimethyl disulfide and methyl mercaptan concentrations in the cheese increased as the propionic to acetic acid ratio increased.

Further studies to determine the role of different starter organisms in the formation of sulfur compounds in Swiss cheese will be conducted.

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Chapter 9

Volatile Compounds of the Genus *Allium* L. (Onions)

Prof. Dr. Michael Keusgen*

University of Marburg, Institute of Pharmaceutical Chemistry, Marbacher Weg 6, 35032 Marburg, Germany
***E-mail: keusgen@staff.uni-marburg.de.**

Garlic (*Allium sativum* L.) and common onion (*A. cepa* L.) are used by mankind since ancient times as vegetable, spice and medicine. If bulb material is disrupted, volatile sulfur compounds with a characteristic smell and taste will be liberated. Most prominent are the thiosulfinate allicin from garlic and the 'lachrymatory factor' LF (propanethial *S*-oxide) of common onion. Both compounds are formed by the action of the enzyme alliinase. For LF, the enzyme LF synthase is additionally required. Allicin as well as LF are instable and decompose to a high variety of so called 'secondary aroma compounds". For garlic, allyl (poly)sulfides, vinyl dithiins and ajoenes are typical volatile compounds. For onion, the LF dimmer, cepaenes and zwiebelanes are frequently reported. Besides these cultivated species, sulfur compounds are also available in many wild *Allium* species.

Introduction

Species of the genus *Allium* L. (onions) have been used by mankind since many thousand years. About 6,000 years ago, garlic (*A. sativum* L.) and common onion (*A. cepa* L.) were taken into cultivation and used as vegetable, spice and medicine. Flavoring as well as medicinal properties of these plants are mainly related to volatile sulfur compounds, which typically contain two or even more sulfur atoms. First attempts regarding the sulfur chemistry of garlic were summarized by the German chemist T. Wertheim in 1844 (*1*). By aqueous distillation of garlic bulbs a strongly smelling, sulfur containing oil was found.

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Finally, this compound could be determined as diallyl sulfide ($C_6H_{10}S$). The name 'allyl' was derived from the name '*Allium*'. Later on, similar experiments were performed by F. W. Semmler with onions, but distillation was performed under reduced pressure. A compound with the molecular formula $C_6H_{12}S_2$ was found (*2*), which can be suggested to be 1-propenyl propyl disulfide. By the used technology, only 233 g of onion oil could be obtained out of 5.000 kg of fresh onions. In our days, steam-distilled oils of both garlic and onion are of strong commercial interest and mainly used as flavoring agents by the food industry (*3*).

After World War II, a number of researchers started to elucidate the 'primary' flavoring compounds ('primary aroma compounds') of garlic and onion as well as the mechanism of their formation. Remarkable is the work of C.J. Cavalito, who isolated the allicin ($C_6H_{10}S_2O$) from garlic (*4*). Similar investigations were undertaken for common onion. In 1963, first descriptions of the so called 'lachrymatory factor' (LF, C_3H_6SO) were delivered by the group of Finish Nobel Laureate A. Virtanen (*5*). Also at the same time, it got evident that enzymes must be involved in the formation of volatile compounds. Therefore, much emphasis was put on the analysis and characterization of the precursor compounds for the main enzyme named 'alliinase'. This theory was proved by A. Stoll and E. Seebeck in 1948 with the discovery of the precursor molecule alliin, a derivative of the amino acid cysteine (*6*). Alliin was isolated, synthesized and first enzymatic experiments were performed (*7*, *8*). Analogously, the isoallin as precursor of the LF was found by C.-G. Spåre and A. I. Virtanen in 1963 (*5*).

After the basic findings were made, intensive research activities started in order to elucidate the rather complex chemistry of *Allium* volatile compounds, mainly to find 'secondary aroma compounds' and to elucidate health benefits of these compounds. It has been demonstrated that the sulfides of the distilled oils of garlic and onions are belonging to these 'secondary aroma compounds'. Formation of these compounds strongly depends on the treatment of bulb material. Research on these molecules is still ongoing. Comprehensive reviews regarding these topics were given by Block, Lawson and Keusgen (*3*, *9*, *10*).

Many wild *Allium* species were used in a similar manner as common onion and garlic. Only little is known until now about the entire chemical composition of these plants. First attempts to get an overview of the chemistry and the usage of *Allium* plant material were made by M. Keusgen and R.M. Fritsch, mainly in Central Asia (*11–13*). It must be mentioned that latter activities were generously funded by the VolkswagenStiftung, Hannover, Germany.

Cysteine Sulfoxides, the Precursors of Volatile Sulfur Compounds of *Allium*

The history of the sulfur compounds of *Allium* in general has been briefly explained above. Cysteine sulfoxides do not directly contribute to the flavor of *Allium* plants, but they are the precursors of volatile compounds giving the special aroma and taste to *Allium*. Cysteine sulfoxides itself are nearly lacking of any taste or smell. Without cysteine sulfoxides and without the enzyme alliinase, which will be explained later on, no aroma will occur.

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Cysteine sulfoxides are amino acid derivatives and can be understood as alk(en)yl cysteine derivatives, which are oxidized at the sulfur atom. It is worth to note, that besides the chiral center of the cysteine at carbon 2 (L-cysteine), the sulfur is also chiral. Only L-cysteine sulfur derivatives were found in *Allium*. The chirality of the sulfur atom is a quite unique fact. Before the discovery of cystine sulfoxides, only cyclic organosulfur compounds were known carrying a chiral sulfur atom. In *Allium*, only the (+)-sulfoxides were found until now in signifiacant amounts. Interestingly, *Marasmius* species, which are mushrooms, form (-)-cysteinesulfoxides, *e.g.* **66a**, Figure 13 (*14*). This special case will be explained at the end of this chapter.

Most important aliphatic cysteinen sulfoxides contributing significantly to the flavor of commonly used *Allium* species are listed below. Usually, an individual mixture of these compounds occurs in each *Allium* species. These compounds are (Figure 1): (+)-*S*-methyl-L-cysteine sulfoxide (methiin **1**), (+)-*S*-propyl-L-cysteine sulfoxide (propiin **2**), (+)-*S*-(2-propenyl)-L-cysteine sulfoxide (alliin **3**), (+)-*S*-(1-propenyl)-L-cysteine sulfoxide (isoalliin **4**), (+)-*S*-butyl-L-cysteine sulfoxide (buttin **4**) and (+)-*S*-(1-butenyl)-L-cysteine sulfoxide (homoisoalliin **6**). Additionally, (+)-*S*-ethyl-L-cysteine sulfoxide (ethiin) was found in trace amounts (*15*). Synthetic, but naturally not occurring cysteine sulfoxides were named analogously (*e.g.*, pentiin, hexiin). The above given chemical nomenclature is the commonly used one, but is not absolutely correct. For instance, the correct name for alliin **3**, which is the trivial name of this substance, is (R_cR_s)-(+)-*S*-2-propenylcysteine sulfoxide and for isoalliin **4** (R_cR_s)-(+)-*S*-1-propenylcysteine sulfoxide. In the following text, the common names will be used, because they are still well established in literature. The chemistry of the *Allium* subgenus Melanocrommyum is somewhat special and will be discussed below into detail.

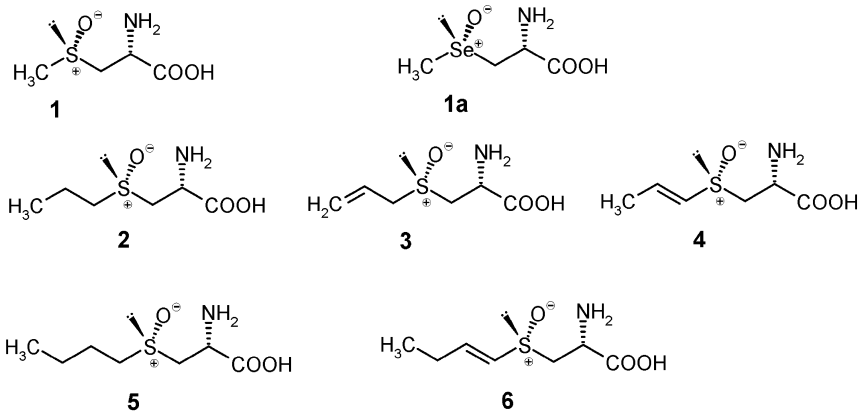

*Figure 1. Chemical structures of the main aliphatic cysteine sulfoxides, which contribute to the flavor of Allium plants. It was proposed that the sulfur atom can be replaced by selenium (**1a**).*

In nature, it is also possible that a sulfur atom as part of an organosulfur compound can be replaced by a selenium molecule (*16*). Theses compounds usually occur in trace amounts. One still hypothetic substance is 'selenomethiin' **1a**. However, *S*-methylcysteine ('deoxyselenomethiin') has already been reported in *Allium*.

The distribution of these substances inside the genus *Allium* shows high variations. Alliin **3** is typical for garlic and isoalliin **4** for common onion. Methiin **1** occurs ubiquitous with mostly high concentrations in wild onions (*11*, *17*). The chemistry of these three compounds is very well investigated and will be explained in more detail below. Butiin **5** and homoisoalliin **6** are typical substances of species belonging to the subgenus Nectaroscordum. It must be mentioned that **4** and **6** can also undergo an intramolecular cyclization (*18*, *19*).

The biosynthesis of cysteine sulfoxides is not fully understood until now. It is proposed, that the sulfur atom is derived from sulfate (*20*). Therefore, sulfate content of the soil has an influence of the organosulfur compounds of *Allium*. One intermediate is proposed to be glutathione, from which γ-glutamyl-desoxycysteine sulfoxides are bio-synthesized by several steps. The glutamyl-derivatives were subjected to a stereoselective oxidation. As the last step, the γ-glutamyl residue is removed by γ-glutamyl transpeptidase. γ-Glutamyl derivatives of cysteinesulfoxides could be frequently isolated from different *Allium* species and were discussed as storage forms of them. Further on, γ-glutamyl-cysteine sulfoxides were not accepted by the alliinase.

Allinase as a Key Enzyme in the Biosynthesis of Volatile Sulfur Compounds

The enzyme alliinase (EC 4.4.1.4) belongs to the group of C-S lyases and plays a key role in the formation of volatile compounds of the genus *Allium*. As mentioned above, enzymatic precursors are nearly without any odor. If cell material of *Allium* plants gets disrupted, the alliinase, which is stored in the vacuole of cells, came in contact with cysteine sulfoxides (*7*, Figure 2), which are stored in the in the cytoplasm. This situation is rather unique because usually low-molecular secondary metabolites are stored in the vacuole.

Further on, alliinase and cysteine sulfoxides **7** are concentrated in different cell types. In detail, the alliinase is located in vascular bundle sheat celles, were as the cysteine sulfoxides **7** are concentrated in the abundant storage mesophyll cells of bulbs. But alliinase enzymes as well as precursors are additionally available in nearly all other parts of the plant. Good overviews about the current stage of knowledge are given in ref. (*3*, *9*, *20*).

Key steps in the modern analysis of the alliinase were DNA sequencing and expression of the recombinant enzyme of *e.g.*, the alliinase of garlic (*21*), and crystallographic studies including elucidation of the mode of action (*22*, *23*). It must be mentioned, that the DNA sequence of alliinase differs from species to species (*20*). The DNA deduced amino acid sequence of the enzyme shows a rather high homology between common onion, shallot and garlic (> 90%), were as the Chinese chives alliinase was only 66-69% homologous to other alliinase

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sequences. Alliinase of garlic is a glycoprotein, consists of two equal subunits, each of them having 448 amino acids, and has a total molecular weight of 103 kDaltons. It could be demonstrated, that the garlic alliinase is only little affected by drying of bulbs and storage (*24*).

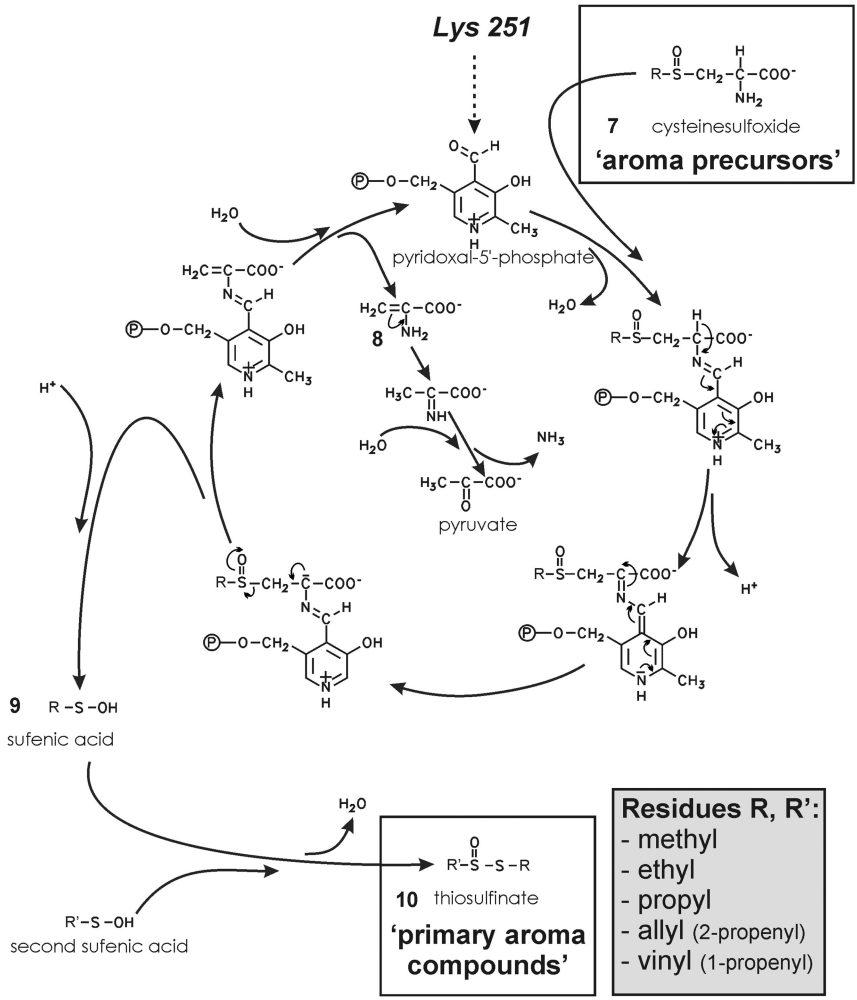

*Figure 2. Enzymatic cleavage of aliphatic cysteine sulfoxides **7** by the enzyme alliinase (EC 4.4.1.4, C-S lyase). Two molecules of cysteine sulfoxides **7** are necessary to give one molecule of thiosulfinate **10**. Pyridoxal phosphate acts as a cofactor, which forms an 'internal' aldime with the lysine 251 of the protein part of the enyme. Cysteine sulfoxides **7** were bound as 'external' aldimines. Further intermediates are aminoacrylate **8**, which decomposes into pyruvate and ammonia, and sulfenic acid **9**, which is an intermediate. Two molecules sulfenic acid **9** will give one molecule thiosulfinate **10**. Thiosulfinates with 'mixed' residues (different R and R') are also possible.*

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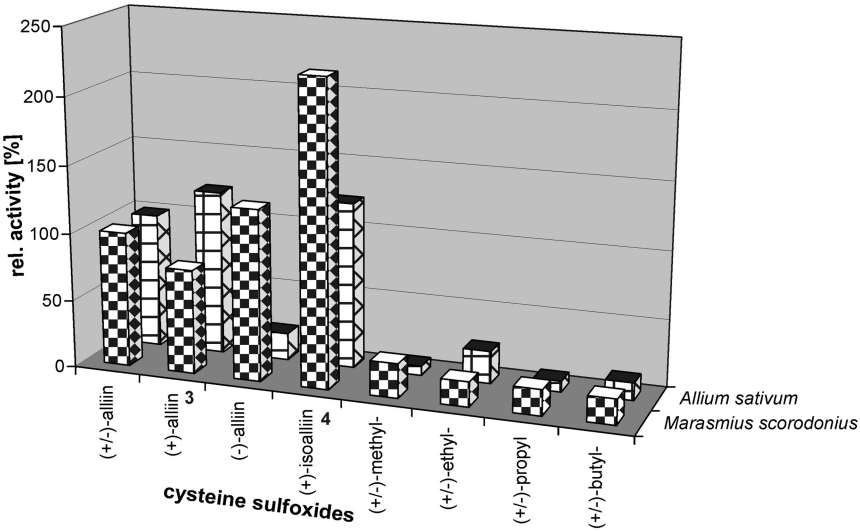

Figure 3. Relative activities and substrate specificity of alliinases isolated from Allium sativum and the mushroom Marasmius scorodonius. The activity towards racemic (+/-) alliin is set to 100 %. Names of residues of cysteine sulfoxides 7 (methyl, ethyl, propyl, butyl) are given on the X-axis.

The action of alliinase is schematically given in Figure 2. The cofactor pyridoxal-5′-phosphate is essentially for the enzymatic activity. If this cofactor gets lost, no activity will remain. However, a lost cofactor can be usually replaced by a surplus of pyridoxal-5′-phosphate in the storage buffer (*25*). The pyridoxal-5′-phosphate is bound to the amino acid lysine at position 251 of the amino acid sequence in form of an internal aldimine. Upon the contact with a cysteine sulphoxide **7**, which fits to the active center of the enzyme, an external aldimine will be formed. The main step of this reaction is the C-S cleavage in the manner of a α,β-elimination reaction. The final products will be sulfenic acid **9**, which will be liberated from the enzyme, and the aminoacrylate **8**, which is liberated from the pyridoxal-5′-phosphate and hydrolyzed into pyruvate and ammonia. In effect, one molecule of **7** will result in one molecule of **9** as well as one molecule pyruvate and ammonia.

Sulfenic acid **9** is a highly instable intermediate and will react with a second molecule sulfenic acid to give a so called thiosulfinate **10** by the loss of one molecule water. Because the alliinase is not strictly acceptable to one substrate (*e.g.*, alliin **3** or isoallin **4**), 'mixed' thiosulfinates **10** with different residues R and R′ are also possible, if the enzyme came in contact with different cysteine sulfoxides **7** at the same time. Because *Allium* species typically do lead more than one cysteine sulfoxides **7**, mixed thiosulfinates **10** should occur frequently. The final composition of thiosulfinates **10** depends on the relative cysteine sulphoxide pattern and the substrate specificity of the alliinase.

Differences between alliinase activities regarding the substrate specificity are depicted in Figure 3. Alliinase of garlic (*A. sativum*) and an alliinase from the mushroom *Marasmius scorodonius* were compared. Interestingly,

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the genus *Marasmius* has a very similar sulfur chemistry in comparison to *Allium*. In both organisms, only cysteine sulfoxides derived from L-cysteine were synthesized. However, the chirality at the sulfur atom is different. The (R_S)-isomers [*e.g.*, (+)-alliin **3**] occur in the genus *Allium*, were as *Marasmus* produces the (L_S)-isomers with a high selectivity [*e.g.*, (-) marasmin) **66a**, Figure 13]. D-Cysteine sulfoxides, which do not occur in nature, were not accepted by alliinase.

As shown in Figure 3, different cysteine sulfoxides were offered to both enzymes and the activity towards racemic (+/-)-alliin was set to 100% (*25*). It is easily visible, that the alliinase from garlic prefers the (+)-alliin **3** (relative activity of about 120%), were as the alliinase from *Marasmius* prefers the (-)-alliin (relative activity also about 120 %). This fits perfectly with the situation in both organisms. *Allium* mainly produces (+)-cysteine sulfoxides, were as Marasmus produces the (-)-isomers.

A further interesting fact is that isalliin **4** and alliin **3** are accepted by the garlic alliinase in nearly the same manner. In contrast, the enzyme from *Marasmus* prefers (+)-isoalliin **4** very much. It could be also found by some wild *Allium* species that (+)-isomers were highly preferred (*26*). For instance, the alliinase of *A. subhirsutum* L. has a relative activity toward (+)-alliin **3** of 223% in comparison to (+/-)-alliin. As shown for *A. ursinum* L., isoalliin was accepted in the same manner as (+)-alliin **3** (*27*). For other wild species, a comparable situation can be expected, because a high number of theses species do lead (+)-isoalliin **4** (*11*).

The aliphatic cysteine sulfoxides without any double bond were much less preferred by the alliinase as alliin **3** or isoalliin **4**. For instance, methiin **1**, which is common for nearly all *Allium* species, is accepted by the garlic alliinase by 7% relative activity, were as *A. ursinum*, which is very rich in methiin **1**, shows a relative activity towards methiin **1** of 27%. As a consequence, thiosulfinates **10** carrying a 1-propenyl or/and a 2-propeny moiety, were produced in a higher amount as those thiosulfinates carrying a methyl residue. In many cases, mixed thiosulfinates **10** carrying one methyl and one propenyl residue were found. As also demonstrated by Figure 3, cysteine sufloxides **7** having an aliphatic residue longer as three carbons were poorly accepted by the alliinase.

Residues up to four carbons were frequently found in nature. Garlic alliinase also seems to prefer saturated cysteine sulfoxides with an even number of carbon atoms (*e.g.*, relative activities towards methiin **1** 7%, propiin **2** 8%, ethiin 24%, buttiin **5** 13%). In summary, the final composition of thiosulfinates **10** can be hardly predicted from the pattern of found cysteine sulfoxides **7**. Alliinase substrate specificity as well as relative amounts of the cysteine sulfoxides **7** of individual plant species play an important role.

Volatile Compounds Related to Garlic (*Allium sativum*)

As explained in the introduction section, the chemistry of garlic has been investigated for 150 years. First experiments were carried out by performing garlic distillation, meaning that the 'primary aroma compounds' get discomposed and ally sulfides occurred in the final volatile oil. The name 'allyl', used for the

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CH_2=CH-CH_2 residue is not perfectly correct; the valid name for this would be '2-propenyl' residue. Because the name 'allyl' is commonly used, it will be also used here.

The most important sulfur compounds related to garlic are summarized in Figure 4. Alliin **3** is rapidly converted by the enzyme alliinase to give the allylsulfenic acid 11 (or chemically more correct: 2-propensulfenic acid), which is highly instable, but could be recently directly proven by mass spectrometry (*3*). Two molecules of **11** will react to give the thiosulfinate allicin **12**. In contrast to cysteine sulfoxides **7**, allicin **11** is not chiral. This can be simply explained as chirality is lost during the step catalyzed by alliinase and the allylsulfenic acid **11** does not show any chirality. Consequently, allicin, which is simply formed by condensation, can not be chiral.

It must be considered that garlic also contains methiin **1** and isoalliin **4** as minor components. The pattern of these cysteine sulfoxides **7** is highly variable and the total amount of cysteine sulfoxides can be more than one percent, related to the fresh weight of bulbs. All theses cysteine sulfoxides were accepted as substrate by the garlic alliinase (compare Figure 3). In addition to allicin **12**, a broad variety of thiosulfinates **10** containing one or two methyl- ore 1-propenyl residues is formed by the alliinase reaction. These minor thiosulfinates **10** will affect the aroma of crunched garlic significantly. Especially methyl-containing thiosulfinates **10** have a somewhat unpleasant smell and a 'hard' taste (*12*). Besides the total concentration of cysteine sulfoxides **7**, also the relative composition of these compounds has a significant influence on the aroma of crunched garlic. Varieties leading higher levels of methiin **1** were probably not well accepted by most people, especially in western countries. However, methiin-rich species were often used in Asia and therefore the tolerance against the 'hard' taste of methiin-deduced volatile compounds seems to be much higher.

Allicin **12** and related thiosulfinates **10** are responsible for the typical smell and taste of freshly crunched garlic. It is also believed, that allicin is the most active compound of garlic (for details see below). But unfortunately this compound is highly instable. A simple experiment can demonstrate this: if fresh garlic juice is kept for one day at room temperature, the taste and smell will change significantly. Allicin **12** itself has a rather aromatic smell and taste. After one day of standing at room temperature, the smell and taste is still remaining to garlic, but is somewhat unpleasant and interpreted as 'strong'.

The reason for this observation is that allicin **12** undergoes a number of different reactions at room temperature, especially at increased temperature, which can be explained as reactions of allicin with itself under mild acid catalysis. The major product of hydrolysis of allicin **12**, which can be increased by boiling, is diallyl trisulfide **19** besides diallyl disulfide **18** and other polysulfides (*28–30*). These products are named 'secondary aroma components'. Hydrolysis of allcin results in 2-propenethiol and 2-propene-1-sulfenothiolic acid, also known as 'allylperthiol'. The reaction of 2-propenethiol with allicin **12** gives diallyl disulfide **18** and one molecule of allylsulfenic acid **11**, which is subjected to further reactions (*e.g.*, rearrangement of two molecules of **11** to one molecule of **12**). 2-Propene-1-sulfenothiol will also react with allicin **12** to give one molecule diallyl trisulfide **19** and one molecule allylsulfenic acid **11**.

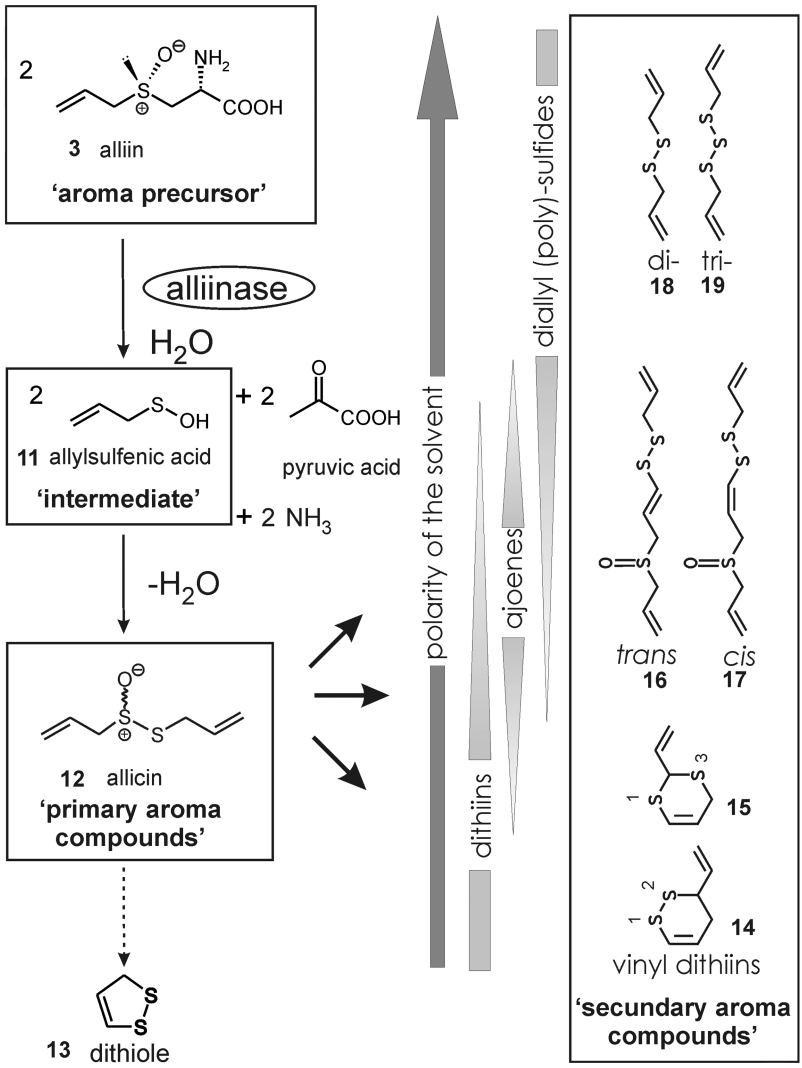

*Figure 4. Volatile sulfur compounds derived from the precursor alliin **3**. After enzymatic cleavage, the primary aroma product is allicin **12**, which is formed via the intermediate allysulfenic acid **11**. Allicin **12** can be degraded to 3-H-1,2-dithiole **13**, e.g. by thermal decomposition. Mainly in dependence on the polarity of the solvent, various volatile compounds **14** – **19** have been observed.*

As shown in Figure 4, the formation of these 'secondary aroma components' depends on the kind of solvent. If *e.g.*, alcohol is used instead of water, three molecules of allicin **12** will formally give two molecules of (*E/Z*)-ajoene **16** and **17** by the loss of one molecule water (*31*, *32*). However, this simple equation does not fully reflect the rather complex mechanism of the ajoene formation. According to Kice (*33*), two molecules of allicin 12 will react with each other in a kind of 'S-thiolation' by the loss of one molecule allylsulfenic acid **11**. The result will be

a thiosulfonium ion, from which a second allylsulfenic acid **11** will be eliminated by internal rearrangement. The formed disulfinde carbocation is highly reactive; the positive charge can be located to the terminal carbon allowing a 'γ-attack' or 'vinylogous addition' of one molecule allylsulfenic acid giving (*E/Z*)-ajoene **16** and **17**. In summary, 1.5 molecules of allicin **12** will give one molecule of (*E/Z*)-ajoene **16** and **17**. It is worth to mention, that the ajoenes do have a chiral sulfinyl group (S=O), from which two stereoisomers do exist like alliin **3**. Ajoenes **16** and **17** have slightly different bioactivities.

A further reaction occurs in unpolar solvents, *e.g.*, oily macerates of garlic. Interestingly, the same reaction was observed, when allicin **12** is analyzed under GC conditions. By these experiments, the 'secondary aroma compounds' 3-vinyl-*4H*-1,2-dithiin **14** and 3-vinyl-*4H*-1,3-dithiin **15** were formed in a ratio of about 1:4 (*31*, *32*). Interestingly, pure dithiine **14** has a rather pleasant smell, which remains to garlic, were as dithiin **15** has a really unpleasant smell remaining to burned gum. Dithiins can be easily synthesized out of two molecules of thioacrolein. In unpolar solvents, allicin **12** can decompose in one molecule allyl sulfenic acid **11** and one molecule thioacrolein (*34*). In hydrogen-bond-donating solvents, *e.g.* water, the allicin **12** is not so susceptible for this reaction.

Finally, thermal decomposition can also lead to *3H*-1,2-dithiole **13** (*35*, *36*). This product can be also seen as a thermal artifact during GC analysis. Probably, the dithiole **13** is formed by a rearrangement of allicin **12**, leading to the loss of one molecule allylalcohol.

Concerning the development of the flavor of garlic preparations, another fact must be additionally taken into account: it is well known, that allicin **12** can directly react with amino acids like cysteine (*3*). In order to see if there are further effects of this kind, incubation experiments with different amino acids were undertaken by the author (*37*). A much unexpected result occurred, when isolated alliinase from garlic was incubated with alliin **3** and the amino acid cystine: the analysis of the volatile compounds by HPLC did not yield any allicin **12**, but therefore di- and polysulfides (Figure 5). Interestingly, allyl trisulfide **19** was the main compound, which can be expected as major product by decomposition of allicin **12** under aqueous solutions. Besides the sulfides, elementary sulfur could be detected.

The proposed mechanism of this reaction is schematically drawn in Figure 6. Besides the alliinase activity described above (C-S- lysis of cysteine sulfoxides **7**), the enzyme catalyses the C-S lysis of cystine in a manner of a cystine lyase. Ammonium, pyruvate and elementary sulfur but not cysteine could be detected as primary reaction products. The ratios between the enymatic products cystine, ammonia and pyruvate are 1:1.9:1.9 suggesting a new type of reaction mechanism. Thiocysteine and disulfine were assumed as intermediates. The pH-optimum of the cystine lyase activity was found at pH 7.5 and the temperature optimum was at 44 °C. The K_M-value for the homogeneous enzyme was at 2.65 mM and V_{max} was at 4.12 nkat/mg using cystine as substrate. This value is much lower as that found for the alliinase activity (about 2.5 μkat/mg).

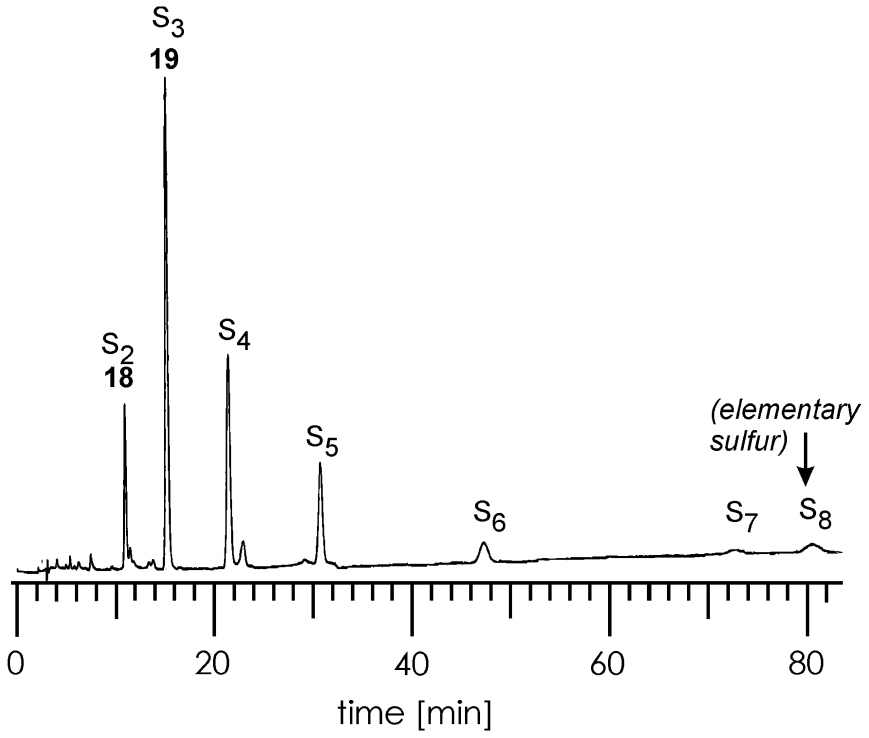

*Figure 5. HPLC chromatogram showing diallyl (poly)sulfides **20** resulting from a parallel incubation of alliin **2** and cystine with garlic alliinase. The number of sulfur atoms is indicated above every peak. At 81 min, elementary sulfur was observed.*

During the parallel incubation of alliin **3** and cystine, a pale yellow residue was observed even after a few minutes of incubation. It is proposed, that formed disulfine can discompose to give sulfur directly or can act on allicin **12**. A direct action on the intermediate allylsulfenic acid **11** seems to be also possible. But because of the big differences in the V_{max} values of both alliinase activities, it can be assumed that allicin **12** is synthesized first followed by increasing amounts of disulfine.

In summary, the presence of cystine will alter the composition of garlic volatile compounds significantly toward allyl (poly)sulfides. Thus, the significance of alliinase and its enzymatic products has to be newly considered in terms of ecological, pharmacological, and biochemical aspects. As also described above, the used solvent for garlic preparations as well as temperature have a strong influence on the pattern of the 'secondary aroma compounds'. In addition to cysteine sulfoxides **7**, amino acids like cysteine have to be considered as reaction partners. It can be expected that even more 'secondary aroma compounds' of garlic and related species will be discovered in future.

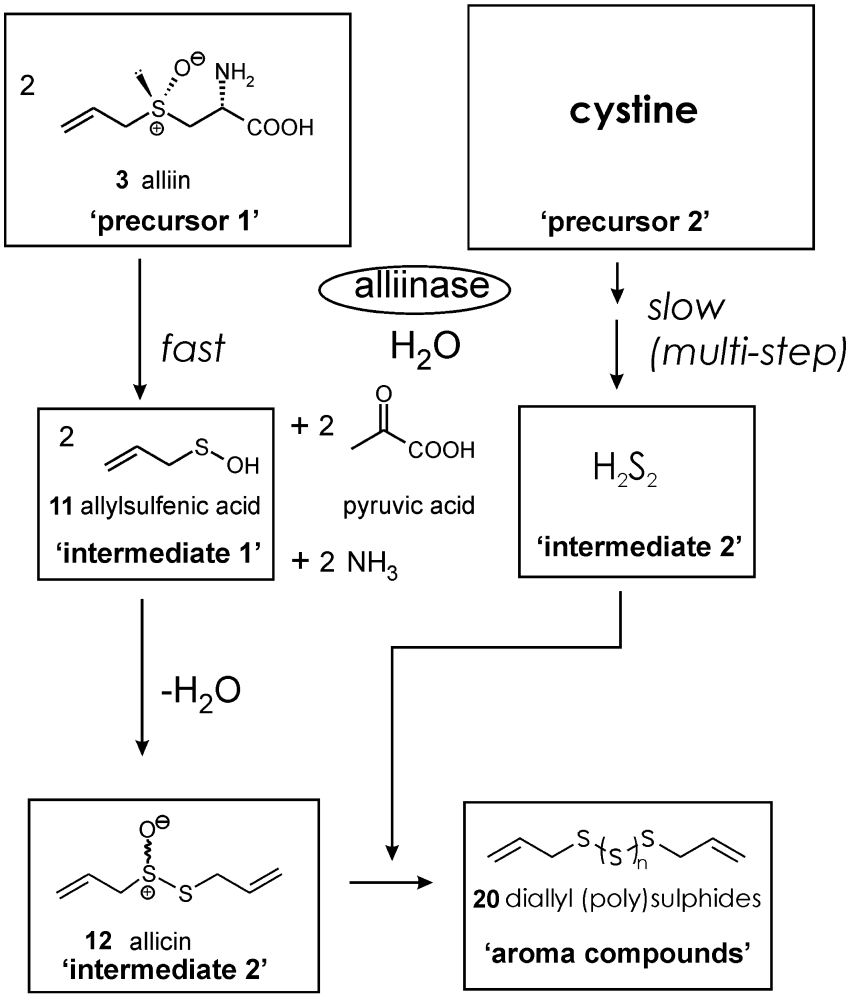

*Figure 6. Proposed mechanism for the direct formation of diallyl (poly)sulfides **20** out of alliin **3** and the amino acid cystine. Cystine is converted to the highly reactive disulfine, which directly acts on allicin **12**. As shown in Figure 5, allicin **12** could not be detected by HPLC methods and seems to be only an intermediate in this reaction.*

Volatile Compounds Related to Common Onion (*Allium cepa*)

The chemistry of common onion is in many parts different from that of garlic. Formation of aroma components is schematically given in Figure 7. Both, garlic and onion, have cysteine sulfoxides **7** as precursors of aroma compounds. Methiin **1** occures ion both species in more or less high amounts. Hower, instead of alliin **3** in case of garlic, common onions contains isoalliin **4**. This different will lead to a complete diverse pattern of aroma compounds. Further on, very hot garlic contains

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about 1.2 % (fresh weight basis) cysteine sulfoxides **7** while a very pungent onion contains about 0.25 % cysteine sulfoxides **7**. As explained in the introduction section, high amounts of onions are necessary for yielding 'onion oil' by steam distillation.

In both garlic and onion, cysteine sulfoxides **7** are subjected to the enzyme alliinase when plant material is crunched. As explained above, alliinase isoenzymes from onion and garlic are different, but do basically catalyze the same reaction. 'Primary aroma compounds' of alliinase reaction are thiosulfinates **10**, *e.g.*, dimethyl thiosulfinate **24**, probably accompanied by mixed thiosulfinates **10** carrying one 1-propenyl moiety.

However, there is a significant difference in the biochemistry of garlic and onion: common onion is eye-irritating and garlic is not. What is the reason for this difference? A simple experiment demonstrates this: purified isoalliin **4** was incubated with purified alliinase from garlic. After a few seconds, a smell appeared which remained to onion, but was much more 'smother'. After a while, the obtained solution was only very slightly eye-irritating. If, in contrast, isoalliin is incubated with a purified proteine extract of onions, which contains further proteins besides alliinase, the 'lachymatory factor' LF causing a strong eye irritation occurred immediately.

The explanation for this observation is that an additional enzyme in onion, the 'LF synthase' is necessary, to form LF (*38*). This enzyme is responsible for a fast (!) conversion of (*E*)-1-propenesulfenic acid **21** into (*Z*)-propanethial *S*-oxide, the onion LF **23**. Recently, the homologous compound (*Z*)-butanethial *S*-oxide **51** (Figure 10) has been discovered. This compound is typical for species of the subgenus Nectaroscordum (*19*). This means that also species producing the thial *S*-oxide **51** must have a LF synthase.

But there is still one point, which needs further investigations: after the enzymatic generation of the sulfenic acid **21**, spontaneous formation of the corresponding di-(1-propenyl) thiosulfinate must be prevented by some mechanisms. It is most likely, that the LF synthase is closely placed to the alliinase that sulfenic acid **21** can be directly hand over to the active center of the LF synthase. But on the other hand, methylsulfenic acid **22** must be released from the alliinase without blocking the LF synthase. The detailed mechanism for this is still unclear.

In summary, the enzymatic system of onions and some other species of the genus *Allium* will lead to LF **23** as well as to thiosulfinates **11**. This makes the chemistry of the secondary aroma compounds, which is summarized in Figure 8, much more complex as this of garlic. A similarity to garlic is the occurrence of sulfides like disulfides **32** and polysulfides **31**. As explained above for allicin **12**, dimethyl thiosulfinate **24** can undergo the same reaction, mainly in aqueous solution or during a distillation process. Also 'mixed' di- and polysulfides are possible.

It must be mentioned, that volatile organoselenium compounds related to the amino acid cysteine occur in onion and other *Allium* species (*16*). Compound **31a** is an example for a mixed sulfur-selenum volatile, which has an analogous structure to allyl trisulfide **19**. The number of selenium molecules is variable. The residues R and R′ are mainly methyl- and/or propyl-groups.

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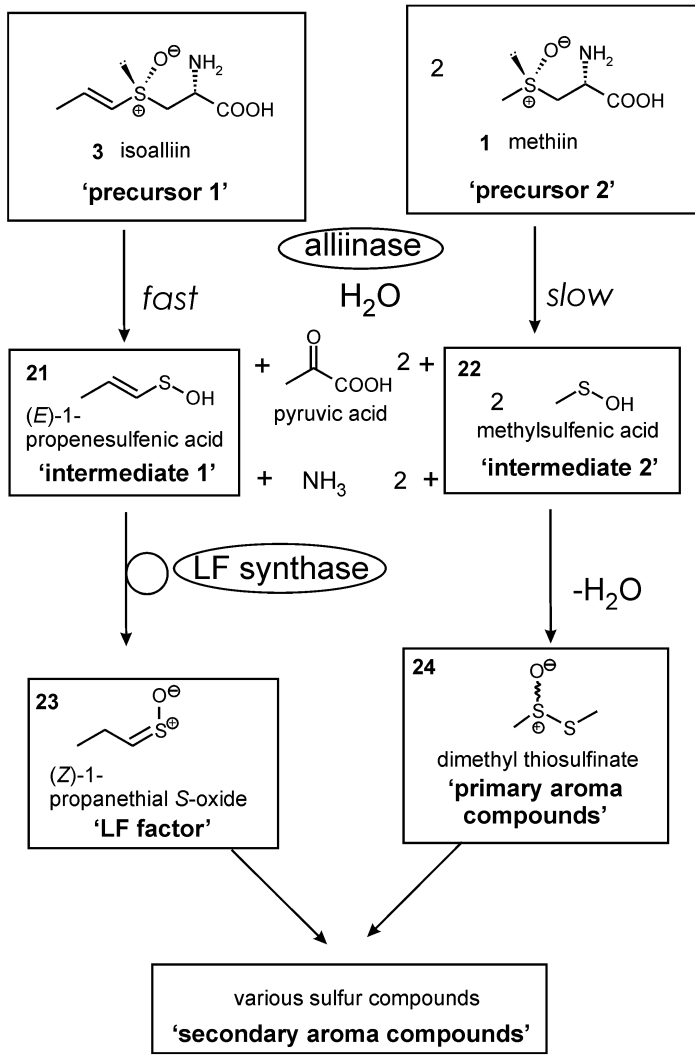

*Figure 7. Enzymatic formation of the LF factor **23** out of isoalliin **3**. This reaction is catalyzed by the enzyme alliinase and the LF synthase. Alternatively, the alliinase can convert further cysteine sulfoxides (e.g., methiin **1**) into the corresponding thiosulfinates. As demonstrated in Figure 2, mixed thiosulfinates are also possible. All of these primary aroma components are instable and react rapidly to a number of secondary aroma components (see Figure 8).*

The organoselenium compounds occur only in very small amounts in *Allium* plants. Concentration can be increased by fertilization with selenium salts. It can be assumed that alliinase and non-enzymatic steps are involved in the formation of these compounds. However, the required precursor selenomethiin **1a** has not been discovered until now. Therefore, it is more likely that non-enzymatic processes are responsible for the formation of sulfur-selenium compounds like **31a**.

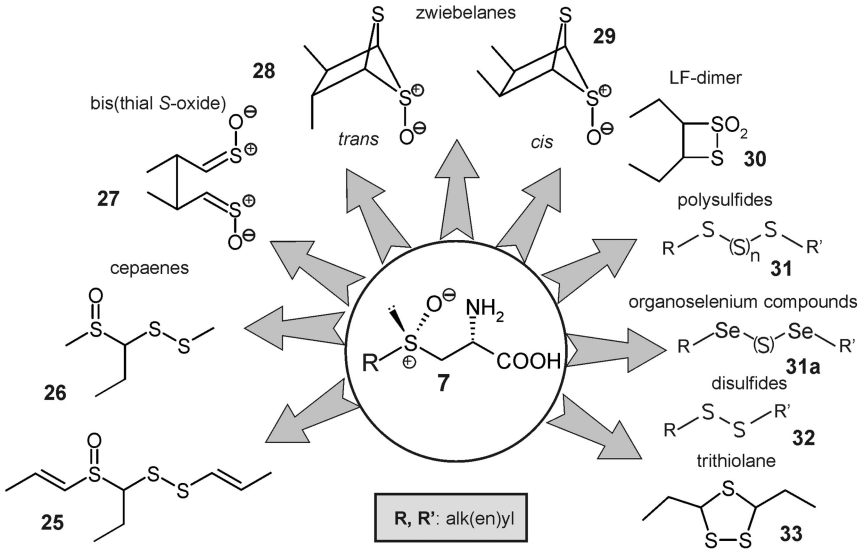

*Figure 8. Summary of 'secondary aroma compounds' derived from isoalliin **7**. Also further aliphatic cysteine sulfoxides can be involved. Main products are cepaenes (**25**, **26**), bis(thial S-oxide) **27**, zwiebelanes (**28**, **29**), the LF-dimer **30**, polysulfides **31**, disulfides **32** and the (cis/trans) 3,5-diethyl-1,2,4-trithiolane **33**. Organoselenium compounds **31a** were found in various Allium species, where R, R' are mostly methyl residues.*

There are several possibilities for secondary reactions of LF **23**. If LF is kept in a unpolar, water free solvent, the slightly yellow, non-lachrymatory compound trans-3,4-diethyl-1,2-dithietane 1,1,-dioxide, named 'LF-dimmer' **30** will occur. The LF dimmer has a strong onion-like odor (*39*). From onion extracts, a rather similar compound could also be identified as bis(thial *S*-oxide) **27**. The correct name for **27** is (*Z,Z*)-*d,l*-2,3-dimethyl-1,4-butanedithial 1,4-dioxide (*40*, *41*). It is proposed, that **27** is formed via a bis-sulfoxide, which is also a dimmer of LF **23** (dimerization between the sulfur atoms and shift of the double bond between C1 and C2).

A very prominent group of onion aroma compounds are cepaenes. Two examples were given in Figure 8: 1-propenyl 1-(1-propenylsulfinyl)propyl disulfide **25** and methyl 1-(methylsulfinyl)propyl disulfide **26** (*42*, *43*). It was proposed, that sulfenic acids **9**, resulting from the alliinase reaction on methiin **1** (in this case, the sufenic acid would be methylsulfenic acid **22**) or on isoalliin **4** (1-propensulfenic acid **21**), make a 'carbophilic attack' on C1 of the LF **23**. In a second step, a further sulfenic acid **9** is condensed by the loss of one molecule water. Because various sulfenic acids **9** can be involved in the formation of cepaenes, many different structures are thinkable. More examples were given in Figure 10. In summary, usually one molecule of LF **23** and 2 molecules of sulfenic acids **9** with different residues are involved. It also must be considered, that thiosulfinates **10** can be hydrolyzed to give two molecules of sulfenic acid **9**.

Also another group of LF dimmers can be isolated from freshly pressed onion juice: the 'zwiebelanes' **28** and **29** (*41*). Firstly, it was suggested that the alliinase reaction would also lead to 'allicin-like' condensation products of 1-propenesulfenic acid **21**. In deed, a pair of molecules with the formula $C_6H_{10}S_2O$ could be isolated, but the spectroscopic data did not fit to a thiosulfinate **9**. Instead of this, the isomeric zwiebelanes *trans*-2,3-dimethyl-5,6-dithiabicyclo[2.1.1]hexane 5-oxide **28** and *cis*-2,3-dimethyl-5,6-dithiabicyclo[2.1.1]hexane 5-oxide **29** could be identified. Zwiebelanes are rather stable and can be analyzed by GC.

Finally, the LF can also be hydrolyzed. The final products are propanal, H_2S and SO_2 (*44*, *45*). It is proposed, that two molecules of propanal and three molecules H_2S can condensate to give *cis/trans*-3,5-diethyl-1,2,4-trithiolane **33**. In summary, three molecules of LF **23** must be hydrolyzed to give one molecule of trithiolane **33**.

With the examples listed above it could be demonstrated that the onion chemistry – but also the chemistry of closely related species – is rather complex. It can be expected that variation of extraction condition of onion material will even increase the number of 'secondary aroma components'. Additionally, the number of cysteine sulfoxides **10** and 'primary aroma components' has been increased by hybridization of common onion with various wild *Allium* species (*46*, *47*). In total, hybrids obtained by the crossbreeding of *Allium cepa* (onion) as the mother plant and seven taxonomically distant wild species obtained by embryo rescue, were investigated with special respect to their individual profiles of cysteine sulfoxides as well as enzymically and nonenzymically formed aroma substances. Cysteine sulfoxides **7** as well as alliinase activity were found in all investigated samples at different levels, but methiin **1** was the most abundant sulfoxide present. Isoalliin **4**, coming from the mother plant (onion), was found in all investigated hybrids. The pattern of the other cysteine sulfoxides **7** depended strongly on the parent plants used. The profile of aroma components corresponded with the related pattern of aroma precursors. Successful hybridization was proven by randomly amplified polymorphic DNA analysis.

However, one remaining question from these investigations is about the presence and the activity of the LF synthase, which was not investigated. In case that the LF synthase is still active, a fairly high number of condensation products, especially those of the cepaene-type, can be expected. Also, the number of different thiosulfinates 10 should be rather high in these hybrids, but individual compound were not analyzed. But even from the gained results it can the concluded, that crossbreeding by embryo rescue is an excellent method to modify aroma properties of common onion.

Finally, it is worth to summarize the common features and the differences between garlic and onion: both have cysteine sulfoxides **7**. Both have methiin **1**; the characteristic cysteine sulfoxide **7** of garlic is alliin **3**, were as isoallin **4** is characteristic for onion. Both have the enzyme alliinase, but onion has additionally the LF synthase. Therefore, an unique feature of onions is the LF **23** ('primary aroma compound' of onion). For garlic, thiosulfinates **10**, mainly allicin **12**, are the characteristic 'primary aroma compounds', were as the zwiebelanes **28**, **29** are the analogous structures of onions. They even will be formed, when the LF

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synthase is inactivated (for details see below). Trimeric structures of allylsulfenic acid **11** and the LF **23** are ajoenes (**16**, **17**) and cepaenes (**25**, **26**), respectively. Di- and polysulfides can be found as 'secondary aroma compounds' for both species. In unpolar solvents, vinyl dithiins (**14**, **15**) and the LF-dimer **30** were formed as 'secondary aroma compounds' in garlic and onion, respectively.

Tearless Onions

It is a well known observation that onions and garlic cultivated at different locations do have different flavors and also can significantly differ in pungency. However, even at the same location, differences can be large from year to year (*20*). As one factor, the sulfur content of the soil in relation to the pungency has been well investigated. Usually, normal soil contains sufficient sulfur, meaning that the onion can reach maximum production of isoalliin **3**. If the soil does contain sufficient sulfur above the 'saturation point', additional fertilization with sulfur does not show any effect.

Fort the production of mild onions it is necessary to grow them on soils with sulfate content not higher as 50 ppm. Because sulfate is a leachable ion, lighter soils and especially sandy soils are preferred for onion production. Heavy, loamy soils and highly organic soils have to be avoided because they can store sulfate over a longer time. On the other hand, restricted amount of sulfate also resulted in lower bulb yields.

An alternative way to get tearless onions can be archived by modern gene technologies. After genetic transformation of plants, the LF synthase gene can be suppressed by using RNA interference silencing (*48*). This modification reduced LF synthase activity by up to 1,544 fold, meaning that 1-propenesulfenic acid **21** can not be converted into LF **23**. Instead of LF **23**, higher yields of di-1-propenyl thiosulfinate ('primary aroma compound') were observed.

As a consequence, further production of 'secondary aroma compounds' was altered. The production of zwiebelane isomers (**28**, **29**) and production of disulfides **32** was increased. Additionally, 2-mercapto-3,4-dimethyl-2,3-dihydrothiophene was observed, which had previously been reported only in trace amounts or has not been detected in onion. In summery, the whole pattern of volatile compounds was changed also affecting odor, taste and health benefits of genetically modified onions.

Sulfur Compounds of *Allium ursinum* L.

The sulfur chemistry described in the chapters above is also valid for most wild *Allium* species (*11*). Great exceptions are those species belonging to the subgenera Melanocrommyum and Nectaroscordum. These exceptions in sulfur chemistry will be explained in detail below.

But it must be pointed out that a correct identification of wild plant material with reference to existent herbarium vouchers and/or plants is absolutely necessary before starting chemical work. Further on, according to good scientific practice, GPS (global positioning system) coordinates of natural plant material

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should be recorded. It is an observation of the author that plant material coming from botanical gardens does not fit to the given scientific name in many cases. Because only few botanical gardens are supervised by curators familiar with the complex taxonomy of *Allium*, unproved material coming from those gardens should be avoided. It also must be mentioned, that material from plant breeders can be only accepted for scientific work, if the natural origin of the material is carefully documented and hybridization can be excluded.

As a good example that main parts of garlic chemistry – and also partially onion chemistry - can be applied to a wild species, research on *Allium ursinum* is described below. Leaves of ramson (also named wild garlic or bear's garlic, *Allium ursinum* L., a wild-growing *Allium* species in the forests of Europe and northern Asia, are wildly used in traditional medicine and as a spice. Consequently, attempts are currently undertaken to cultivate *A. ursinum*. The herbaceous plants grow up to a height of 50 cm and feature pseudoumbels with white flowers as well as elongated bulbs not exceeding 6 cm in size. Ramson bears trichotomic capsules with black seed as fruits (*27*). Similar to garlic (*A. sativum* L.), ramson contains high amounts of cysteine sulfoxides as well as the enzyme alliinase (EC 4.4.1.4). Mainly methiin **1** and alliin **3**, but also propiin **2** and isoalliin **4** were found in all parts of the plants. Consequently, numerous volatile sulfur-containing compounds would result from alliinase reaction on these cysteine sulfoxides. The 'primary aroma compounds', the thiosulfinates **10**, should carry methyl, propyl, 1-propeny and 2-propenyl residues in all thinkable combinations. That means, in total 16 different thiosulfinates, but in different concentrations, can be expected making detailed analysis nearly impossible. In this case, allinnase follow-up products are a kind of 'combinatorial sulfur chemistry'.

But chemistry got even more complex. These thiosulfinates will be rapidly converted into 'secondary aroma compounds' with an even higher variation. By means of GC-MS, the following substances could be detected ((*27*), Figure 9): methyl 2-propenyl sulfide **34**, propylthiol **35**, dimethylthiophene **36**, dimethyl disulfide **37**, (*E*)-methyl 1-propenyl disulfide **38**, (*Z*)-methyl 1-propenyl disulfide **39**, methyl propyl disulfide **40**, methyl 2-propenyl disulfide **41**, dipropyl disulfide **42**, 2-propenyl propyl disulfide **43**, (*Z*)-1-propenyl propyl disulfide **44**, (*E*)-1-propenyl propyl disulfide **45**, (*E*)-1-propenyl 2-propenyl disulfide **46**, di-2-propenyl disulfide **18**, dimethyl trisulfide **47**, methyl propyl trisulfide **48**, dipropyl trisulfide **49** and di-2-propenyl sulfide **50**. Most detected substances were disulfides **32**.

The composition and concentration of cysteine sulfoxides **7** as well as volatile compounds was monitored over a period of 3 months. Highest amounts of cysteine sulfoxides **7** in bulbs were reported in the dormant phase (about 0.3%). With the appearance of the first leafs, which were also very rich in cysteine sulfoxides **7** (about 0.4%,), sulfur compounds from bulbs were probably moved into leaves, so that total concentration of cysteine sulfoxides **7** inside the bulbs felt down below 0.1% to get higher again in the late vegetative phase of the plant.

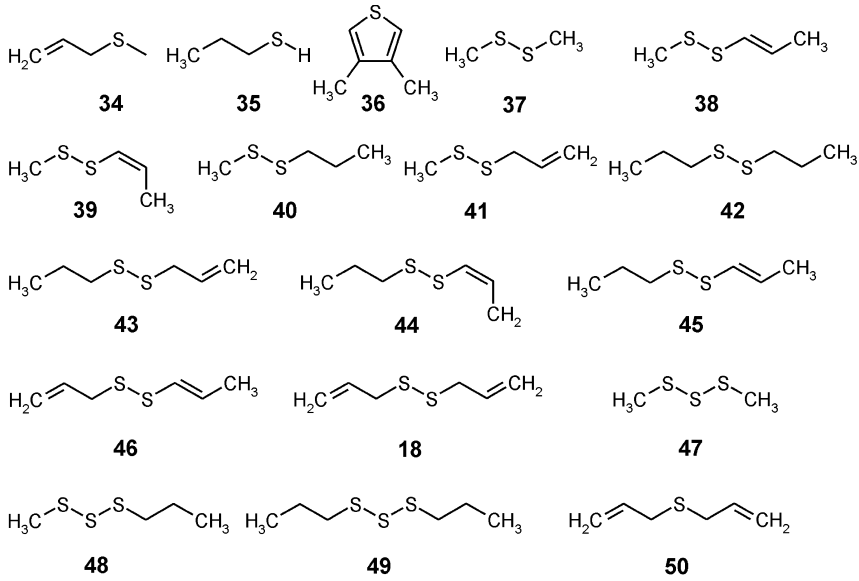

Figure 9. 'Secondary aroma compounds' found in A. ursinum. For details see text.

But also the pattern of cysteine sulfoxides **7** changed during vegetation period. In both, bulbs and leafs, methiin **1** is the main cysteine sulfoxide **7**. But the amount of alliin **3** steadily increased until week 8 of the study. In leafs, isoalliin **4** showed the same tendency. Propiin **2** is only a minor constituent over the whole course of investigations.

These changes were also reflected by the analysis of volatile compounds. Methyl 2-propenyl disulfide **41** and di-2-propenyl disulfide **18** were the main volatiles in the investigated plant parts. Volatile **18**, which is derived from alliin **3**, increased until week 8, whereas disulfide **41**, which is derived from methiin **1** and alliin **3**, decreased over the same period of time. Dimethyl disulfide **37**, coming from two molecules of methiin **1** via dimethyl thiosulfinate **24** (compare onion-biochemistry Figure 7), was also high in the beginning of investigations, but disappeared until week 8 in leafs.

Some conclusions can be drawn, which are also valid for further wild *Allium* species showing a broad variation of cysteine sulfoxides **7**: i) the pattern of primary and secondary aroma compounds is very complex ('combinatorial sulfur chemistry'). The pattern is not stable over the vegetation period. That means, time of harvesting significantly affects odor and taste of *Allium* material. ii) The bulb shows highest concentrations in the dormant phase. These bulbs should be very pungent. iii) Leaves showed highest concentration at early spring. After that, concentration of cysteine sulfoxides **7** and by that concentration of sulfur volatile compounds continuously decreased.

Sulfur Compounds Related to *A. tripedale* Trautv. and *A. siculum* Ucria

Allium tripedale Trautv. belonging to the subgenus Nectaroscordum grows naturally in the mountainous areas of northwest Iran. The fresh leaves, which were sometimes transported over hundreds of kilometres inside Iran, are highly prized by the local populations as a spice vegetable and were mostly used for the preparation of a special bread (*49*). In the area of the Iranian city Yasuj, the leafs of *A. tripedale* were also used as medicine and are named 'khargriv', which means 'donkey-cry'. The leafs are so hot that even a donkey will cry when eating them.

If plant material of *A. tripetale* Trautv. is crunched, a very strong and somewhat unpleasant smell occurs immediately, which is accompanied by a slight eye irritation. The taste of *A. tripedale* is very pungent. In Iran it is believed that *A. tripedale* is the hottest *Allium* species of all. Especially the irritation of eyes led to the assumption that the sulfur chemistry of this plant is related to that of common onion. In a closely related species, *A. siculum* Ucria, *S*-butylcysteine sulfoxide (butiin, **5**, Figure 1) has been already reported (*50*). This compound could be also detected in *A. tripedale*. Besides this compound, *S*-methylcysteine sulfoxide (methiin, **1**, Figure 1), an ubiquitous cysteine sulphoxide, was found in both species. These compounds have been also reported as their corresponding γ-glutamyl dipeptides. Also γ-glutamyl dipeptides were frequently reported for common onion.

However, isoalliin **5** as the dominant cysteine sulphoxide of *A. cepa* could not be detected. Instead of this, (+)-*S*-(1-butenyl)-L-cysteine sulfoxide (homoisoalliin **6**, Figure 10) was found as the leading compound, but mostly as the corresponding γ-glutamyl derivatives. Analogously, no LF **23** was detected, but butanethial *S*-oxide **51**, which is a homologues compound to the propanethial *S*-oxide (LF **23**) of common onions (*19*). Additionally, several 1-butenyl thiosulfinates could be detected by DART (direct analysis in real time) mass spectrometry. This method was newly applied to *Allium* extracts allowing rapid detection of 'primary aroma compounds'. These compounds can be explained by the action of alliinase.

All these products are highly instable. It was possible to determine a number of 'secondary aroma compounds' from *A. tripedale* (Figure 10). Bulb material was crunched to allow digestion of cysteine sulfoxides **7**. After 30 min, enzymatic products were extracted by ethyl acetate and were subjected to HPLC-MS/MS. The obtained yields were extremely low (below 1 mg) so that analysis was only possible by MS/MS experiments and HR-MS (high resolution mass spectrometry). Obtained mass spectra have been selectively screened for a fragment ion at 87 amu in positive ionization mode representing a butenyl-thio moiety. This strategy was chosen to find specifically alliinase products of homoisoalliin **6**. About 14 substances could be detected by a fragment ion at 87 amu. Out of this, the structure of compounds **52** – **55** could be identified.

Di-(1-butenyl) disulfide **52** was an expected aroma compound, which is probably directly derived from di-(1-butenyl) thiosulfinate. Substances **53** – **55** are cepaenes and are homologues structures to cepaenes **25** and **26**. For cepaene **53**, enzymatic degradation of two molecules of homoisoallin **6** and two molecules of methiin **1** are necessary. Cepaene **54** can be explained by alliinase digestion of

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two molecules homoisoallin **6**, one molecule methiin **1** and one molecule butiin **5**. Analogously, three molecules homoisoalliin **6** and one molecule methiin **1** are required for **55**.

In conclusion, *A. tripedale* and also *A. siculum* seem to have a rather similar chemistry to common onion, *A. cepa*. Both species (*A. cepa* and *A. tripedale*) have been used as vegetable, spice or even as medicine. Isoalliin **4** is the most important cysteine sulfoxide of *A. cepa*, whereas *A. tripedale* and *A. siculum* contain the homologous compound homoisoalliin **6** and the corresponding butane LF **51**, which were reported in nature for the first time. But already the Finnish Nobel Laureate Artturi Ilmari Virtanen predicted this compound in nature (*51*). This theory of Virtanen could be proven nearly 45 years later being a nice example that research on *Allium* volatile sulfur compounds is rather time expensive and strongly depends on the development of new analytical methods (*e.g.*, DART mass spectrometry). Described similarities between the chemistry of *A. cepa* and *A. tripedale* as well as *A. siculum* are unique in nature and are helpful for the understanding of the complex sulfur chemistry of the genus *Allium*.

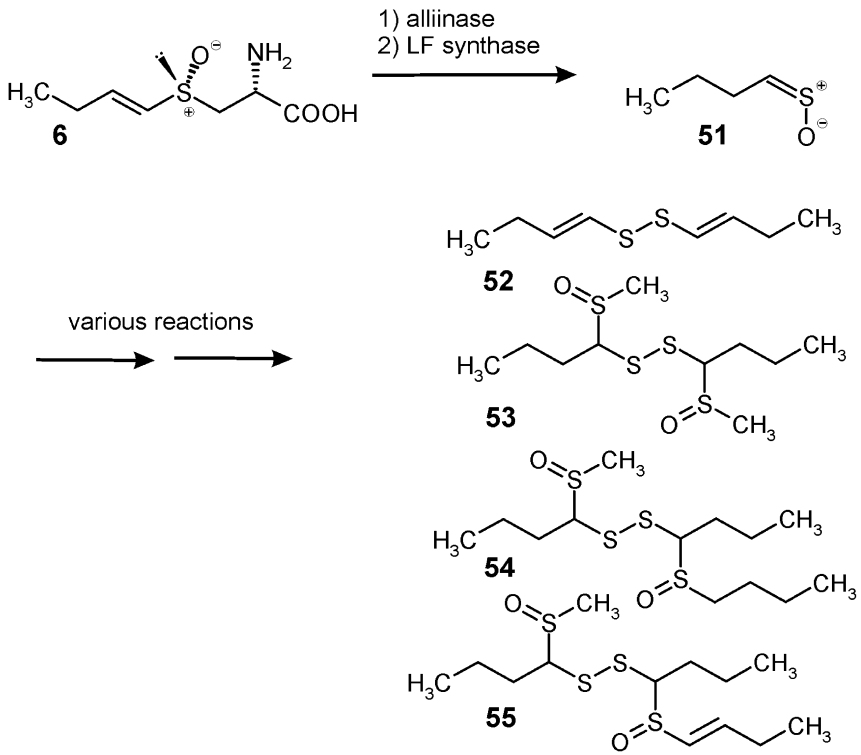

*Figure 10. Enzymatic conversion of the homoisoalliin **6** into the butanethial S-oxide (butane LF **51**). This compound rapidly discomposes into di-(1-butenyl) disulfide **52** and a whole set of cepaene-like substances (**53** – **55**).*

Despite the small differences in the chemistry of *A. cepa* and *A. tripedale*/*A. siculum*, both plants have a completely different smell and taste. The smell and taste of *A. tripetale*/*A.siculum* are absolutely unique and not comparable to those of other *Allium* species. Basically, taste is very pungent with a strong sulfur note. These findings might give new input to plant breeders. It is also thinkable that carefully dried bulbs and leaves of *A. tripedale* can be merchandized as a spice also in the Western World.

As a last aspect of these investigations, it could be demonstrated that the sulfur chemistry of *Allium* species located in South West and Middle Asia is much more complex and diverse than the chemistry of those species which were traditionally used as vegetable, spice or medicine in the Western World. *Allium* species from Asia seems to be an excellent source for new sulfur compounds and aroma constituents as it also was reported previously (*13*, *52*, *53*). These findings should have a significant impact on plant breeders, especially those located in Asia.

Sulfur Compounds Related to *A. stipitatum* Regel

Allium species of the subgenus Melanocrommyum, to which *A. stipitatum* Regel belongs, exhibit a great variability (>200 species are known until now), and the center of distribution is in Middle and in Southwest Asia. Countries with a high diversity of this subgenus are Iran, Tajikistan, and Uzbekistan. Some of these species are known as ornamental plants in the western hemisphere, the so-called "drumstick onions". Genetic analysis displayed a high similarity of most of these ornamental plants. These species show a high polymorphism. By using genetic analysis, these plants could be related to the subgenus *Melanocrommyum* (*54*).

In Middle Asia, *Allium* species of the subgenus *Melanocrommyum* have a large range of usage. The characteristic smell and taste make the bulbs and leaves of these plants favored vegetables of the native populations. In several cases, the amounts of cysteine sulfoxides and their metabolites are rather high, so that the plants are used as spicy vegetables or are even not edible. *A. stipitatum* is a very common edible *Allium* species in Central Asia and is intensively used by local populations as a spicy vegetable and medicinal plant. In Iran, the plant is named "Mu-sir", and in countries of the former Soviet Union, it is known as "Anzur". Beside *A. stipitatum*, several species of the subgenus Melanocrommyum are used in folkmedicine.

As an example, leaves and bulbs of *Allium severtzovioides* R.M. Fritsch are applied against stomach and duodenum diseases (*12*). *Allium motor* Kamelin et Levichev leaves are served as a tonic soup. *Allium komarowii* Lipsky is used as an anabolic for horses. Beside these extraordinary uses, it is applied against anemia and bad blood circulation. *Allium suworowii* Regel is used against early forms of bronchitis and tuberculosis.

In addition to common sulfur compounds described above, new steroid saponins of the spirostan series were isolated and identified (*55*, *56*) in the bulbs of *A. stipitatum* and *A. suworowii* but no reports about unusual cysteine sulfoxides were found. However, the alliinase activity of *A. stipitatum* was found to be

similar to other *Allium* species. The pH optimum (pH 7.5) and the temperature optimum (38 °C) were typical values (*26*). Beside these findings, O'Donnell *et al*. suggested three new volatile compounds in samples of *A. stipitatum*, which showed antimicrobial activity (*57*). These compounds were identified as N-oxides, also described for basidomycetes (*58*). Extracts showed an activity against *Mycobacterium tuberculosis*.

Based on these findings, authentic *A. stipitatum* and also *A. altissimum* from natural origin were re-investigated (*49*, *59*). Methiin **1** was found as the main cysteine sulphoxide in concentrations up to 0.8%, related to the fresh weight of bulbs. Beside this well known substance, (+)-*S*-(2-pyridinyl)-L-cysteine sulfoxide (pyridinylcysteine sulphoxide **56**, Figure 11) was isolated for the first time and was found in concentrations up to 0.4%, related to the fresh weight of bulbs. If this compound is subjected to alliinase, a thiosulfinate **10** caring two pyridinyl residues can be expected. After incubation of crunched bulb material at room temperature for 30 min, the thiosulfinate **58** could be detected. Because also methiin **1** is present, the finding of the mixed thiosulfinate **60** was an expected result. Surprisingly, numerous other compounds were found. The main product was the di-sulfoxide **57**, a class of compounds, which was never observed in nature before. Substance **57a**, which is commercially available, could be excluded by spectroscopic methods. However, volatile compounds with a R-SO-SO-R structure are already described in literature (*60*). Normally, these compounds are intermediate products. The relatively high amount in the samples of this compound is a surprising observation.

Further on, dipydridyl disulfide **59** is a 'secondary aroma compound', which was probably formed from the thiosulfinate **58**. As explained by Figure 4, disulfides **18** are very common for preparations made of *Allium* material. Thiosulfinate **61** was also surprising and firstly no explanation for this unique molecule was available. But later on, it was found that many species belonging to the subgenus Melanocrommyum do contain the cysteine sulphoxide marasmin **66** (compare Figure 13). By a mixed alliinase incubation of **56** and **66**, the methylthiomethyl-residue can be fully explained.

In conclusion, *A. stipitatum* is widely used as a spicy vegetable and as a traditional medicine. However, no information is available about possible toxicity of the pyridine derivatives. The observed strong antibiotic effects as mentioned above are an interesting finding, but it can be assumed that the sulfur compounds do also show further bioactivities. This is of importance, because bulbs of *A. stipitatum* are eaten by several million people in Asia.

Only a limited number of pyridinyl compounds of *A. stipitatum* could be elucidated. Analogously to *A. sativum* and *A. cepa*, it can be expected that different post-harvest treatments of the crude bulb material will lead to different patterns of pyridinyl sulfur compounds. The proposed enzymatic cleavage of the pyridinylcysteine sulphoxide **56** leads to thiosulfinates like **58**, **60** and **61**. The alliinase theory only allows thiosulfinates as primary enzymatic products, also mixed thiosulfinates. Sulfides **57** and **59** are probably 'secondary aroma compounds'. The primarily formed thiosulfinates are highly reactive and are probably subjected to secondary and tertiary modifications. It is unclear if further enzymatic steps are involved in this procedure. Nevertheless, the results

strongly emphasize that N-oxides were not formed during or directly after alliinase reaction. The already described N-oxides are probably caused by further fermentation processes. Because of the complicated taxonomy of *A. stipitatum*, it can also be assumed that investigations described in ref. (*57*) were performed with a different species.

Sulfur Compound Related to *Allium giganteum* Regel

As already mentioned above, many species of the subgenus Melanocrommyum were widely used in traditional medicine of the countries of South West and Middle Asia. It was a surprising observation, that many of these species showed a red discoloration if plant material is wounded. This coloration occurs at all parts of the plant. For instance, a deeply red pigment is formed if bulbs or leafs of *A. giganteum* Regel are damaged. Interestingly, this red pigmentation turns to dark green after some time. This can be easily observed at herbarium vouchers of *A. giganteum*.

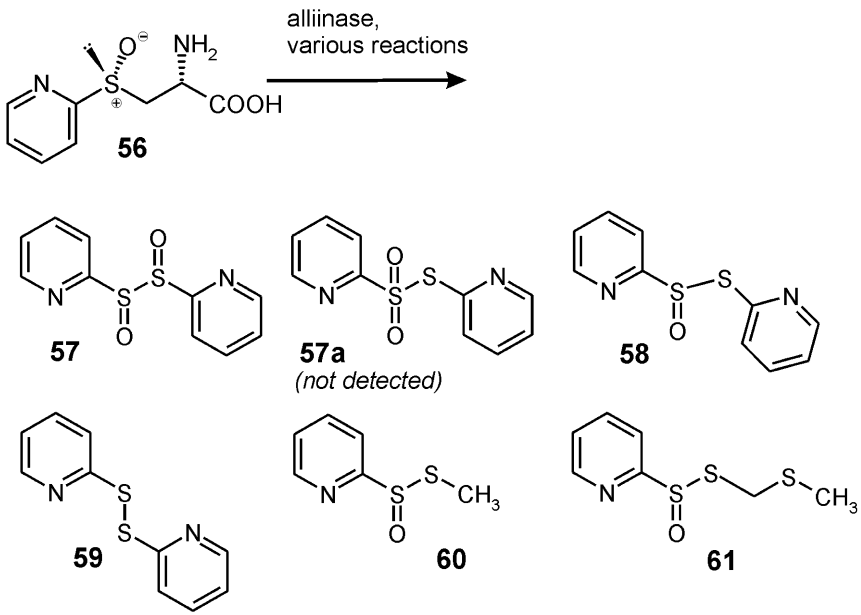

*Figure 11. Pyridinylcysteine sulfoxide **56** is degraded by alliinase into the thiosulfinate **58**, which undergoes several further reactions finally giving substances **57** and **59**. The mixed thiosulfinates **60** and **61** can be explained by simultaneous incubation of the cysteine sulfoxides **1** and **56** or **56** and **66** (Figure 13).*

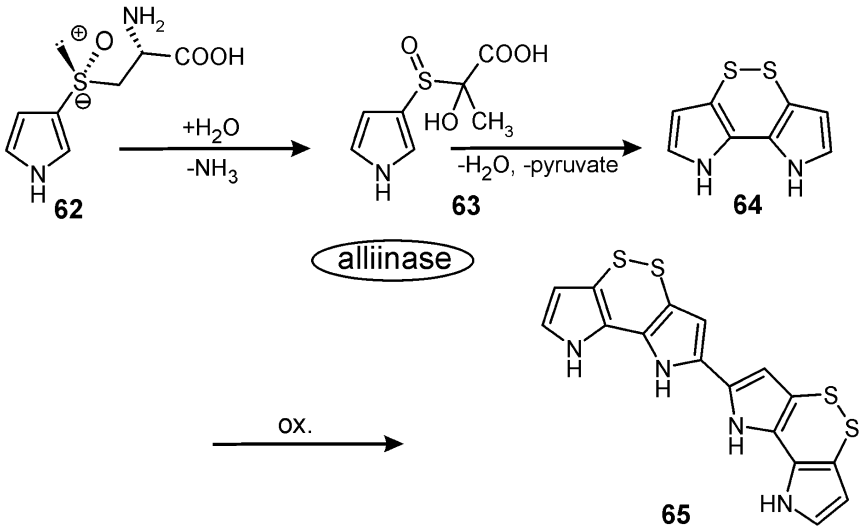

*Figure 12. Enzymatic decomposition of the pyrrolylcysteine sulphoxide **62** into red colored 3,3'-dithio-2,2'-dipyrrole **64**. Substance **64** is a rather stable intermediate and seems to be an addition product of pyrrolylsulfenic acid and pyruvate. Substance **64** is instable and is rapidly oxidized to substance **65** and similar compounds.*

No former knowledge did exist about this red dye, which was sometimes used for coloring of textiles. There were no hints that this pigment has a relation to sulfur chemistry. One of these *Allium* species showing the red pigment, *Allium rosenbachianum* auct., has been analyzed in terms of sulfur compounds but only dimethyl thiosulfinate has been detected (*61*).

In 2001, research on this red dye started but turned out to be extremely difficult because of the instability of these compounds. Already this behavior was a link that the pigment chemistry may be related to sulfur compounds. Finally, the reaction cascade could be elucidated and it got visible that the reaction scheme is close to that of other *Allium* species (Figure 12) (*53*). A hypothetical biogenetic scheme was proposed in which (+)-*S*-(3-pyrrolyl)-L-cysteine sulfoxide (pyrrolylcysteine sulfoxide **62**) is enzymically degraded. The resulting 2-lactyl-3′-pyrrolyl sulfoxide **63** is condensed readily to the red pigment 3,3′-dithio-2,2′-dipyrrole **64**. The dipyrrole **64** forms polymers with a dark red color as given by the dimmer **65**. All compounds are chemically unstable, rendering the analysis extremely difficult. Correlation NMR in combination with diffusion NMR allowed the identification of these low molecular weight compounds. For the first time, the compounds involved in the coloring process of *Allium* plant material have been identified from native plant material.

All *Allium* species having significant amounts of the red pigment do also have a characteristic smell. Nearly all of these species do also lead methiin **1** resulting in volatile methylsulfur compounds. It is not clear yet if only these compounds cause the characteristic smell or if also 'mixed' volatile thiosulfinates are formed. All attempts in order to prove these 'mixed' compounds failed until

now. Pharmacological and toxicological properties of these pyrrolyl compounds are also completely unknown until now. Research on these questions is ongoing.

Further Sulfur Compounds Related to *Allium* Species Belonging to the Subgenus *Melanocrommyum*

During numerous expeditions through the mountains of Middle Asia, the author got aware, that many species, which are used for medicinal reasons, do exhibit a very characteristic smell. One of the strongest smelling species is *Allium suworowii* Regel, which was formerly present in whole Middle Asia. It was told that this plant was intensively used by local tribes and plant populations were extinguished at many places. Because *A. stipitatum* has a similar habit and also a rather intensive smell, people turned over to use *A. stipitatum* instead of *A. suworowii*. In our days, *A. suworowii* can be only found at places, which have not been used by man, especially those places, which have not been used as grassland for animals

Isolation and full structure elucidation of the aroma precursor got the structure of a (+)-*S*-(methylthiomethyl)-L-cysteinesulfoxide **66** (*62*). This compound has previously reported from *Tulbaghia* species, which also belong to the Alliaceae family (*63*). The substance was named 'marasmin' (**66**, Figure 13). The reason for this name is the fact, that (-)-*S*-(methylthiomethyl)-L-cysteinesulfoxide **66a** was originally described as its γ-glutamyl derivative for the mushroom *Marasmius* spec. (*14*). This is an unique example in nature, that two completely different organisms – a higher plant and a fungus – produces nearly the same compound, but with the opposite stereochemistry! It is also an unique fact, that the alliinase from *Marasmius scorodonius* as shown in Figure 3 preferes the (-)-isomeres of the cysteine sulfoxides, a fact, which was never observed for *Allium* species.

The thiosulfinate marasmicin **67** results from the action of alliinase as "primary aroma compound' and was firstly described for *Tulbaghia* spec (*63*). A similar chemistry can be assumed for *A. suworowii*, especially because of the high amount of marasmin **66** (up to 1.2% related to the fresh weight of bulbs. For *Tulbaghia*, about 0.2% was reported). Marasmicin **67** is a unique thiosulfinate **10** carrying 4 sulfurs. Most likely, 'mixed' primary and secondary aroma compounds will also result in thiosulfinates **10** with 3 sulfur atoms. Research on these questions is also ongoing. It is worth to mention that a bioactivity against *Mycobacterium* as well as an anticancer activity was reported for *Tulbaghia* (*64*, *65*). Similar effects and because of the higher amount of marasmin **66** even stronger biological properties can be expected for *A. suworowii*. Again, it must be pointed out, that the usage of *A. suworowii* was replaced by the usage of *A. stipitatum*, which also has an anti-tuberculosis activity! It could be demonstrated by ongoing research of the author, that marasmin **66** is rather common in subgenus Melanocrommyum, mostly as a minor constituent. However, also minor compounds can significantly contribute to the flavor of *Allium* extracts.

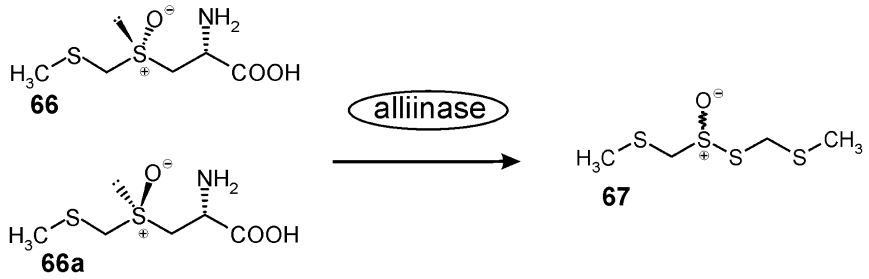

Figure 13. Enzymatic conversion of the cysteine sulphoxide (+)-marasmin ***66*** *into the thiosulfinate marasmicin* ***67****. The (-)-marasmin* ***66a*** *has been discovered in the mushroom genus Marasmius. Marasmin* ***66*** *could be also involved in the formation of* ***61****.*

Health Benefits Related to Sulfur Compounds of *Allium*

Several hundreds of studies regarding the health benefits of garlic, onion and related species were undertaken until now (*3*, *10*). In many cases it is difficult to match results of different studies which were performed in order to proof a specific effect. There are three main reasons for this problem: firstly, plant extracts are a complex mixture of many compounds as described above. *Allium* species do not only contain sulfur compounds, which account for 1-5% of the mature bulbs (*3*, *9*), but also flavanoides. Especially common onion is very reach in derivatives of the flavanoids, which can be up to 2.1% (*10*). Additionally, significant amounts of saponins are present also exhibiting some bioactivity. The amount and pattern of all this compounds is not stable and depends on many factors, *e.g.*, variety of plant material and cultivation conditions. It also makes a big difference, if test were performed with isolated compounds or with whole bulb extracts.

Secondly, primary and secondary aroma compounds are instable. Primary compounds have to be used directly after isolation or synthesis. A test preparation containing volatile sulfur compounds has to be characterized carefully before applied to the pharmacological test system. If test compound were not stored properly before usage, decomposition products are most likely.

Thirdly, many different test systems were used for proving pharmacological effects. To give an example: antibiotic activity, which is well known for garlic extracts, can be easily demonstrated by using a set of isolated bacteria strains. However, results can significantly differ from those obtained by using an animal model. Especially studies dealing with anticancer properties were mainly performed with cell models. Because of this, conclusions regarding anticancer therapy for humans are rather difficult.

The antibiotic effect of both, onions and garlic, is well described. Even in Egyptian Papyrus Eberts, onion containing remedies are mentioned to be active against worms, diarrhea, other infections and inflammatory diseases (*66*). Similar, but stronger effects were observed for garlic. Allicin **12** was found to be a very potent antibiotic (*67*, *68*). A 93% bactericidal effect against *Staphylococcus epidermidis* was apparent after 1 h of incubation. A comparable effect was

observed for *Salmonella typhi* within 3 h of incubation. Yeasts and also other pathogenic fungi are highly sensitive to garlic extracts. *Candida* species seems to be sensitive towards diallyl disulfide **18** (*69*), but garlic preparations are also active against *Aspergillus* species (*70*). Aqueous extracts mainly containing diallyl (poly)sulfides **20** as well oily extracts containing vinyl dithiins (**14**, **15**) seems to be active against fungi. Because results are very promising, garlic extract and its preparations should be also considered as plant protecting agents against plant pathogenic fungi (*71*). Comparing all literature data dealing with the antibiotic activity of garlic of onion, it seems to be that garlic is much more active as common onion. Garlic extracts do show highest activity against fungi followed by activity against gram positive bacteria. Gram negative bacteria were less attacked. But nevertheless, Garlic might be an alternative in the fight against multi-resistant bacteria strains. But further research is required in order to find the best pharmaceutical formulation for the treatment of infections.

Another interesting point of concern is the anti-larval, anti-insecticidal and use as repellent (against mosquitoes) of garlic and its preparations (*72–74*). Fresh garlic extract is sufficiently active against mosquito larvae (*75*). These effects are from special interest, because volatile garlic compounds show a low environmental persistence and can be considered as alternative to chemical fumigants, repellents and insecticides. Garlic compounds can be also considered, if resistance of insects against established chemical insecticides occurs.

The antiasthmatic activity of common onion is also well investigated. Onion extracts as well as isolated or synthesized sulfur compounds were tested (*66*). For example, thiosulfinates **10** and cepaenes (**25**, **26**) were investigated by *in vitro* tests. They exhibited a dose dependent inhibitory effects at 0.25 to 100 μM. Cepaenes (**25**, **26**) inhibited both cyclooxigenase and 5-lipoxygenase by more than 75% at 10 and 1 μM concentrations, respectively (*76*).

Antioxidant and radical scavenger activity were investigated for some *Allium* species (*77*, *78*). It was observed that volatile and non-volatile compounds do have a significant effect. Obviously the number of sulfur atoms must be taken in account. Best results regarding the antioxidant activity were obtained for vinyl dithiins (**14**, **15**). There are several hints that antioxidant activity might prevent from cancer. Ajoene is also reported as a potent agent to induce apoptosis (*79*).

There are numerous reviews concerning prevention from cancer in recent years, sometimes among those of other vegetables (*80–84*). As one interesting point of concern, onion and garlic are rich in organoselenium compounds, which may also prevent from cancer. Selenium is usually fixed to cysteine derived amino acids and can replace the sulfur atom (*e.g.*, in case of 'selenomethiin'). But also quercetin and its derivatives, which are typical constituents of onions, have to be considered in terms of anticancer properties.

Besides the antibiotic effects, the lipid lowering action of garlic is one of the best investigated effects in modern phytotherapy. Exceptionally large numbers of animal and human studies have been carried out (*10*). Moreover, the effect of lipid lowering actions, mainly lowering of the blood cholesterol level, has been thoroughly elucidated in detail (*85–88*). The synthesis of cholesterol is mainly inhibited by allicin, diallyl disulfide and ajoene. However, it must be mentioned

that patients suffering from genetically induced high cholesterol levels, can not be sufficiently treated with garlic preparations.

Increased cholesterol levels are a well known risk for atherosclerosis. However, reduction of antiplatelet activity, hypotension and the antioxidative effects of garlic may also contribute to the prevention of atherosclerosis. Antiplatelet activity of garlic was studied in humans and in isolated tissues. Diallyl disulfide **18** and diallyl trisulfide **19** were found to be the active compounds, which are typically found in garlic oil (*89*). Raw garlic seems to be more active as boiled bulb material (*90*). A dose-dependent inhibition of cyclo-oxygenase was observed in rabbit tissues treated with raw garlic. Additionally, water and alcoholic extracts are potent inhibitors of platelet aggregation, which is induced by adrenaline and adenosine diphosphate (ADP). Further on, these garlic extracts showed the ability to increase nitric oxidase synthase activity intracellularly, resulting in the relaxation of blood vessels, as well as in the inhibition of platelet aggregation (*91*). These results suggest that the intake of garlic is of some risk in terms of getting accidentally wounded, because wound healing can be interrupted.

In summary, numerous biological activities were reported for garlic, onion and related species. But precise dose-finding curves are missing in most of these investigations. Therefore it is hard to say which concentration is necessary in order to get the claimed health benefits. As a very rough calculation about a daily intake of about 2.5 to 4 g of garlic and an intake of about 50 g common onion are necessary to meet active serum levels of sulfur compounds.

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Chapter 10

Sulfur Compounds in Still and Sparkling Wines and in Grappa: Analytical and Technological Aspects

Bruno Fedrizzi,[*,1,2] Giuseppe Versini,[2] Roberto Ferrarini,[3] Fabio Finato,[2] Giorgio Nicolini,[4] and Franco Magno[1]

[1]University of Padova, Chemical Sciences Dept., via Marzolo 1, 35135 – Padova, Italy
[2]Unione Italiana Vini Soc. Coop., Viale del Lavoro 8, 37135 – Verona, Italy
[3]University of Verona, Wine Science & Technology Dept., Via della Pieve 70, 37029 – Verona, Italy
[4]IASMA Research Center, Agrifood Quality Department, via Mach 1, 38010 – San Michele all'Adige, Italy
[*]E-mail: bruno.fedrizzi@unipd.it.

Grape products are really important in the cultural and dietary Italian traditions. Oenological products have been largely studied since the early '70s, even if the lack of biochemical and microbiological knowledge and poor sensitivity of the analytical techniques prevent from carrying on a deep studies on sulfur compounds. The topic discussed in the present work is the development of HS-SPME/GC-MS methods to quantify fermentative sulfur compounds (*i.e.* molecules produced both by yeast metabolisms from amino acidic precursors and via chemical reactions from other sulfur compounds) and the following application of these procedures on still wines, sparkling wines and distillates (*e.g.* Italian Grappa). 13 and 10 sulfur compounds were quantified in still and sparkling wines and Grappas, respectively. Influence of variety, aging, yeast strain and other technological practices on the level of these fermentative sulfur compounds was also investigated.

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Sulfur compounds represent the most intriguing species present in oenological matrices both for their extremely low sensory threshold and for their implication in yeast microbiology, plant physiology and winemaking technology (*1*, *2*). Moreover the very different sensorial characteristics (*3*), according to their chemical structure (*i.e.* physical-chemical proprieties, position of the sulfur atom along the molecule, stereochemistry), makes them one of the species less studied in wine chemistry.

In wine products, only a few sulfur compounds have been studied since early '70s, and they were mostly investigated because of their connections with negative scents such as "reduction, putrescence and rotten eggs" (*4*, *5*). Recently the improvement of analytical techniques and the availability of more sensitive methods enabled to identify new species and permitted to revaluate sulfur compounds contribution to wine typicality and traceability (*6–8*).

Fermentative sulfur compounds derive from yeast metabolisms converting amino acidic precursors into the relevant sulfur compounds. In particular it is possible to recognize for all these molecules a common origin; L-methionine and L-cysteine play a primary role in fermentative sulfur compounds biogenesis.

Typical biosyntheses are those of 3-methylthiopropanol (methionol, MTP) and 2-mercaptoethanol (ME) via Ehrlich mechanism starting from methionine and cysteine, respectively, both present in juice or made up in the yeast cell during fermentation.

The main fermentative sulfur compounds investigated in the current paper are depicted in Figure 1.

The impact of these molecules on grape products is impressive and, differently from "good thiols", it is ubiquitous in all grape varieties/products. To gain a better understanding on their formation and evolution it is necessary to obtain a significant improvement in wine quality assessment and to fulfill the lack of information in this field.

Aroma of Italian sparkling wines, produced according to *Champenoise* or the so-called *Classic* method, are deeply influenced by the second fermentation occurring in bottle (*9–11*). Furthermore, typical long aging on yeast lees could have a strong impact on final quality. Significant influence of yeast autolysis have been shown by Wisser (*12*), leading to a variation of total thiols level.

Marc storage and distillation methods significantly improved in the last decades to significantly improve the grape pomace distillates quality *i.e.* grappa (*13*). Nonetheless, some off-flavors are still present (*14*), often recalling sulfur compound taints described as "sauerkraut, cabbage, onion, burnt straw" scents (*15*). Up to now, only dimethyl sulfide (DMS) has been quantified in grappas (*16*). Other sulfur compounds were evaluated in other distillates like whiskies (*17–21*) and Calvados and Cognac products (*22*).

Investigation on the factors influencing fermentative sulfur compounds level are fairly scarce. So far, only Chatonnet et al. (*23*) and Rauhut (*24*) investigated the effects of some winemaking practices and yeast influence, respectively. Minor attention has been paid on the effect of vintage, grape variety, and yeast lees contact in wines and of distillation procedures on the level and formation of fermentative sulfur compounds

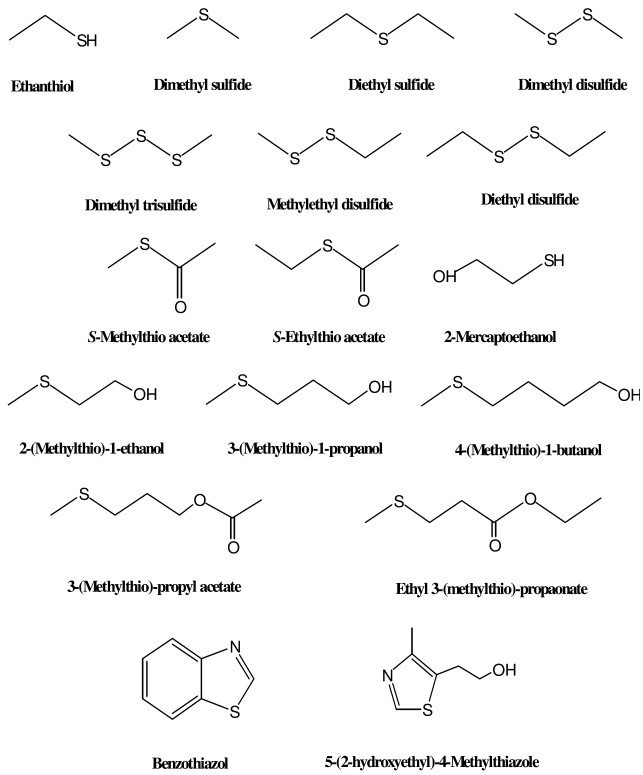

Figure 1. Main fermentative sulfur compounds investigated in still and sparkling wines and grappas.

Aiming at defining markers able to assess wine and distillates typicality and traceability and at addressing wine technology and microbiology requests, we focused our attention on the design of analytical methods useful to investigate fermentative sulfur compounds in grape-derived products. The main goal we accomplished is the definition of sensitive, robust and easily applicable procedures to study fermentative sulfur compounds which furnished interesting information on the effect of sulfur compounds in different grape products.

Experimental

The low concentration of the investigated analytes requires the use of a preconcentration step before instrumental analysis. Many techniques are available to sample and concentrate volatile analytes; a technique commonly applied in wine chemistry is the headspace solid phase microextraction (HS-SPME) (*1*, *2*, *25*). An important tool for selective analysis in complex matrices is gas chromatography coupled with mass spectrometry (GC-MS).

HS-SPME was performed on 20 mL of wine added with 5 g of $MgSO_4$ $7H_2O$, and spiked with IS solution. The sampling took place in 30 mL vial stirred at 500 rpm for 30 min at 35 °C. The SPME fiber adopted was a 2 cm long

Carboxen-Polydimethylsiloxane-Divinylbenzene (CAR-PDMS-DVB; 50/30 μm, Supelco, Bellefonte, PA, USA). GC-MS analyses were carried out on a Perkin Elmer Autosystem XL gas chromatograph coupled with a TurboMass Gold mass spectrometer (Perkin Elmer; Boston, MA, USA) equipped with a 30 m x 0.32 mm I.D. x 0.25 μm film thickness Innowax (PEG) fused-silica capillary column (Agilent Technologies; Palo Alto, CA, USA). (*26*).

The sulfur compounds studied in still and sparkling wines were: ethylmercaptan (EtSH), dimethyl sulfide (DMS), diethyl sulfide (DES), dimethyl disulfide (DMDS), diethyl disulfide (DEDS), *S*-methyl thioacetate (MTA), *S*-ethyl thioacetate (ETA), 2-mercaptoethanol (ME), 2-(methylthio)-1-ethanol (MTE), 3-(methylthio)-1-propanol (MTP), 4-(methylthio)-1-butanol (MTB), benzothiazole (BT) and 5-(2-hydroxyethyl)-4-methylthiazole (HMT). Dimethyl sulfide-d6 (d6-DMS), dipropyl disulfide (DPDS), 3-(methylthio)-1-hexanol (MTH) and 4-methylthiazole (MT) were used as internal standards (I.S.).

As for grappa analyses, due to the naturally occurring high ethanol content, raw samples were unsuitable to be analyzed by HS-SPME and therefore a dilution had to be made. In particular grappa samples were diluted 8 times with MilliQ water. The HS-SPME sampling was carried out on 5 mL of solution in 20 mL vials, added with 2 g of NaCl and spiked with 25 μL of internal standard solutions (methyl heptanoate and d_6-dimethylsulfide). Sampling was managed by MPS2 Twister autosampler (GERSTEL Inc., 701 Digital Drive, Suite J, Linthicum, MD, USA) using a 2 cm long DVB/CAR/PDMS, 50/30 μm SPME fiber (Supelco Inc., Bellefonte, PA, USA). Sampling time and sampling temperature were 30 min and 40°C, respectively. GC-MS analyses were carried out on a 6890N Network GC system (Agilent Technologies; Palo Alto, CA, USA), equipped with a DB-WAX (50 m x 0.32 μm x 0.25 film thickness, Agilent Technologies; Palo Alto, CA, USA) capillary column, coupled to a 5975B XL EI/CI MS mass spectrometer (Agilent Technologies; Palo Alto, CA, USA).

Identification of sulfur compounds was achieved via either reference standards or NIST library. The compounds considered in the grappa samples were: CS_2, DMS, DMDS, DEDS, ethylmethyl disulfide (EMDS), dimethyl trisulfide (DMTS), dihydro-2-methyl-3-(2H)-thiophenone (DMTP), ethyl 3-(methylthio)-propionate (EMTP) and 3-(methylthio)-propyl acetate (MTPA). d6-DMS was used as internal standard to evaluate CS_2, DMS and X (a tentatively identified compound), while methyl heptanoate as internal standard was used for DMDS, MEDS, DEDS DMTS, DMTP, EMTP and MTPA.

The data were statistically evaluated and plotted using STATISTICA v7.1 (Statsoft Italia S.r.l., Padova, Italy).

The 80 still wine samples involved in this study were produced in the experimental winery of the IASMA Research Center (Italy). Vinification occurred in stainless steel tanks, following traditional winemaking protocols. Several commercial yeast strains were adopted (*8*), Four wines per four varieties (three red-fruited, Teroldego, Marzemino, Merlot and a white-fruited Chardonnay) and per vintage year (1998, 2001, 2002, 2003, 2004) were selected (*8*).

Basic analytical data of the grape juices processed and of the final wines obtained are reported in Table 1 (*8*, *27*).

To study the evolution of fermentative sulfur compounds in sparkling wines, 15 Italian sparkling wines (with the relevant replicated samples) produced according to the "Classic" method, from different vintages and aging on lees, were analyzed in 2007. We chose two renowned wineries from two different neighboring grape-growing areas (*i.e.* Trentino and South Tyrol).

Finally the sulfur compounds profile in Grappa was for the first time evaluated. In this case about 30 raw grappa samples from four neighboring Italian Provinces (*i.e.* South Tyrol, Verona, Padova and Treviso) and 2007 vintage were analyzed The grappa samples were representative of the production systems (pot still and continuous distillation) and derived both from red-fruited (marc ensiled after fermentation in winery) and white-fruited (marc fermented in silage tanks) pomaces. Alcoholic proof varied between about 72 and 85 % Vol..

Results and Discussion

Still and Sparkling Wines

The presence of significant differences (*28*) due to aging and variety effects on each sulfur compound, was checked by applying the Tukey test to the data collected for the 80 wines (Table 2).

It is known that some fermentative sulfur compounds change with storage (*7*, *29*); in particular DMS level increases with aging (*7*, *29*, *30*), and *S*-methyl- and *S*-ethyl thioacetate (MTA and ETA) can undergo to hydrolysis during the first months with consequent increase of the relevant thiols and disulfides (*24*). The present research, on the basis of the balanced sampling plan and of the statistical approach adopted, shows that the level of some sulfur compounds is strongly affected by aging. This fact results to be quite important and useful in the definition and in the understanding of the wine aroma evolution.

Table 1. Main Basic Data of the Grape Juices Processed and of the Wines Obtained

	Grape Juice						Wine								
	YAN (mg/L)			Sugar (g/L)			Alcohol Concentration (% Vol.)			pH			Titratable Acidity[a]		
Variety	Median	Min	Max	Median	Min	Max	Median	Min	Max	Median	Min	Max	Median	Min	Max
Teroldego	156	44	285	208	183	233	12.28	10.79	13.7	3.805	3.4	3.98	4.9	4.05	6.8
Marzemino	99	19	233	205	186	221	12.04	10.94	13	3.775	3.55	3.93	5.1	4.2	5.4
Chardonnay	184	60	324	203	153	237	12.3	9.24	14.3	3.305	3.05	3.57	6.85	5.2	10
Merlot	85	29	251	217	194	247	13.005	11.41	14.5	3.72	3.38	3.98	4.7	4.1	6.3

[a]Titratable acidity is expressed as tartaric acid in g/L.

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Table 2. Vintage Year Dependence of the Mean Content of the Analytes. Adapted from ref (*8*). Copyright 2007 American Chemical Society

Analyte (µg/L)	1998		2001		2002		2003		2004	
	mean	S.D.	mean	S.D.	mean	S.D.	mean	S.D.	mean	S.D.
EtSH	0.7 ^{b}	0.26	1.5 ^{ab}	0.77	1.5 ^{ab}	0.48	1.8 ^{ab}	1.09	2.5 ^{a}	1.74
DMS	53.4 ^{a}	9.42	35.6 ^{b}	8.86	22.5 ^{c}	7.83	15.1 ^{d}	6.61	8.0 ^{e}	5.77
DES	8.9 ^{a}	2.55	7.4 ^{ab}	2.26	4.9 ^{bc}	1.86	5.7 ^{bc}	2.38	3.5 ^{c}	1.79
DMDS	8.7 ^{a}	8.23	4.0 ^{ab}	3.62	3.5 ^{b}	3.27	5.3 ^{ab}	4.33	4.5 ^{ab}	3.60
DEDS	6.0 ^{a}	4.22	3.4 ^{b}	2.19	3.5 b	2.12	3.7 ^{b}	2.81	2.8 ^{b}	1.15
MTA	8.2 $^{n.s.}$	3.14	8.3 $^{n.s.}$	3.55	10.4 $^{n.s.}$	3.19	10.3 $^{n.s.}$	4.82	11.6 $^{n.s.}$	4.55
ETA	1.8 ^{b}	0.64	2.3 ^{ab}	0.82	2.8 ^{ab}	1.44	2.7 ^{ab}	1.49	3.2 ^{a}	2.09
ME	4.8 ^{d}	2.39	10.9 ^{c}	8.30	13.8 ^{c}	4.36	21.0 ^{b}	12.04	27.7 ^{a}	11.24
MTE	26.4 ^{a}	14.14	25.5 ^{ab}	18.00	20.8 ^{ab}	15.58	23.2 ^{ab}	14.90	18.9 ^{b}	13.04
MTP	3386 ^{a}	840.5	2807 ^{b}	881.4	2543 ^{bc}	588.2	2170 ^{cd}	521.5	1850 ^{d}	596.9
MTB	38.7 $^{n.s.}$	24.84	42.6 $^{n.s.}$	32.53	41.0 $^{n.s.}$	22.29	41.1 $^{n.s.}$	26.81	30.2 $^{n.s.}$	16.00
BT	6.6 ^{a}	3.21	4.4 ^{ab}	3.16	3.8 ^{b}	2.35	5.9 ^{ab}	4.24	5.1 ^{ab}	3.69
HMT	2.1 $^{n.s.}$	0.95	3.0 $^{n.s.}$	1.29	2.5 $^{n.s.}$	1.14	2.6 $^{n.s.}$	0.99	2.6 $^{n.s.}$	1.01

Values with the same letter do not differ significantly at the Tukey's test, $p < 0.05$. n.s. not significant.

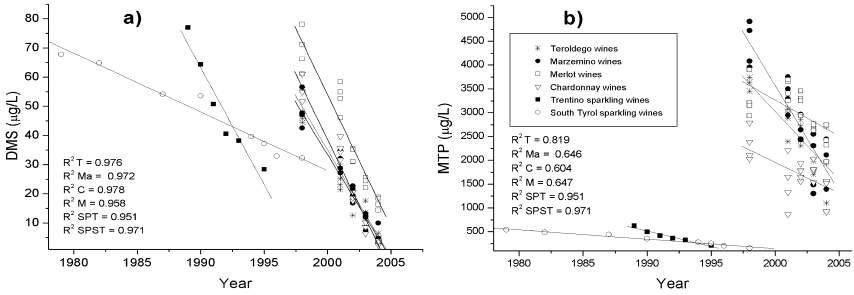

Figure 2. Time evolution of DMS (a) and MTP (b) for the considered still and sparkling wines.

Table 3. Variety Effect on the Mean Concentration of the Considered Sulfur Compounds. Adapted from ref (*8*). Copyright 2007 American Chemical Society

Analyte (μg/L)	Merlot		Teroldego		Marzemino		Chardonnay	
	mean	S.D.	mean	S.D.	mean	S.D.	mean	S.D.
EtSH	0.7 ^{b}	0.35	3.2 ^{a}	2.00	1.5 ^{b}	1.05	0.8 ^{b}	0.32
DMS	39.9 ^{a}	17.67	22.1 ^{b}	14.83	21.9 ^{b}	14.20	23.8 ^{b}	15.62
DES	7.2 ^{ab}	3.43	5.2 ^{bc}	1.95	7.5 ^{a}	4.68	4.3 ^{c}	2.05
DMDS	9.0 ^{a}	6.96	0.7 ^{b}	0.18	1.1 ^{b}	0.49	10 ^{a}	8.35
DEDS	4.9 ^{a}	2.40	2.2 ^{b}	0.13	2.3 ^{b}	0.22	6.0 ^{a}	4.08
MTA	8.5 ^{b}	3.21	13.7 ^{a}	6.57	7.6 ^{b}	5.01	9.2 ^{b}	2.94
ETA	2.1 $^{n.s.}$	0.66	2.7 $^{n.s.}$	2.03	2.7 $^{n.s.}$	1.58	2.7 $^{n.s.}$	1.10
ME	11.5 ^{b}	6.27	20.7 ^{a}	12.91	22.3 ^{a}	12.82	8.0 ^{b}	5.61
MTE	44.7 ^{a}	8.90	11.5 ^{c}	3.70	24.2 ^{b}	9.40	11.4 ^{c}	7.05
MTP	3024 ^{a}	493	2569 ^{b}	755	2861 ^{ab}	1041	1749 ^{c}	453
MTB	75.1 ^{a}	20.02	28.9 ^{b}	7.27	26.0 ^{b}	7.85	24.9 ^{b}	13.58
BT	9.0 a	3.29	2.9 ^{c}	2.40	3.2 ^{c}	2.14	5.5 ^{b}	1.82
HMT	2.3 ^{b}	0.83	2.0 ^{b}	0.56	2.4 ^{b}	1.08	3.6 ^{a}	1.16

Values with the same letter do not differ significantly at the Tukey's test, $p < 0.05$. n.s. not significant.

Besides DMS, also MTP and ME contents change in the course of time, increasing and decreasing respectively (*26*). Figure 2 shows the evolution of DMS and ME, using a straight line model, confirming the data reported in the literature.

In this research the capability of some fermentative sulfur compounds to discriminate wines according to the variety was investigate. To date, evidence of the dependence of sulfur compound concentrations on grapes variety have been never reported in literature with the exception of DMS (*29*) and for some thiols resembling tropical fruit scents (*31*), here not considered. Data in Table 3 show that some sulfur compounds are more abundant in some wine varieties than in others.

In particular, as shown by the Tukey test, the concentration of DMS and MTB in Merlot wines is significantly higher than in all the other varieties, thus supporting the important grassy/truffle-like scent for DMS (*6*, *7*, *29*) and the earthy-like scent for MTB (*32*), used commonly as descriptors for the Merlot aroma. Furthermore, a clear difference for DMS and DES between the groups Merlot/Chardonnay and Teroldego/Marzemino is found, having the first group a higher content in such compounds; the opposite behavior was aboserved for ME level.

Finally, to highlight possible varietal dependence, the data were submitted to Principal Component Analysis (PCA) (Figure 3). To point out differences

linked to the variety, temporal correlation of wines was eliminated by performing a centering on each variable for each variety. The variance explained by the first two functions increased to about 67%. We recognized the big difference existing among the scores of Merlot, Chardonnay and Marzemino plus Teroldego, these last ones resulting as two partially overlapped groups (data not shown). In particular, focusing only on the three red wines it is possible to notice the total overlap of the two Trentino-native grape varieties. This finding could address the similarities perceived in the sensory analysis of these two wines (*33*). The only analysis of the YAN data, both from the literature (*27*) and from more recent 6-year long investigations (Teroldego: 60 samples; YAN mean = 160 mg/L, 20.3 °Bx; Merlot: 94 samples; YAN mean 131 mg/L, 20.6 °Bx; Marzemino: 84 samples; YAN mean 117 mg/L, 18.8 °Bx), as well as of the amino acid profiles of grape juices (*34*), does not seem to provide a thorough explanation for our findings, suggesting that more investigations are required in this filed.

The effect of some technological parameters on the evolution profile of sulfur compounds was investigated in sparkling wines.

Comparing the sparkling wine data with those found in still wines we can notice that DMS, DES, DMDS, MTP and MTB increase with aging in both matrices (*8*).

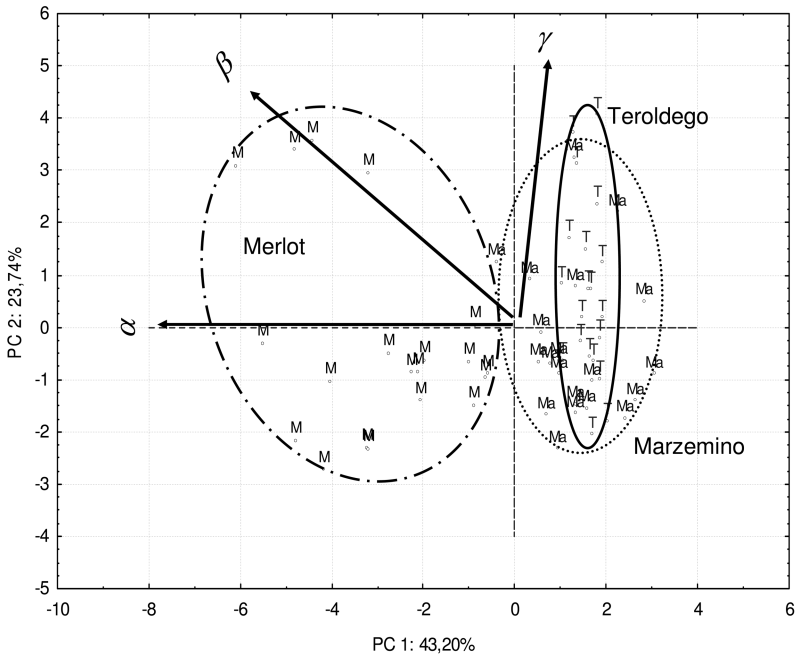

Figure 3. PCA biplot of the mean loadings and scores for the red wine analyzed after removing vintage effect. α: DMS, DES,DMDS, DEDS, MTE, MTP and MTB; β: BT and HMT; γ: EtSH, ME, MTA and ETA.

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Table 4. Effect of Lees Contact on Considered Sulfur Compounds. ‡ Sparkling Wines Disgorged in 2001 (6 years of contact). † Sparkling Wines Disgorged at the Analysis (14 years of contact).

Vintage	DMS* (ppb)	DES* (ppb)	MTA (ppb)	DMDS* (ppb)	ETA (ppb)	DEDS* (ppb)	ME* (ppb)	MTE (ppb)	MTP (ppb)	MTB (ppb)	BT* (ppb)	HMT (ppb)
1995 †	29.28	3.63	5.95	1.18	0.98	2.10	2.68	4.40	212.13	2.43	3.71	4.73
SD	1.20	0.54	0.12	0.23	0.05	0.35	0.47	0.55	0.71	0.01	0.30	0.40
1995 (01) ‡	23.48	2.44	4.06	1.50	0.89	1.17	4.16	4.35	169.74	2.72	3.11	4.34
SD	1.48	0.61	1.35	0.37	0.33	0.22	0.40	0.41	33.45	0.92	0.15	0.47
t	3.19	3.61	2.06	3.28	1.59	11.06	7.65	0.22	0.08	0.68	10.48	2.02

* sulfur compounds level affected by yeast lease contact according to *t*-test ($\alpha \leq 0.05$, $t_c = t_{\alpha/2}$, $v = 4 = 2.78$). SD: standard deviation

Interesting increasing evolutions for DMS (Figure 2a) and MTP (Figure 2b) levels during aging are evident; the different profiles for the two wineries considered are likely due to some different winemaking conditions adopted. Among those, storage temperature (8°C vs. 16°C) might play a pivotal role as it is strictly correlated to evolution kinetics.

The effect of yeast lees contact was investigated for six samples of Ferrari Co. produced in 1995: three sparkling wines kept on lees till the analysis (lees contact of 14 years) and three sparkling wines disgorged from the lees in 2001 (lees contact of 6 years) were analyzed. Table 4 shows the results of this study; the data of the two groups were submitted to *t*-test to check for lees contact time effects.

Lees contact duration does not seem to affect the evolution of thioalcohols (ME, MTP, MTB and MTE), S-thioacetates (MTA and ETA) and one heterocyclic compound (HMT). On the other hand BT, sulfides and disulfides slightly increase with longer lees contact. This finding agrees with the report of Vasserot et al. (*35*) who suggested a possible involvement of yeast lees in the methanethiol and ethanethiol oxidation, producing the relevant disulfides.

Grappa

Finally, the results obtained via a new HS-SPME/GC-MS method, were reported. Several sulfur compounds were quantified on a significant number of Venetian grappa samples. In particular a possible "distillery effects" were investigated.

In Table 5 concentration ranges, average, standard variation and median were listed.

DMTP is generated from methionine metabolism like EMTP and MTPA (*36*), as so as DMS (*37*), even if it mostly originates from *S*-methylmethionine (*38*). Decomposition of DMS in methanol and H_2S could also happen in the distillation, thorugh copper catalysis (*39*). DMS content in grappa is in the same ranges found by Cardoso et al. (*39*).

Mean DMDS value of 40 µg/L of raw distillate resulted close to DMS while DEDS is a little higher. No data relevant to other sulfur compounds level in grappa or similar distillates are available from the literature.

Table 5. Variability Ranges of the 10 Sulfur Compounds Analyzed in Grappa Samples. Data reported as µg/L.

	Mean	Median	Standard dev.	Min	Max	25% Percentile	75% Percentile
CS_2	4725	1087	8741	0.03	34356	43.6	4126.4
DMS	117.6	4.5	364	0.09	1772	1.1	20.9
X	617.3	167	1132	4.22	5186	69.2	541.3
DMDS	56.0	25.4	71.6	1.43	272	5.9	81.3
MES	14.0	6.2	19.5	1.16	75.8	2.7	16.0
DEDS	69.7	2.6	323	0.01	1713	0.0	4.9
DMTS	1.3	1.0	1.16	0.06	4.1	0.4	2.1
DMTP	0.26	0.02	0.45	0.00	2.0	0.0	0.3
EM	544.4	459	472	0.18	2076	232.8	699.9
MA	102.3	55	150	0.16	630	1.5	127.6

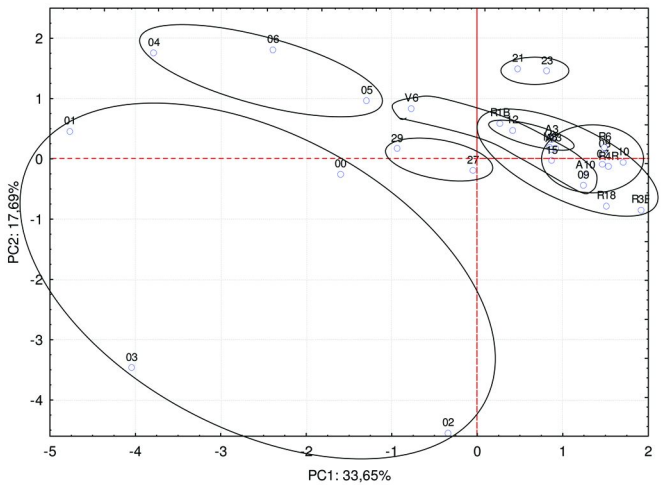

Figure 4. PCA biplot of the scores for the Venetian grappa samples examined.

By plotting DMTS vs. DMDS and MEDS vs. DEDS, different correlations were observed, mostly according to the distillery and likely not depending on the distillation technique. In particular, for some distilleries the independence between the variables was observed. Dependence of DMTS from DMDS is in agreement with the results of Prentice at al. (*21*); the independence could be related to a different contribution of H_2S in relation with a different level of Cu catalysis (*19*). According to these authors, both compounds are considered important flavor contributors in whisky. Generally, whiskies with a "light flavor" show low levels of DMDS, while those with higher contents are "slightly bitter, roasted" or "with heavy characteristics".

Further, the different correlation between DEDS and MEDS values could be linked to different EtSH level in marcs compared to MeSH.

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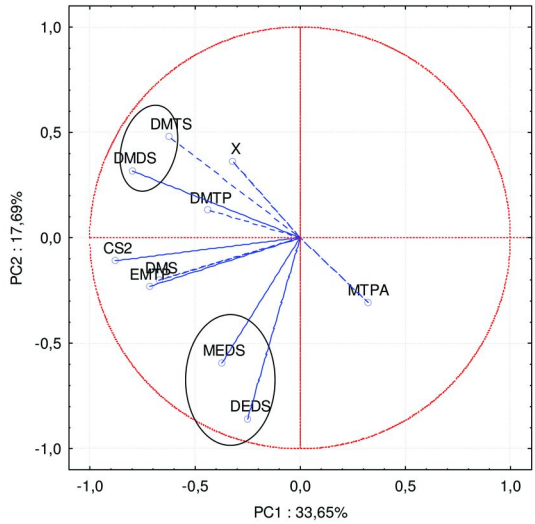

Figure 5. PCA biplot of the loadings for the Venetian grappa samples examined.

The data relevant to the 10 sulfur compounds quantified were submitted to PCA data treatment whose results are represented in Figure 4. It can be noticed that about 51% of total variance is explained by the first's two principal components with evidence of score subgroups according to the distillery.

Possible groupings of variables involved DMDS with DMTS and MEDS with DEDS in reason of a different origin. CS_2 loading is located in an intermedium position, close to DMS and EMTP. MTPA and EMTP are located in opposite direction. X (the tentatively identified compound) and DMTP are less charged on these two principal components and result located rather close to the DMDS and DMTS group Figure 5.

A sulfur compound not yet identified and likely connected with cabbage-burnt off-flavor (Versini, unpublished results) has been evidenced. The MS data were (*m/z* and relative intensity): 33 (8), 43 (16), 45 (18), 61 (81), 69 (28), 85 (6), 89 (100), 101 (24), 115 (16) and 145 (<1). The putative molecular weight is 146, likely corresponding to a $C_7H_{14}OS$ formula. The tentatively identified molecule could be a sulfur acetal, like 1-ethoxy-1-ethylsulfanyl-propenal. This hypothesis would be supported by the presence of the following fragments: M-1, M-45 and M-61 and at *m/z* 33, 61 and 89 (*40*). Furthermore the non-linear variation of its concentration by performing serial dilutions of the distillate with acidified water strengthens our hypothesis. According to our chromatographic conditions, its relative retention time, in respect to diethylacetal of isovaleraldehyde, was 0.90.

Finally, an important role of this class of compounds, also on the typicity of grappa, can be demonstrated by re-distilling the product adding AgCl: the resulting distillate appear "clean" but lose every typicality characteristics.

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Chapter 11

Determination of Volatile Sulfur Compounds Formed by the Maillard Reaction of Glutathione with Glucose

Sang Mi Lee and Young-Suk Kim*

Department of Food Science and Engineering, Ewha Womans University, Seoul 120-750, South Korea
***E-mail: yskim10@ewha.ac.kr.**

The volatile compounds formed from the thermal reaction of glutathione with glucose were analyzed by gas chromatography-mass spectrometry (GC-MS). Sulfur-containing compounds dominated the volatiles in glutathione-Maillard reaction products (GSH-MRPs) and included 1 thiazole, 12 thiophenes, 2 polysulfides, 1 sulfur-substituted furan, and 1 miscellaneous. The carbohydrate module labeling (CAMOLA) experiment was employed to evaluate the relative importance of precursors to the formation pathways and elucidate the origin of the carbon skeleton for sulfur-containing compounds in GSH-MRPs. The isotopomeric distribution patterns showed that 2-ethylthiophene, 2,5-dimethylthiophene, 1-thiophen-2-ylethanone, 5-methylthiophene-2-carbaldehyde, and 1-thiophen-3-ylethanone can be formed from the intact carbon skeleton of a C-6 glucose chain, whereas 3-methylthiophene-2-carbaldehyde occurs via the recombination of fragments that may originate from both GSH and glucose.

Volatile sulfur-containing compounds have been found in diverse foods such as vegetables, roasted coffee, roasted seeds, wheat bread, cooked meats, and many thermally processed foods (*1–4*). These sulfur-containing compounds are known to play an important role in contributing meaty flavor, in particular, to roasted and cooked meats. Sulfur-containing amino acids, such as cysteine, cystine,

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and methionine, are major precursors for the formation of the sulfur-containing compounds. During the thermal processing, reactive intermediates such as hydrogen sulfide are liberated from the sulfur-containing amino acids and participate in the Maillard reaction and Strecker degradation to form volatile sulfur-containing compounds (*4*, *5*).

Glutathione (γ-L-glutamyl-L-cysteinylglycine, GSH), a tripeptide, can form diverse volatile sulfur-containing compounds through the Maillard reaction during the heating processing. In earlier publications, Zheng et al. (*6*) reported that hydrogen sulfide is released from the cysteine residue in GSH and involved in the generation of volatile sulfur-containing compounds during thermal reactions. It can therefore lead to diverse sulfur-containing components, such as thiols, thiophenes, thiazoles, and polysulfides, which are related to savory and meaty-type flavor notes, through the Maillard reaction and Strecker degradation (*1*, *2*, *6*).

Volatile compounds generated from the thermal decomposition of glutathione were compared to those of cysteine when heated under same conditions. In that study, Zhang et al. (*7*) reacted glutathione in an aqueous solution at 180°C and identified 17 compounds, including isomers of 3,5-dimethyl-1,2,4-trithiolane as the major compounds. Also, Ho et al. (*8*) determined the reactivity of Maillard volatile compounds generated from the thermal reaction of four cysteine-containing peptides, GSH, γ-glu-cys, cys-gly, and gly-cys, with glucose. Volatiles found in those model systems were mainly thiophenes, thiazoles, and cyclic polysulfides whereas pyrazines were minor components. The results might be due to the fact that hydrogen sulfide is released more easily than ammonia, and hydrogen sulfide inhibits the Strecker degradation and pyrazine formation (*8*).

The use of isotopically labeled compounds proposed as precursors or intermediates in the formation of certain target molecules is a powerful technique to elucidate complex reaction pathways (*9*). The carbohydrate module labeling (CAMOLA) technique was developed to evaluate relative importance of different pathways that lead to a certain target molecule. In particular, this technique employes a combination of $^{13}C_6$-labeled and unlabeled carbohydrates such as glucose and fructose at equal ratio to explain the extent of fragmentation of the sugar skeletons and the formation of key transient intermediates involved in the formation of flavor molecules (*9*). If these transient intermediates combine, isotopomers of the respective product are formed, being ruled statistically, from these modules. This approach has been used to clarify formation pathways and gain insight into the fragmentation of precursors of Maillaed reaction products (MRPs) (*9*, *10*). Using CAMOLA technique, Schieberle demonstrated that the reaction conditions influence the formation pathway of furaneol from the thermal reaction of glucose and proline (*9*). Cerny and Davidek also performed CAMOLA approach to show that the carbon skeleton remains intact in the formation of 2-methyl-3-furanthiol, 2-furfurylthiol, and 3-mercapto-2-pentanone during the reaction of ribose/[$^{13}C_5$]-ribose with cysteine (*11*). Some sulfur-containing compounds, such as 2-methyl-3-furanthiol, 3-mercapto-2-pentanone, furfurylthiol, and 4,5-dihydro-2-methyl-3-furanthiol, were reported to be key odorants from the reaction of cysteine and thiamine with xylose. When [$^{13}C_5$]-xylose was used in the Maillard reaction instead

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of xylose, the carbon atoms of furfurylthiol were found to be completely labeled by ^{13}C. This could demonstrate that the whole carbon skeleton of furfurylthiol was originated from xylose as the carbon precursor. In contrast, 4,5-dihydro-2-methyl-3-furanthiol was virtually unlabeled, indicating thiamine as the carbon source (*12*).

Although GSH plays an important role in the formation of diverse volatile sulfur-containing compounds during the heating processing, sulfur-containing compounds from Maillard reaction products (MRPs) of GSH and the relative importance of their formation pathways have not been systematically studied yet. Therefore, the objective of this study was to elucidate the formation of volatile sulfur-containing compounds in GSH-MRPs, which can be formed from the interaction of GSH and glucose during the thermal reaction. The CAMOLA approach was employed to evaluate the relative contribution of precursors to the formation pathways and elucidate the origin of the carbon skeleton for sulfur-containing compounds .

Experimental

Chemicals

L-glutathione (GSH), D-glucose ([$^{12}C_6$]-D-glucose), n-alkane standards (C_8-C_{22}), sodium sulfate, and an internal standard compound (ethyl trans-2-octenoate) were purchased from Sigma-Aldrich (St. Louis, MO). Dichloromethane of HPLC grade was obtained from Fisher Scientific (Seoul, South Korea). All authentic standard compounds used in this study were obtained from Sigma-Aldrich. For the CAMOLA study, [U-$^{13}C_6$]-D-glucose was obtained from ISOTEC (Milwaukee, WI).

Model Maillard Reaction Systems

GSH (0.01 M) and D-glucose (0.01 M) were dissolved in 100 mL of HPLC grade water (Fisher Scientific). The reaction mixtures were adjusted to pH 7.5 and then sealed in a 200 mL stainless steel cylinder. The cylinder was heated in a 160 °C drying oven for 2 h. After the thermal reaction, the cylinder was cooled in cold water before the cap was opened.

Extraction of Volatile Maillard Reaction Products

After the reaction mixture was cooled, the volatile components were extracted using a simultaneous steam distillation and solvent extraction (SDE) method with a Likens-Nickerson (L-N) apparatus with 50 mL of dichloromethane. Before the SDE, an internal standard compound (50μL of 100 ppm ethyl trans-2-octenoate in dichloromethane, w/v) was added for quantification. After the sample started boiling, SDE was run continuously for 2 h. The extract was dehydrated using anhydrous sodium sulfate and filtered on Advantec 110 mm filter paper (Toyo Roshi Kaisha, Tokyo, Japan) before concentrated to a final volume of 0.1 mL using a gentle stream of nitrogen gas.

Analysis by Gas Chromatography-Mass Spectrometry (GC-MS)

The volatile extracts from Maillard reaction products (MRPs) were analyzed by GC-MS, using a gas chromatograph and mass selective detector (6890N and 5975, respectively; Agilent Technologies, Palo Alto, CA) equipped with a DB-5 ms column (30 m length × 0.25 mm i.d. × 0.25 mm film thickness; J&W Scientific, Folsom, CA). Helium was run as a carrier gas at a constant column flow rate of 0.8 mL/min. A 1 μL aliquot of the MRP extract was injected into the GC column using the splitless injection mode. The oven temperature was initially held at 40 °C for 4min, raised to 200 °C at a rate of 2 °C/min, and then held there for 10 min. The temperatures of the injector and detector transfer line were 200 °C and 250 °C, respectively. The mass detectorwas operated in electron impact mode with ionization energy of 70 eV, a scanning range of 33-550 amu, and a scan rate of 1.4 scans/s.

Identification of Volatile Compounds

Volatile compounds were positively identified by comparing their mass spectral data, and linear retention indices (RIs) with those of authentic compounds. The RI of each compound was calculated using n-alkanes C_8-C_{22} as external reference (*13*). Otherwise, tentative identification was made based on mass spectra in on-line Wiley database. The semiquantitative analysis of volatile compounds was performed by comparing their peak areas to that of the internal standard compound (50μL of 100ppm ethyl trans-2-octenoate in dichloromethane, w/v) on the GC-MS total ion chromatograms.

Carbohydrate Module Labeling (CAMOLA) Experiment

Equimolar amounts of 0.01 M fully labeled [$^{13}C_6$]-D-glucose and 0.01 M unlabeled D-glucose ([$^{12}C_6$]-D-glucose) were reacted with 0.01 M GSH in a drying oven at 160 °C for 2 h. The reaction mixture was then extracted using the SDE method as described above. The extracts were dehydrated over anhydrous sodium sulfate, concentrated to 0.1mL of final volume under a gentle stream of nitrogen gas, and then subjected to GC-MS analysis.

Calculation of Isotopomer Proportions

The isotopomer ratios were calculated using the relative signal intensities of analyzed ions in the mass spectrum of the respective compound. The values of the calculated isotopomer proportions for sulfur-containing compounds were corrected by subtracting the naturally occurring percentages of ^{13}C (1.1%), ^{33}S (0.76%), and ^{34}S (4.20%). The loss of hydrogen observed with the molecular ion signal was determined in the labeled molecular ions by the ratio $[M^+ - 1]/[M^+]$. Additional data processing was required for 5-methylthiophene-2-carbaldehyde and 3-methylthiophene-2-carbaldehyde; calculation of the isotopomer ratio was based on the $[M^+ - 1]$ ion signal instead of $[M^+]$ because the former was

more intense than the molecular ion signal. After correction, any isotopomer percentages below 1% were taken to be 0%.

Results and Discussion

Volatile Compounds in Glutathione-Maillard Reaction Products

Table I lists the volatile compounds identified in glutathione and glucose (GSH-GLU) Maillard reaction products (MRPs), considering their mass spectral data, relative peak areas, and RIs on the DB-5 column, respectively. A total of 29 volatile compounds, including 1 thiazole, 12 thiophenes, 7 furans and furanones, 2 polysulfides, 1 pyrazine, 5 other nitrogen-containing heterocyclics, and 1 miscellaneous, were identified in GSH-GLU MRPs. The volatile compounds in GSH-GLU MRPs were primarily composed of sulfur-containing compounds. The chemical structures of these sulfur-containing compounds are shown in Figure 1. The major volatile compounds formed in the GSH-GLU MRPs were thiophene and its derivatives, the abundance of which could be due to the hydrogen sulfide easily released from the cysteine residue in GSH. It was reported that the release of hydrogen sulfide from GSH is much faster than that of ammonia (*8*). Therefore, thiophene derivatives dominated the sulfur-containing compounds, whereas thiazole derivatives, which need additional nitrogen for their formation, were almost undetectable in GSH MRPs. When Ho et al. (*8*) investigated the Maillard volatile products generated from cysteine-containing peptides, GSH, γ-glu-cys, cys-gly, and gly-cys, with glucose, they found that GSH produced larger amounts of thiophenes compared to thiazoles.

Our study identified thiophenes and polysulfides as major components in GSH-GLU MRPs. Among thiophene derivatives, 2-ethylthiophene, 2,5-dimethylthiophene, thiolan-3-one, thiophene-2-thiol, 1-thiophen-2-ylethanone, 5-methylthiophene-2-carbaldehyde, 3-methylthiophene-2-carbaldehyde, and 1-thiophen-3-ylethanone were detected as major components. These thiophenes have been identified in a wide range of food systems in which they significantly contribute to the characteristic odor properties (*14*). In particular, 2,5-dimethylthiophene has been identified in cooked beef, chicken, and pork liver (*15*). On the other hand, 3-methylthiophene-2-carbaldehyde was identified in chicken, whereas 5-methylthiophene-2-carbaldehyde, which has a roasted odor note, was found in cooked beef (*16*).

Elucidation of Volatile Sulfur-Containing Compounds Formation

GSH and glucose (equimolar amounts) were reacted at pH 7.5 in aqueous system at 160 °C for 2 h. The thermal reaction was carried out using a mixture (1:1) of unlabeled and $^{13}C_6$-labeled glucose. Diverse sulfur-containing compounds were found in GSH MRPs. The mass spectra of volatile sulfur-containing compounds identified from the Maillard reaction of [$^{12}C_6$]-glucose/[$^{13}C_6$]-glucose and GSH were analyzed on the basis of the mass-to-charge ratios (m/z) of the molecular ions of the isotopomers, which exhibited signals with mass differences of up to M^{+}+ 6 as compared to those obtained from GSH-unlabeled

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glucose MRPs. Table II lists the identified volatile sulfur-containing compounds and the proportions of their ^{13}C-labeled isotope molecules. The isotopomers indicated that the molecules comprise either unlabeled carbons, fully ^{13}C-labeled carbons, or a mixture of labeled and unlabeled carbon fragments. In the case of 2-methylthiophene, 2,5-dimethylthiophene, thiophen-2-ylmethanol, 5-methylthiophene-2-carbaldehyde, and 3-methylthiophene-2-carbaldehyde, the values for the labeled $[M^+-1]$ ions were corrected by the ratio $[M^+]/([M^+ - 1]$ due to the significant loss of hydrogen.

Table I. Volatile compounds formed from the thermal reaction of glutathione with glucose

No	*RI*[a]	*Possible compounds*	*Relative peak area*[b]	*ID*[c]
		Thiazoles		
1	<800	1,3-thiazole	0.015±0.001	A
		Thiophenes		
2	<800	2-methylthiophene	0.013±0.001	A
3	856	2-ethylthiophene	0.272±0.040	A
4	862	2,5-dimethylthiophene	0.382±0.033	A
5	948	thiolan-3-one	1.295±0.200	A
6	965	thiophene-2-thiol	0.442±0.040	A
7	1024	thiophen-2-ylmethanol	0.132±0.007	B
8	1038	2-methyl-2H-thiophen-5-one	0.029±0.002	C
9	1048	2-methylthiophene-3-thiol	0.028±0.005	A
10	1081	1-thiophen-2-ylethanone	0.533±0.088	A
11	1108	5-methylthiophene-2-carbaldehyde	0.293±0.004	A
12	1109	3-methylthiophene-2-carbaldehyde	0.321±0.027	A
13	1263	1-thiophen-3-ylethanone	3.325±0.245	C
		Furans and Furanones		
14	803	2-methyloxolan-3-one	0.138±0.061	B
15	823	furan-2-carbaldehyde	0.659±0.043	A
16	847	furan-2-ylmethanol	0.280±0.031	B
17	860	2-methylfuran-3-thiol	0.139±0.006	A
18	905	1-furan-2-ylethanone	5.148±0.745	A
19	955	5-methylfuran-2-carbaldehyde	3.985±0.282	A
20	1217	5-(hydroxymethyl)furan-2-carbaldehyde	0.428±0.023	A

Continued on next page.

Table I. (Continued). Volatile compounds formed from the thermal reaction of glutathione with glucose

No	*RI*[a]	*Possible compounds*	*Relative peak area*[b]	*ID*[c]
		Pyrazines		
21	820	2-methylpyrazine	0.011±0.002	A
		Other nitrogen-containing heterocyclics		
22	<800	1-methylpyrrole	0.033±0.007	B
23	831	2-methyl-1H-pyrrole	0.039±0.001	B
24	1004	1H-pyridin-4-one	1.393±0.210	C
25	1026	2-methyl-4-methylene-tetrahydropyran	0.078±0.003	C
26	1061	1-(1H-pyrrol-2-yl)ethanone	2.480±0.228	A
		Polysulfides		
27	813	1-methylsulfanylpropan-2-one	1.448±0.132	C
28	1161	dithian-4-one	1.967±0.266	C
		Miscellaneous		
29	<800	S-methyl ethanethioate	1.756±0.130	B

[a] Retention indices were determined using n-paraffins C_7-C_{22} as external references. [b] Average of relative peak areas to that of internal standard (n=3)±standard deviation. [c] Identification was performed as follows: A, mass spectrum and retention index were consistent with those of an authentic standard; B, mass spectrum was identical with that of Wiley 275 I mass spectrum database (1995, Hewlett Packard Co., Palo Alto, USA) and retention index were consistent with those of the literature (Kondjoyan and Berdague 1996); C, mass spectrum was consistent with that of Wiley 275 I mass spectrum database or by manual interpretation (tentative identification).

As indicated in Table II, 2-ethylthiophene, 2,5-dimethylthiophene, 1-thiophen-2-ylethanone, 5-methylthiophene-2-carbaldehyde, and 1-thiophen-3-ylethanone had the isotopomers of $[M^+]$ and $[M^{+}+6]$ ions, indicating that the unlabeled and the 6-fold-labeled molecules were present at approximately 1:1. These data suggest that these compounds can be formed from the intact carbon skeleton of a C-6 glucose chain without the recombination of glucose fragments.

In addition, the isotopomeric distribution patterns of thiolan-3-one, 1-methylsulfanylpropan-2-one, and dithian-4-one showed that unlabeled isotopomer and isotopomer with complete 4-fold labeling appeared at 1:1. This shows that these compounds can be formed from glucose by the loss of two carbon atoms, but the exact positions of carbon units lost from glucose was not confirmed. For 2-methyl-2H-thiophen-5-one, only unlabeled isotopomer and 5-fold labeled isotopomer were found at approximately 1:1, supporting that 2-methyl-2H-thiophen-5-one can be formed from glucose through the loss of one carbon atom.

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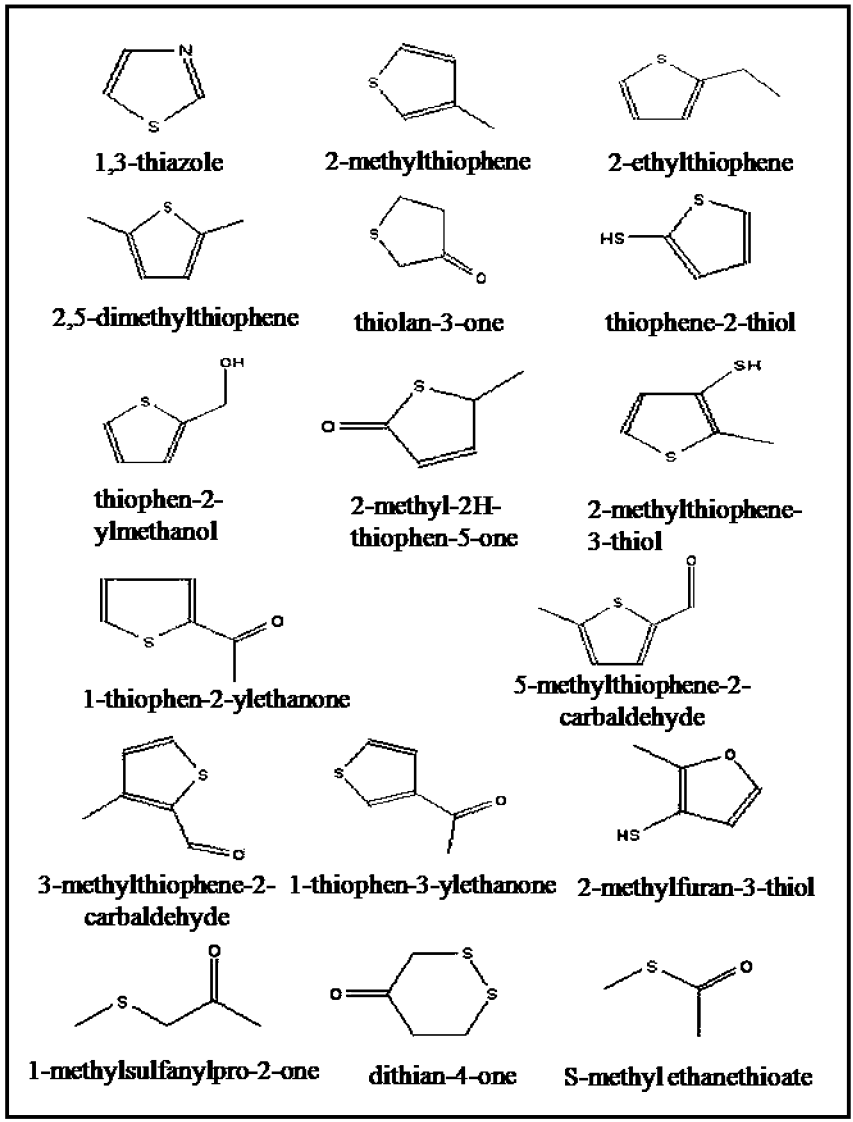

Figure 1. Volatile sulfur-containing compounds formed from the thermal reaction of glutathione and glucose.

Table II. Proportion of isotopomers of volatile sulfur-containing compounds formed from the reaction of glutathione with a mixture of [$^{12}C_6$]-glucose and [$^{13}C_6$]-glucose

No [a]	*Sulfur-containing Compounds*[b]	*m/z* (M^+)	*Proportion of labeled carbon atoms in the molecule* [c] *(%)*						
			0[d]	*1*	*2*	*3*	*4*	*5*	*6*
1	1,3-thiazole	85	52	34	9	5			
2	2-methylthiophene	98	35	6	15	12	1	31	
3	2-ethylthiophene	112	47	4	2	0	0	10	37
4	2,5-dimethylthiophene	112	46	6	3	1	3	0	41
5	thiolan-3-one	102	46	3	3	4	44		
6	thiophene-2-thiol	116	57	5	12	3	23		
7	thiophen-2-ylmethanol	114	48	4	6	3	13	26	
8	2-methyl-2H-thiophen-5-one	114	59	0	0	0	0	41	
9	2-methylthiophene-3-thiol	130	33	5	12	29	6	15	
10	1-thiophen-2-ylethanone	126	47	3	3	3	3	1	40
11	5-methylthiophene-2-carbaldehyde	126	42	7	4	0	6	0	41
12	3-methylthiophene-2-carbaldehyde	126	45	5	4	0	41	4	1
13	1-thiophen-3-ylethanone	126	45	3	1	0	1	4	46
17	2-methylfuran-3-thiol	114	32	4	12	6	12	34	
27	1-methylsulfanylpropan-2-one	104	47	3	2	1	47		
28	dithian-4-one	134	47	2	8	0	43		
29	S-methyl ethanethioate	90	48	1	3	48			

[a] No represented in Table I. [b] Sulfur-containing compounds were identified in the thermal reaction of glutathione and glucose by comparing the mass spectra and retention indices. [c] Values were corrected by subtracting the naturally occurring percentages of ^{13}C (1.10%), ^{33}S (0.76%), and ^{34}S (4.20%) in $M^+ + 1$ and $M^+ + 2$. The loss of hydrogen observed with the molecular ion in EI-MS was also corrected in the labeled molecular ions by the ratio $(M^+ - 1)/M^+$. [d] Number of ^{13}C atoms in the molecule.

Although two methylthiophene-2-carbaldehydes (5-methylthiophene-2-carbaldehyde and 3-methylthiophene-2-carbaldehyde) were present as isomers with almost identical mass spectra, these compounds showed different isotopomeric distribution patterns. 5-Methylthiophene-2-carbaldehyde had the isotopomers of [M^+] (*m/z* 126) and [M^++6] (*m/z* 132). Whereas, other mixed isotopomers at *m/z* 127-131 were almost undetectable, indicating that the unlabeled and the 6-fold-labeled molecules were present at approximately 1:1. That is, the carbon skeleton of glucose chain remained intact for the formation of 5-methylthiophene-2-carbaldehyde. Recently, we reported that the intact carbon skeleton of glucose via 3-deoxyhexosone is incorporated into 5-methylthiophene-2-carbaldehyde, with hydrogen sulfide released from GSH (*17*). On the other hand, the isotopomeric distribution patterns of 3-methylthiophene-2-carbaldehyde demonstrated that unlabeled isotopomer and isotopomer with complete 4-fold labeling appeared at 1:1, whereas other labeled isotopomers (*m/z* 127, 128, 129, 131, and 132) were practically undetectable. This suggests that C-4 skeleton can be derived from glucose and the C-2 fragment come partly from GSH in the carbon skeleton of 3-methylthiophene-2-carbaldehyde. On the basis of the isotopomeric distribution results, a possible formation pathway for 3-methylthiophene-2-carbaldehyde in GSH-GLU MRPs was shown in our previous study (*17*). 3-Methylthiophene-2-carbaldehyde could be produced via recombination and cyclization of mercaptoacetaldehyde and glucose C-4 fragment.

The use of labeled and unlabeled precursors in the thermal reaction was proposed to gain insight into the fragmentation of precursors for the formation of volatile sulfur-containing compounds. The CAMOLA approach demonstrated that formation of 5-methylthiophene-2-carbaldehyde and 3-methylthiophene-2-carbaldehyde occurs via the recombination of fragments that may originate from both GSH and glucose.

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Chapter 12

Volatile Sulfur Compounds in Foods as a Result of Ionizing Radiation

Xuetong Fan,*,[1] Eun Joo Lee,[2] and Dong Ahn[2]

[1]USDA, Agricultural Research Service, Eastern Regional Research Center, Wyndmoor, Pennsylvania 19038
[2]Department of Animal Sciences, Iowa State University, Ames, Iowa 50011
***E-mail: xuetong.fan@ars.usda.gov.**

Ionizing radiation improves food safety and extends shelf life by inactivating food-borne pathogens and spoilage microorganisms. However, irradiation may induce the development of an off-odor, particularly at high doses. The off-odor has been called "irradiation odor". Substantial evidence suggests that volatile sulfur compounds (VSCs) play an important role in the development of the off-odor. These compounds include hydrogen sulfide, methanethiol, methyl sulfide, dimethyl disulfide and dimethyl trisulfide among others. The formation of off-odor and VSCs due to irradiation in meat, and fruit juices is presented. It is known that irradiation exerts its effect through radiolysis of water in foods where water is a dominant component. Irradiation of water produces three primary free radicals: hydroxyl, hydrogen atoms, and hydrated electrons. Use of specific scavengers in a model system revealed that hydroxyl radicals are involved in the formation of VSCs. Possible mechanisms for formation of VSC are also discussed. Also discussed are possible remedies for formation of VSCs and off-odor, such as use of antioxidants and double packaging.

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Irradiation is a non-thermal processing technology that has been studied for the enhancement of microbial safety, insect disinfestation, sprouting inhibition and shelf-life extension. In general, irradiation at doses for the more common purposes does not affect quality. However, irradiation of many foods at high doses may induce development of an off-odor. The off odor has been called "irradiation odor" and is described as 'metallic', 'sulfide', 'wet dog', and 'wet grain' (*1*, *2*). When beef and pork frankfurters were irradiated at doses of 8 and 32 kGy (irradiation temperature: -34°C), an off-odor and off-flavor were noticed, and the intensity of the off odor increased with radiation dose (*3*). Frankfurters irradiated at 5 and 10 kGy were often scored higher in off-flavor than the non-irradiated ones (*4*). However, ready-to-eat beef luncheon meats irradiated at doses of 2-4 kGy had similar off-flavor as the non-irradiated controls (*5*). Johnson et al. (*6*) showed that the aroma of cooked diced chicken meats and chicken frankfurters irradiated at doses up to 3 kGy (irradiation temperature: 4°C) did not differ from the non-irradiated ones. After 18 days of storage, the aroma of irradiated diced chicken was better than the control, presumably due to inactivation of spoilage microorganisms by irradiation. In a later study by the same group of researchers (*7*), 'wet dog' aroma was detected in chicken frankfurter by panelists immediately after irradiation. However, this aroma decreased and was not present after 7 or 17 days of storage at 4°C. At day 23 after irradiation, 'wet dog' aroma reappeared and received the same low rating as day 2 after irradiation. Hashim and others (*8*) reported that irradiated uncooked chicken thigh had a higher 'blood and sweet aroma' than non-irradiated. Heath and others (*9*) reported that irradiation of uncooked chicken breast and thigh produced 'hot fat', 'burned oil' and 'burned feathers' odors. Ahn et al. (*10*) described the off-odor as 'barbecued corn-like'. Fan (*11*) and Yoo et al. (*12*) found that nonirradiated orange juice was significantly different from irradiated orange juice at doses as low as 0.5 kGy. Sensory panelists described the off-odor in irradiated orange juice as "burning rubber," "chemical," and "alcohol." Other odor descriptions include "bitterness", "medicinal", and "cooked" in irradiated orange juice (*13*). Prakash et al. (*14*) found that irradiated (2.98 and 5.25 kGy) almonds were significantly higher ($p<0.05$) in metallic/chemical/rancid/oxidized/fatty taste than the control samples, but the differences between the two irradiated samples was not significant.

Evidence indicates that VSCs are mostly responsible for the off-odors due to irradiation. This evidence includes: 1) The irradiation odor is different from rancidity, which is believed to be caused mainly by lipid oxidation. 2) Irradiation of the lipid (fat soluble) phase of a meat extract does not produce the characteristic off-odor, while irradiation of the aqueous (water soluble) portion of the meat extract results in a typical irradiation odor (*15*). 3). Irradiation of sulfur-containing amino acids or polypeptides produced a similar off-odor as the irradiation odor (*16*). 4) The amount of VSCs increased with radiation dose, while volatiles from lipids were not always correlated with radiation dose (*17*). 4). Food spiked with VSCs at the amounts similar to those in irradiated samples produced off-odor (*18*).

Formation of Volatile Sulfur Compounds from Various Foods

Raw Meats

Several earlier researchers suggested that hydrogen sulfide (H_2S) and methanethiol (MeSH) were important for the development of the off-odor in irradiated meats (*1*, *15*, *19*). Patterson and Stevenson (*20*), using GC-olfactory analysis, showed that dimethyl trisulfide (DMTS) was the most potent off-odor compound in irradiated raw chicken meats followed by *cis*-3- and *trans*-6-nonenals, oct-1-en-3-one and bis(methylthio-) methane. Ahn and his colleagues (*21*) have identified MeSH, dimethyl sulfide (DMS), dimethyl disulfide (DMDS) and DMTS in different types of irradiated raw meats using GC-FID and GC-MS.

Ready-to-Eat Meats

Du and Ahn (*22*) found that irradiation induced formation of MeSH, DMDS and DMTS in turkey sausage. The low levels and reactivity of volatile sulfur compounds complicated accurate detection of these compounds. A pulsed flame photometric detector (PFPD) has been used to detect VSCs. PFPD is very sensitive to sulfur compounds, detecting VSCs in part per trillion (ppt) ranges. Use of the SPME technique avoids the formation of artifacts due to high temperature as used in many other extraction techniques, however, SPME techniques have low repeatability, resulting in larger variations among replicates. Figure 1 illustrates irradiation-induced VSCs in preccoked turkey breasts using SPME-GC-PFPD (*23*). Six VSCs were identified, including H_2S, CS_2, MeSH, DMS, DMDS and DMTS. Most of the VSCs were promoted by irradiation in a dose dependent manner in the ready to eat turkey meat. CS_2 levels, however, were reduced by irradiation. It appears that irradiation can either increase or decrease the levels of H_2S or DMS depending on meat composition, initial concentration of the compounds, packaging type, and gas composition (*11*, *23*). Many of the VSCs are highly reactive and unstable. H_2S and MeSH decreased rapidly during storage at 4°C even under air-impermeable vacuum packaging (*11*, *23*). The disappearance of the low-boiling-point sulfur compounds may be due to their reactivity and instability. For example, H_2S in aqueous solution becomes elemental sulfur upon reacting with oxygen, while DMDS may convert to DMS and DMTS (Fig. 2).

Fruit Juices

It appears that there are contradictions on whether irradiation induces off-flavors in fruit juice. The type and composition of juice may affect the development of off-flavors. Recently, Yoo et al. (*12*) found that concentrations of methyl sulfide and dimethyl disulfide in orange juice increased with radiation dose. Fan (*18*) identified 2 volatile sulfur compounds (H_2S and CS_2) in nonirradiated orange juice and 5 volatile sulfur compounds in irradiated orange juice, including MeSH, DMS, DMDS, and DMTS. Irradiation induced greater amounts of DMS and MeSH than DMDS and DMTS. CS_2 was reduced by irradiation, while H_2S

was not consistently affected. Sensory evaluation indicated that the odor of irradiated juice differed from that of the nonirradiated samples at 0.5, 1, 2, or 3 kGy. To determine whether these 2 compounds were actually involved in the development of off-odor due to irradiation, fresh orange juice was spiked with MeSH and DMS to levels similar to those in the 3 kGy juice. Sensory evaluation revealed that panelists distinguished between samples spiked with MeSH and DMS and the non-spiked sample (Table 1), indicating that those 2 compounds could be involved in the development of off-odor. However, panelists also distinguished between the spiked sample and the 3 kGy samples, indicating that a difference in odor existed between the irradiated samples and the spiked samples. Therefore, other compounds besides the 2 sulfur compounds may be involved in the development of off-odor.

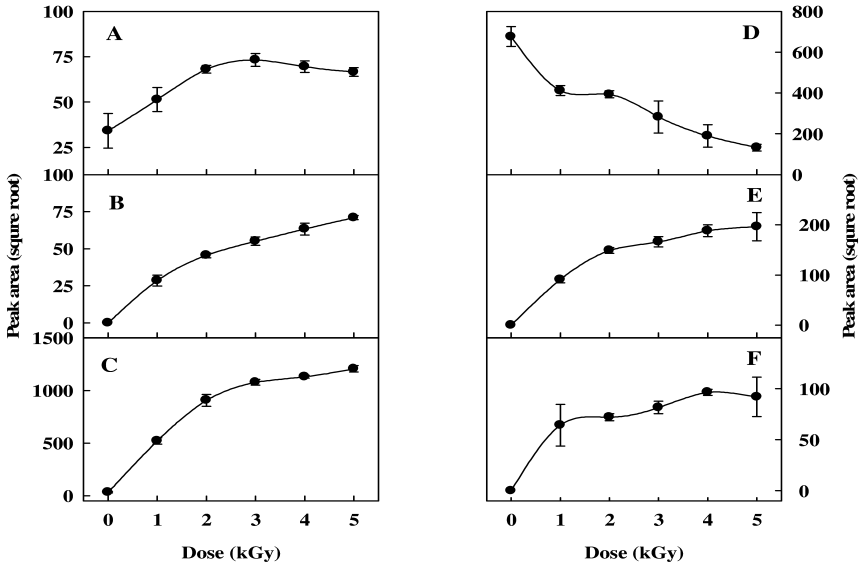

Figure 1. Effect of irradiation dose on the concentration of hydrogen sulfide (A), sulfur dioxide (B), methanethiol (C), carbon disulfide (D), dimethyl disulfide (E), and dimethyl trisulfide (F) of precooked turkey breast. Concentrations of sulfur compounds were expressed as square root of peak area. Vertical bars represent standard deviation of means. (adopted from Fan et al. (23) with permission).

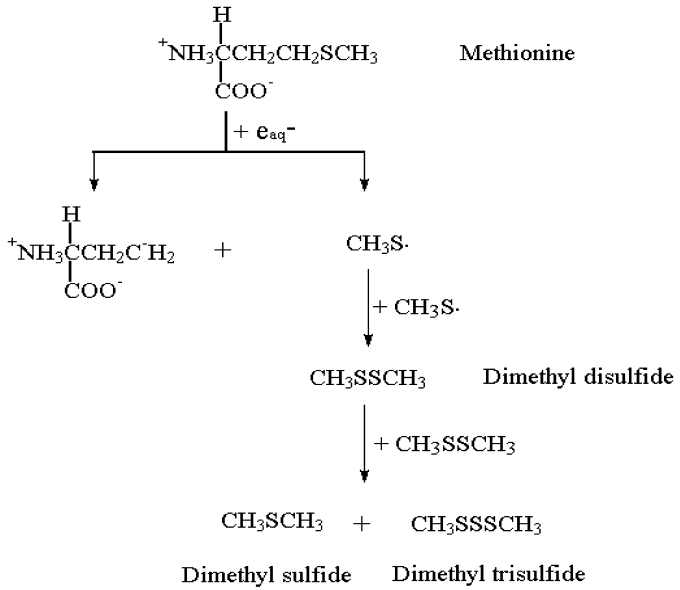

Figure 2. Proposed formation of methyl sulfide, dimethyl sulfide, dimethyl disulfide and dimethyl trisulfide from methionine. (adopted from Yoo et al. (12) with permission).

Mechanism of Volatile Sulfur Compounds Formation

Upon irradiation of water at 25°C, the following reaction occurs: $H_2O \rightarrow e_{aq}^-$ (2.8) + H_3O^+ (2.8) + ·OH (2.8) + ·H (0.5) + H_2 (0.4) + H_2O_2 (0.8). The numbers in parenthesis are the relative amounts expressed as G-values (number of species per 100 eV absorbed) (*24*). The primary free radicals generated from radiolysis of water are hydrated electron (e_{aq}^-), hydroxyl radicals (·OH) and hydrogen atoms (·H). The VSCs found in irradiated meat products and juices are likely formed from sulfur containing compounds reacting with the free radicals generated from the radiolysis of water. These sulfur containing compounds may include amino acids in the form of either free amino acids (methionine, cysteine), peptides (glutathione and cystine) or proteins, and others (thiamine, coenzyme A).

Table 1. Number of panelists correctly identifying the odd juice samples in triangle tests. There were a total of 54 panelists. MeSH and DMS were added into spiked samples. Adopted from Fan (*18*) with permission

Comparison	*Number of correct responses*	
	Exp# 1	*Exp# 2*
0.5 kGy and 0 kGy	29/54 **	29/54 **
1.0 kGy and 0 kGy	33/54 **	31/54 **
2.0 kGy and 0 kGy	40/54 **	43/54 **
3.0 kGy and 0 kGy	38/54 **	40/54 **
Spiked and 0 kGy	26/54 *	37/54 **
3 kGy and spiked	28/54 **	37/54 **

* and ** indicate that the differences are significant at 5% ($P<0.05$) and 1% ($P<0.01$) levels, respectively.

Ahn and Lee (*21*) reported that the majority of volatiles newly generated and increased by irradiation were sulfur compounds. This indicated that sulfur-containing amino acids are among the most susceptible amino acid groups to irradiation. Sensory panels described the odor by the newly produced sulfur compounds as "hard-boiled egg," "boiled sweet corn," "sweet and sulfury," or "steamed vegetable", which was different from lipid oxidation odor but similar to the typical odor of the irradiated meat sample. Ahn suggested that methionine produced far greater amounts of sulfur compounds than cysteine and is the most important amino acid in the production of irradiation off-odor. The sulfur compounds produced from sulfur-containing amino acid dimer or oligomers by irradiation is listed in Table 2.

Ahn (*16*) indicated that more than one site on amino acid side chains was susceptible to free radical attack, resulting in formation of primary VSCs such as MeSH, DMS, DMDS and DMTS. Many more volatiles can be produced by secondary chemical reactions after the primary radiolytic degradation of side chains (Table 2). Furthermore, the amounts and kinds of sulfur compounds produced from irradiated methionine and cysteine indicated that methionine is the major amino acid responsible for irradiation off-odor. The total amount of sulfur compounds produced from cysteine is only about 0.25 to 0.35% of methionine. It has been proposed that formation of DMS, DMDS and DMTS is result of methionine reacting with hydrated electrons (e_{aq}^-) (Fig. 2). Many other free radicals may be involved in the formation of VSCs.

Table 2. Production of volatile compounds from sulfur-containing amino acid dimer or oligomers by irradiation. Adopted from Ahn (*16*) with permission

Volatiles	*0 kGy*	*5 kGy*	*SEM*
	--------- Total ion counts × 10^3 ---------		
Glutathione (γ-Glu-Cys-Gly)			
Carbon disulfide	0[b]	589[a]	24
Hexane	316[b]	496[a]	39
Methyl cyclopentane	0[b]	82[a]	5
Cyclohexane	119[a]	0[b]	2
Dimethyl disulfide	0[b]	214[a]	47
Met-Ala			
2-Methyl-1-propene	614[a]	0[b]	11
Acetaldehyde	0[b]	2910[a]	230
Methanethiol	0[b]	11842[a]	709
2-Propanone	1244[a]	0[b]	456
Dimethyl sulfide	0[b]	166244[a]	6183
2-Methyl propanol	0[b]	114[a]	3
Hexane	281[b]	1146[a]	47
Methyl thiirane	0[b]	4177[a]	174
(Methylthio) ethane	1376[a]	0[b]	47
2-Ethoyxy-2-methyl propane	1299[a]	344[b]	114
Ethyl acetate	3290	4467	415
Cyclohexane	1565[a]	0[b]	13
3-(Methylthio)-1-propene	0[b]	186[a]	11
Methyl thioacetate	0[b]	106[a]	7
2-Methyl-2-(methylthio) propane	86[a]	0[b]	1
Dimethyl disulfide	5043[b]	346229[a]	9385
Methyl benzene	591[a]	0[b]	23
Methyl ethyl disulfide	0[b]	2221[a]	80
2,4-Dithiapentane	0[b]	825[a]	25
Met-Gly-Met-Met			
2-Methyl-1-propene	270[a]	0[b]	8
Acetaldehyde	2264[a]	0[b]	224

Continued on next page.

Table 2. (Continued). Production of volatile compounds from sulfur-containing amino acid dimer or oligomers by irradiation.

Volatiles	*0 kGy*	*5 kGy*	*SEM*
Methanethiol	0[b]	17325[a]	866
Pentanal	0[b]	341[a]	18
Dimethyl sulfide	0[b]	201541[a]	939
2-Propanone	4010[a]	0[b]	289
Acetonitrile	3485[a]	356[b]	414
Hexane	285[b]	780[a]	26
2,2-Oxybis propane	17951[a]	3843[b]	183
(Methylthio) ethane	0[b]	2053[a]	15
2-Butanone	206[a]	0[b]	35
Ethyle acetate	116873[a]	77893[b]	4084
Cyclohexane	988[a]	0[b]	21
Benzene	0[b]	210[a]	1
1-Heptanethiol	0[b]	94[a]	1
3-(Methylthio)-1-propene	0[b]	122[a]	1
Mathyl thioacetate	0[b]	170[a]	8
2-Butanamine	0[b]	156[a]	6
2-Methyl-2-(methylthio) propane	92[b]	149[a]	2
Dimethyl disulfide	1430[b]	351320[a]	1247
Methyl ethyl disulfide	0[b]	1935[a]	15
Ethyl benzene	0[b]	38116[a]	322
1,3-Dimethyl benzene	0[b]	60346[a]	823
1,4-Dimethyl benzene	0[b]	11550[a]	164
Isopropyl benzene	0[b]	725[a]	20

[a,b]Means with no common superscript differ significantly ($p < 0.05$), n = 4. SEM=standard errors of means.

Involvement of Hydroxyl Radicals

Free radical scavengers have been used to study the involvement of the primary species in radiation-induced chemical changes. In the presence of *tert*-butyl alcohol in Ar-purged solutions, ·OH radicals are converted to the non-reative $CH_2(CH_3)_2COH$ radical, via an H atom abstraction process, leaving e_{aq}^- as the dominant reactive species (*25*). A study was conducted to investigate the involvement of hydroxyl radicals generated through water radiolysis in the formation of VSCs. Fifteen g diced turkey breast was added to 29.55 m water

containing 0.45 ml *tert*-butanol, and the mixture was homogenized for 2 min. Then 5 g homogenate was added to 15 ml vials, sealed with septum and caps and flushed with argon for 3 min at 120 ml/min through needles. A control sample without *tert*-butyl alcohol was similarly prepared and flushed with air. Samples were exposed to gamma radiation at a dose of 5 kGy. Immediately after irradiation, internal standards (~1 ppb ethyl sulfide and 1 ppm 2-methyl pentanal) were added. Volatile compounds were then extracted using the solid phase microextraction (SPME) technique. The vials were incubated at 40°C for 35 min before the SPME fiber was inserted and exposed for 30 min. Volatile compounds were analyzed using GC-MS-PFPD. Standard curves were established for DMDS and DMTS in the turkey breast homogenate in the presence of air, and in the presence of the combination of argon and 1% *tert*-butanol. Results showed that irradiation induced formation of volatile sulfur compounds such as DMDS and DMTS. In the presence of *tert*-butanol, the formation of DMDS was reduced by 89% while DMTS was reduced by about 60% (Figure 3), suggesting that irradiation-induced formation of volatile sulfur compounds was partially due to the hydroxyl radicals produced from radiolysis of water. Other VSCs including H_2S and MeSH were also indentified but not quantified. Figure 4 shows a proposed pathway for the formation of volatile sulfur compounds from the reaction of hydroxyl radicals with methionine.

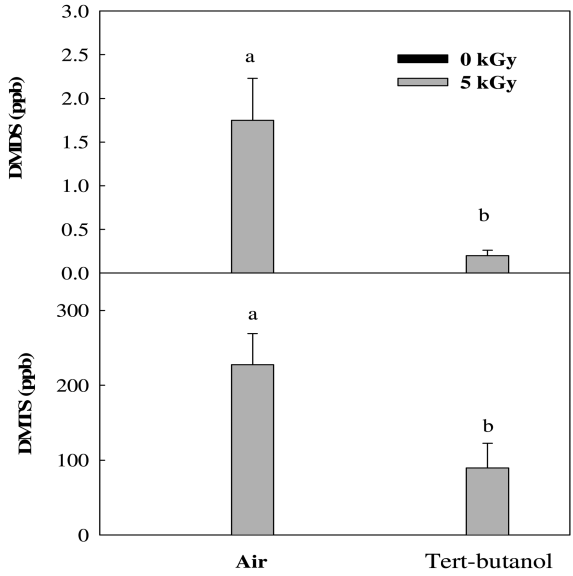

Figure 3. Effect of tert-butanol on irradiation-induced formation of dimethyl disulfide (DMDS) and dimethyl trisulfide (DMTS) in cooked turkey breast homogenates. Turkey breast pieces, homogenized with tert-butanol and flushed with Argon, were irradiated at 5 kGy. Volatile sulfur compounds were measured. Vertical bars represent standard errors (n=3).

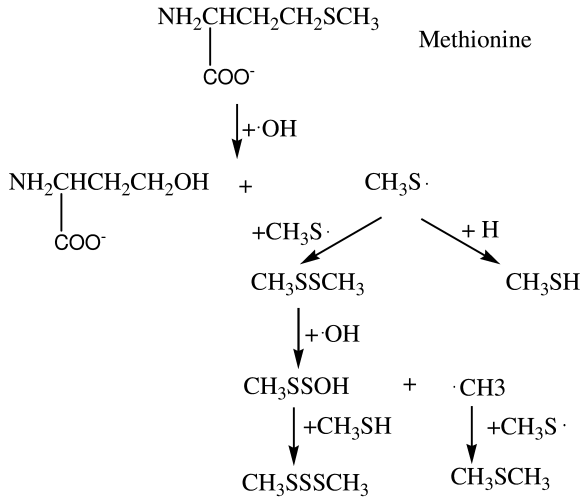

Figure 4. A proposed pathway for the formation of methanethiol, dimethyl sulfide, dimethyl disulfide and dimethyl trisulfide as a result of reaction of hydroxyl radicals with methionine.

Reduction of VSCs and Off-Odor

Developing prevention methods to reduce VSCs and off-odor production in irradiated foods are very important for the adoption of irradiation technology in the food industry. To prevent or minimize VSCs and off-odor production in irradiated foods, various additives and packaging types have been tested.

Use of Antioxidants and Natural Plant Extracts

Many researchers have used and suggested various antioxidants to control off-odor in irradiated meat. Generally, antioxidants interrupt autoxidation of lipids, either by donating a hydrogen atom or quenching free radicals (*26*). Therefore, addition of antioxidants may be effective in reducing the oxidative reactions in irradiated meat by scavenging free radicals produced by irradiation (*27*, *28*). Even though synthetic antioxidants including BHT, BHA and propyl gallate usually show strong antioxidant effects in preventing oxidative rancidity and retarding development of off-flavors (*29*, *30*), natural antioxidants such as ascorbic acid and alpha-tocopherol also have been widely tested in recent years because consumers prefer natural antioxidants (*31*, *32*).

To reduce VSCs and off-odor production of irradiated meats, antioxidants can be added in animal feeds as a dietary supplement or added directly to ground meat and ready-to-eat cooked meat as additives. α-Tocopheryl acetate has been used as dietary supplement of vitamin E in chicken feed (*20*), turkey feed (*33*), and cattle feed (*34*). Dietary antioxidant treatments showed strong effects in stabilizing lipids in membranes and reduced the extent of lipid oxidation in irradiated meat

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during storage, but had marginal effects in reducing sulfur-containing volatiles in irradiated meat (*35*).

Various studies, in which antioxidants were added directly to irradiated raw meat before irradiation, showed stronger effects in preventing oxidative rancidity and retarding off-flavor development than dietary treatments. Antioxidants such as ascorbate, citrate, tocopherol, gallic esters, and polyphenols were effective in reducing the off-odor of irradiated meat after adding directly to irradiated meat (*1*). Ascorbic acid and sesamol (3,4-methylenedioxyphenol) + tocopherol also were reported to reduce the amounts of dimethyl disulfide in irradiated ground beef (*36*). Rice hull extract applied to irradiated turkey breast was as effective in reducing dimethyl disulfide as sesamol or rosemary oleoresin (*37*).

In irradiated cooked meat, antioxidants also showed strong effects in reducing lipid oxidation, but they were not effective in reducing production of VSC's (*22*, *31*). Fan et al. (*38*) manufactured bologna from ground turkey breast containing one of four antioxidant treatments (none, rosemary extract, sodium erythorbate, and sodium nitrite) and then irradiated samples at doses up to 3 kGy. Addition of nitrite, erythorbate, or rosemary extract to raw meat mixtures used for turkey bologna manufacture did not reduce levels of irradiation-induced VSC formation. Some of the VSCs were even promoted by addition of the antioxidants. Dipping diced turkey bologna in antioxidants solutions also did not reduce the production of VSCs due to irradiation (*39*). It appears that antioxidants have very limited effects on irradiation-induced VSCs in ready-to-eat turkey bologna. The limitation of antioxidants suggests that formation of volatile compounds may be resulted in part from direct scission of S-containing amino acids and peptides. Alternatively, antioxidant levels might not be high enough or did not diffuse to places where free radicals were generated.

In conclusion, antioxidants have strong effects in inhibiting lipid oxidation in irradiated meat, but little effect in reducing VSC production. Therefore, instead of using antioxidants to minimize VSC production by irradiation, other approaches such as masking irradiation-induced off-flavor using spices, herbs, or their extracts that reduce sulfur volatiles may be needed.

Packaging

Packaging type and gas compostion (oxygen) are important factors influencing the production of irradiated off-odor (*40*). Irradiation and storage of meat under vacuum-packaging conditions are advantageous in preventing lipid oxidation and aldehyde production. Vacuum-packaged meat, however, retained sulfur volatiles produced during irradiation and maintained the levels during storage (*41*). When irradiated meat was stored under aerobic conditions, significant amounts of volatile aldehydes (propanal, pentanal, and hexanal) related to lipid oxidation were produced (*42*, *43*). Sulfur-containing volatile compounds were highly volatile and disappeared when the irradiated meats were stored under aerobic conditions for a certain period of time. For short-term storage (< 3 days) of irradiated meat in which lipid oxidation is not a great problem, aerobic packaging can be more beneficial than vacuum-packaging, because sulfur volatile compounds responsible for the irradiation off-odor can be significantly reduced

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under aerobic conditions. The reduction of VSCs in air packaged products under aerobic conditions may be due to escapee of highly volatilized sulfur compounds or oxidation to non-volatile end products. For longer-term storage (> 5 days), however, some combination of aerobic and vacuum-packaging may be needed to control both lipid oxidation and VSCs in irradiated meat during storage.

Nam and Ahn (*41*, *44*) developed a new packaging concept called "double-packaging", which combined the merits of aerobic and vacuum packaging. The term "double-packaging" was used to describe a packaging method in which meat pieces are individually packaged in oxygen permeable bags (aerobic condition) first and then a few of the aerobic packages were vacuum-packaged in a larger vacuum bag before irradiation. The outer vacuum bag is removed after certain storage time and then displayed as aerobic condition until the last day of storage. The aerobic packaging promoted lipid oxidation in irradiated turkey meats and vacuum-packaged irradiated samples retained VSC's. Double-packaging, however, was effective in reducing the production of lipid oxidation-dependent aldehydes and minimizing VCS in the meat (*41*, *44*, *45*). This indicated that both lipid oxidation and irradiation off-odor could be minimized without using any additives. However, double-packaging alone was not enough to prevent oxidative changes in meat during storage.

Nam and Ahn (*46*) used the combination of antioxidants with double-packaging and found that this was more effective than double-packaging alone. The beneficial effects of double packaging and antioxidants were more evident in irradiated cooked meat than raw meat. The total amount of sulfur volatiles in double-packaged irradiated turkey meat with antioxidants (sesamol + vitamin E and gallic acid + vitamin E) was only about 5-7% of that in the irradiated vacuum-packaged cooked meat without antioxidants after 10 days of storage. Production of aldehydes (propanal and hexanal for raw meat, and propanal, pentanal and hexanal) in irradiated cooked turkey breast was almost completely prevented by using the antioxidant and double-packaging combination. Therefore, the combination of double-packaging (vacuum for 7 days then aerobic for 3 days) with antioxidants for irradiated raw turkey breast was very effective in reducing total and sulfur volatiles responsible for the irradiation off-odor without any problem of lipid oxidation (*36*). However, the amounts of sulfur compounds in raw meat were not influenced by antioxidants (Table).

A study with ground beef indicated that addition of ascorbic acid at 200 ppm was not effective in inhibiting production of volatile aldehydes in aerobically packaged irradiated beef (*43*). However, vacuum packaging or the combination of double-packaging and ascorbic acid was effective in minimizing the production of volatile aldehydes in irradiated ground beef. The levels of off-odor volatiles in double-packaged irradiated ground beef after 6 d storage were comparable to that of aerobically packaged ones, and the degrees of lipid oxidation and color changes were close to those of vacuum-packaged ones. This indicated that lipid oxidation of irradiated ground beef was highly dependent upon the availability of oxygen to meat during storage. Addition of 200 ppm ascorbate to double-packaged ground beef was helpful in slowing down the development of lipid oxidation in irradiated ground beef.

Table 3. Sulfur compounds and aldehydes of raw and cooked turkey breast with different packaging and antioxidants after 10 d of storage. Adopted from Nam and Ahn (*46*)

	NonIr	*Irradiated*				
Sulfur	*Vacuum*	*Vacuum*	*Aerobic*	*Double pkg*[1]		
compounds	*pkg*	*pkg*	*pkg*	*None*	*S+E*[2]	*G+E*[3]
	----------(Total ion counts × 10^4)----------					
Raw meat						
Dimethyl sulfide	1,304[b]	1,990[a]	140[d]	831[c]	676[c]	546[c]
Carbon disulfide	258[b]	306[a]	0[c]	0[c]	0[c]	0[c]
Dimethyl disulfide	0[b]	22,702[a]	0[b]	32[b]	0[b]	43[b]
Dimethyl trisulfide	0[b]	554[a]	0[b]	0[b]	0[b]	0[b]
Cooked meat						
Dimethyl sulfide	1,008[b]	2,032[a]	451[d]	1,005[b]	689[c]	588[cd]
Carbon disulfide	419[a]	339[ab]	210[b]	271[ab]	278[ab]	374[a]
Dimethyl disulfide	0[b]	17,861[a]	342[b]	940[b]	412[b]	210[b]
Dimethyl trisulfide	0[b]	1,007[a]	0[b]	118[b]	0[b]	0[b]
Propanal	233[d]	2272[c]	8,637[a]	5,962[b]	38[d]	427[d]
Butanal	0[e]	127[d]	592[a]	195[c]	302[b]	226[c]
Pentanal	62[c]	875[c]	3,014[a]	1,667[b]	0[c]	31[c]
Hexanal	0[b]	3,734[b]	37,617[a]	9,686[b]	0[b]	0[b]
3-Methyl butanal	0[c]	100[b]	223[a]	204[a]	131[b]	142[b]

[1] Vacuum packaged for 7 d then aerobically packaged for 3 d. [2] Sesamol (100 ppm) and α-tocopherol (100 ppm) added. [3] Gallic acid (100 ppm) and α-tocopherol (100 ppm) added. [a-e]Different letters within a row of same meat are significantly different ($P < 0.05$). $n = 4$.

Antioxidants reduced lipid oxidation and volatile aldehydes significantly. Packaging was the most critical factor in the development of irradiation off-odor in meat. Combination of antioxidant and double-packaging (V7/A3) was effective in controlling the oxidative changes of irradiated raw and cooked meat. Among the antioxidant and double-packaging treatments, both sesamol+vitamin E and gallic acid+vitamin E, combined with double-packaging, were effective in reducing pink color, off-odor and lipid oxidation of irradiated raw and cooked

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turkey breast, but gallic acid+vitamin E with double-packaging was the most effective in reducing the pink color in cooked turkey breast meat. Because color changes in irradiated ground beef is a major defect, addition of ascorbic acid at 200 ppm (w/w) to ground beef prior to irradiation stabilized color. Ascorbate also significantly slowed down the development of lipid oxidation in ground beef with double-packaging during storage. Therefore, double-packaging in combination with ascorbate can be a good strategy to prevent overall quality changes in irradiated ground beef.

In conclusion, irradiation induces formation of VSCs and VSCs are likely responsible for the development of off-odor. Studies have suggested that VSCs result from reactions of amino acids, peptides and other sulfur-containing compounds with free radicals from water radiolysis such as hydrated electron (e_{aq}^-), hydroxyl radicals (·OH). Use of antioxidants and herbs alone or in combination with double-packaging may reduce, but not eliminate production of VSCs and off-odor. Further research is needed to explore means to negate formation of VSCs in various foods.

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Chapter 13

Volatile Compounds Formed from the Interaction between Organoselenium and Sulfur Compounds

Guor-Jien Wei*,[1] and Chi-Tang Ho[2,3]

[1]Department of Nutrition and Health Sciences, Kainan University, Taoyuan, Taiwan, 33857
[2]Department of Food Science, Rutgers University, 65 Dudley Road, New Brunswick, NJ 08901
[3]Graduate Institute of Food Science and Technology, National Taiwan University, Taipei, Taiwan
***E-mail: gwei@mail.knu.edu.tw.**

Selenium is an element toxic in large quantities, but an essential trace metal for mammals, birds, and many bacteria. Selenium-containing food also contains sulfur, and the ratio of Se: S is over $1:1x10^4$. Selenium can replace sulfur easily, and the interaction between selenium and sulfur occurs easily as well. In order to investigate the potential interaction of organoselenium and organosulfur compounds, a model reaction of selenomethionine, glucose and diallyl disulfide was performed, and volatile products generated were identified by GC/AED and GC/MS. Atomic emission detector is a powerful detector due to its high sensitivity, elemental selectivity, and the ability of multielement analysis. When coupled with GC, it is able to monitor the elemental composition of eluates directly with high elemental specificity, tolerate of non-ideal separation, the selectivity of plasma emission being able to overcome the interference from complex matrixes, and detect multi-element simultaneously for empirical and molecular formula determination.

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Selenium is the 34th element on the periodic table, discovered by Berzelius in 1817. It belongs to Group VI A of the periodic table. Selenium has six stable isotopes: 74 (0.87%); 76 (9.02%); 77 (7.58%); 78 (23.52%); 80 (49.82%); 82 (9.19%) and variable valence within the redox range of biological systems. The 4 natural oxidation states are as follows: (0), elemental selenium, selenodiglutathione; (-2), sodium selenide (Na_2Se), hydrogen selenide (H_2Se); (+4), sodium selenite (Na_2SeO_3), selenium dioxide (SeO_2); and (+6), sodium selenate (Na_2SeO_4), selenic acid (H_2SeO_4).

Selenium is toxic in large quantities, but an essential trace metal for mammals, birds, and many bacteria. It is an essential nutrient with a recommended dose of 50-200 μg/day considered being adequate and safe for adults. 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, thus protecting cell membranes from oxidative damage. Vitamin E is also implicated in this system in which its role is to prevent the formation of lipid hydroperoxides (*1*).

It is believed that the selenium toxicity was first reported by Marco Polo when he described a disease called "hoof rot" in horses in Turkestan. Symptoms of this disease include loss of hooves and hair, liver damage and respiratory failure. The first evidence that selenium may be an anticarcinogenic element was presented by Clayton and Baumann (*2*). They found hepatic tumor incidence induced by azo dye was decreased by a diet containing 5 ppm of selenium. This work was confirmed 28 years later (*3*). In 1973, Shamberger et al. reported that the significantly lower blood levels of selenium have been observed in patients with cancer (*4*). In more extensive studies, selenium intakes, estimated from food consumption data in 27 countries, showed significant inverse correlation with the incidence of cancers of the large intestine, rectum, prostate, breast, ovary and lung (*5*). Selenium has been shown to counteract liver tumors due to 2-acetylaminofluorene under some circumstances (*6*). It was also reported that selenium could reduce liver tumors due to aflatoxins (*7*).

In 1941, Painter was first to propose that selenium toxicity was due to its interaction with thiols (*8*). These selenium reactions were later investigated by Ganther (*9*).

$$4GSH + SeO_2 \rightarrow GSSeSG + GSSG + 2H_2O$$
$$4GSH + SeO_3 \rightarrow GSSeSG + 2OH^- + H_2O$$

In 1982, Hu et al. used scanning electron microscopic (SEM) to examine the damage of red blood cell membranes resulting from selenite, selenocystine, and glutathione peroxidase (*10*). The results suggested that the damage could be due to free radicals. Hu and Spallholz also published data on the lysis of rat erythrocytes by selenium compounds and their sulfur analogs as measured by hemolysis (*11*). In these experiments cellular glutathione was measured and was found to decline as a consequence of selenium cytotoxicity and homolysis. In 1989, Seko et al. suggested that selenite reacted with glutathione and then H_2Se to produce superoxide ($O_2^{\cdot -}$) (*12*). Yen and Spallholz confirmed this observation in 1991 (*13*). Now, it is clear that the toxicity of selenium compounds is due to their

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reactions with GSH and other thiols to form selenotrisulfides that will ultimately react to produce superoxide and hydrogen peroxide. Those selenium compounds (selenate and selenoethers) that do not react with thiols are not toxic.

Selenomethionine is the primary form of organoselenium compounds present in wheat, corn, rice and selenium-enriched yeast. Some vegetables and nuts also contain high level of selenium, such as garlic, onion, and Brazil nuts. The Maillard reaction between selenomethionine and glucose had been studied by a model system, and several organoselenium compounds were identified (*14*).

More evidence shown the cancer prevention properties of selenium compounds depend mainly on their chemical forms. Selenium can exit in various forms. Many kinds of selenium compounds have been studied for their cancer prevention properties. Usually, selenium-containing food also contains sulfur. The ratio of S:Se in food could be over $1x10^4$:1. Selenium possesses similar chemical properties with sulfur and can replace sulfur easily. The interaction between selenium and sulfur occurs easily as well. In order to investigate the potential interaction of organoselenium and organosulfur compounds, the model reaction of selenomethionine, glucose and diallyl disulfide was performed.

Materials and Methods

Sample Preparation

Interaction 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 a nitrogen flow.

GC/Mass Spectrometry Analysis (GC-MS)

GC/MS analysis was performed by an Agilent 5973. Mass spectra were obtained by EI at 70 eV and a mass scan from 40-450 amu. The ion source temperature was 230 °C, and the analyzer temperature was 150 °C.

GC/Atomic Emission Detection Analysis (GC-AED)

An Agilent G2350A GC/AED was used. Oxygen and hydrogen were used as reagent gas with detection at 181, 179, 196, and 174 nm for sulfur, carbon, and selenium. Helium carrier gas was used for all analyses. The cavity temperature was 250 °C. The transfer line temperature was 250 °C. The hydrogen pressure was

8.8 psi. The oxygen pressure was 24 psi, and the auxiliary gas pressure was 30.6 psi.

Results and Discussion

Several organosulfur, organoselenium and mixed organoselenium-sulfur compounds were identified in this model system. The major organoselenium-sulfur compounds identified are CH_3-Se-SH (Figure 1), diallylsulfide, allyl methyl selenide (MeSeAll), dimethyldiselenide, CH_3-Se-S-CH_2-CH=CH_2 (Figure 2), CH_3-Se-Se-S-CH_3 (Figure 3), CH_2=CH-CH_2-S-S-Se-H (Figure 4), CH_3-Se-S-S-CH_2-CH=CH_2 (Figure 5), and CH_3-Se-S-S-S-CH_2-CH=CH_2 (Figure 6). Some of them have previously been identified in garlic (*15*).

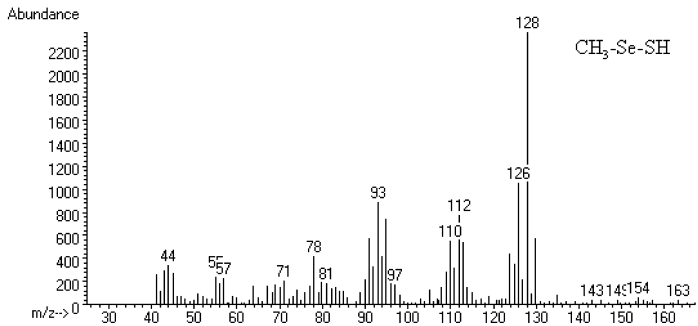

Figure 1. EI/MS spectrum of Me-Se-SH.

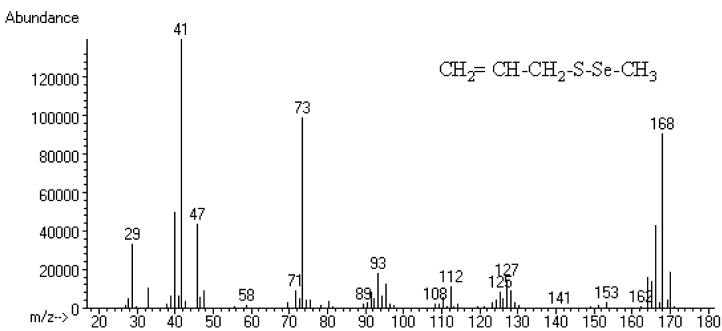

Figure 2. EI/MS spectrum of All-S-Se-Me.

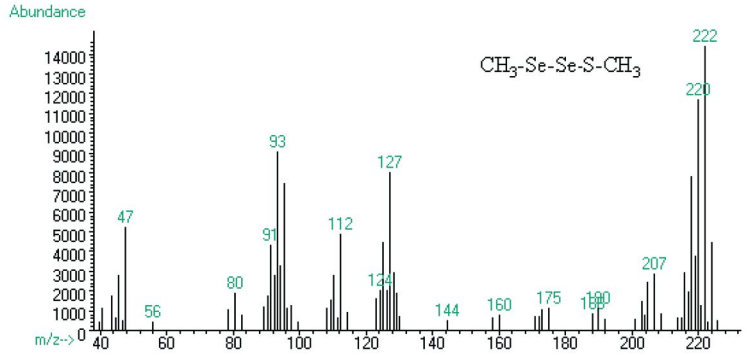

Figure 3. EI/MS spectrum of CH_3-Se-Se-S-CH_3.

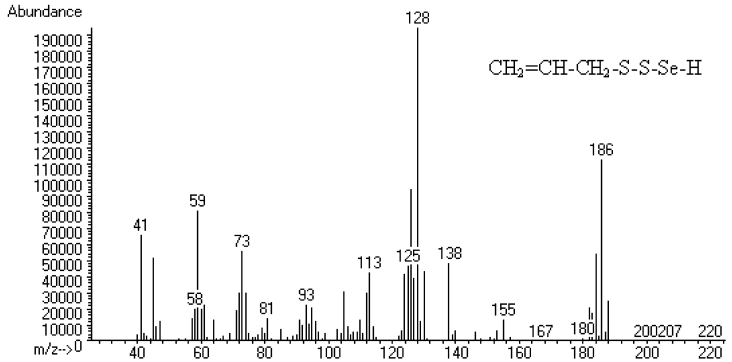

Figure 4. EI/MS spectrum of CH_2=CH-CH_2-S-S-Se-H.

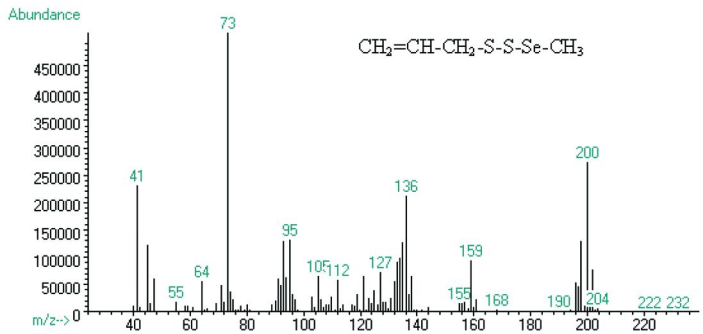

Figure 5. EI/MS spectrum of CH_3-Se-S-S-CH_2-CH=CH_2.

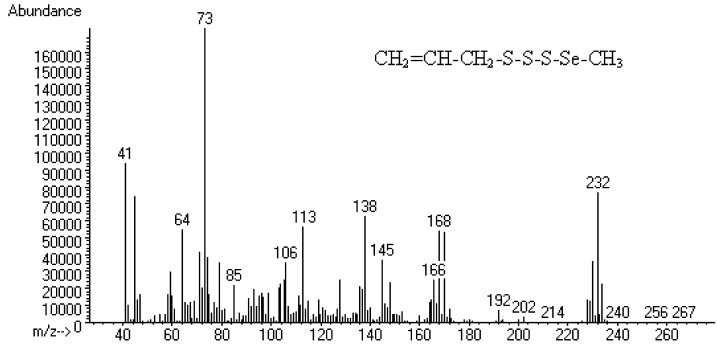

Figure 6. EI/MS spectrum of CH_3-Se-S-S-S-CH_2-CH=CH_2.

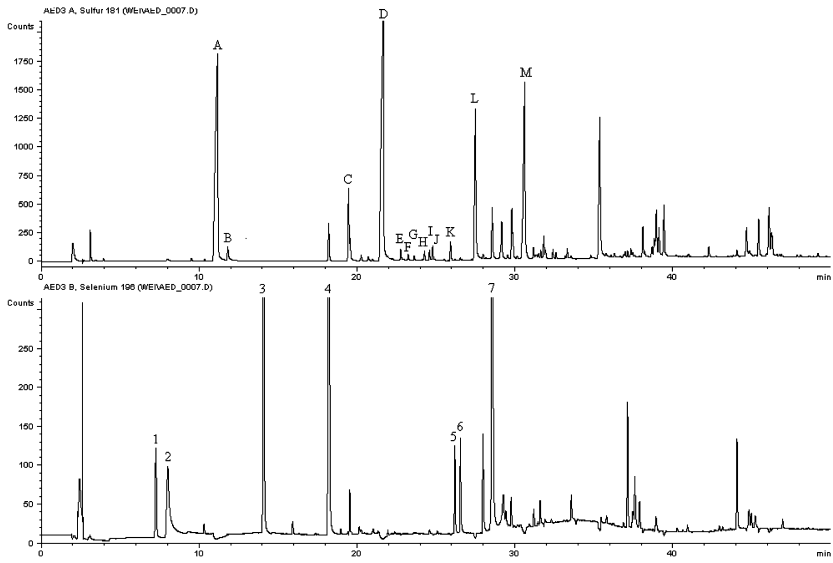

Figure 7. GC/AED profile of volatile compounds formed from glucose-selenomethionine model system with diallyl disulfide (pH3). 1: allyl methyl selenide; 2 : CH_3-Se-S-H; 3 : dimethyl diselenide; 4 : CH_3-Se-S-CH_2-CH=CH_2; 5 : CH_3-Se-Se-S-CH_3; 6 : CH_2=CH-CH_2-Se-CH2-CH_2OH; 7 : CH_3-Se-S-CH_2-CH_2OH A : Diallyldisulfide; B : 3-[(1-methylethyl)thio]-1-propene; C : 3-(methylthio)-thiophene; D : 3-thiophenecarboxaldehyde; E : 1,3-dithiane; F :3-methyl-2-thiophenecarboxaldehyde; G : 2,2-dimethyl-1,3-dithiane; H : propyl-thiophane; I : 1-(2-thienyl)-ethanone; J : trans-3-methyl-2-n-propylthiophane; K : 2-acetyl-5-methylthiophene; L : 1-(2-thienyl)-1-propanone; M : 4-methylthiazole.

The GC/AED profiles are shown in Figure 7. Sulfur-containing compounds generated from heat have been widely studied. Unlike nitrogen-containing compounds, the generation of sulfur-containing compounds is less pH-sensitive. Due to the similar chemical property, selenium can replace sulfur easily, and the interaction between selenium and sulfur occur easily as well. In pH3, the system with diallyl disulfide generated more selenium-containing compounds then that without diallyl disulfide. Actually, most selenium-containing compounds in pH3 system are from selenium-sulfur interaction but Maillard reaction.

Conclusion

The selenium-sulfur interaction is the main reaction for selenium compounds in the presence of sulfur. Even heated with glucose at pH 9, the Maillard heterocyclic reaction product containing selenium was not detected.

The investigation of the toxicity and anticarcinogenic properties of these selenium-sulfur compounds may be needed.

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Chapter 14

Analysis and Formation of Key Sulfur Aroma Compounds in Wine

M. J. Herderich,* I. L. Francis, M. Ugliano, T. E. Siebert, and D. W. Jeffery

The Australian Wine Research Institute, P.O. Box 197, Glen Osmond, SA 5064, Australia
***E-mail: Markus.Herderich@awri.com.au.**

Identification and measurement of sulfur aroma compounds to better describe wine quality and style has been of significant importance to researchers and wine producers for many years. This necessarily requires the development of analytical methods to robustly quantify labile sulfur compounds at trace concentrations. With the ever-growing importance of screwcaps and other alternatives to cork closures, additional focus is on characterizing and minimizing 'reduced characters' during bottle storage. There is also a need to better understand the roles of must composition, yeast nutrients, yeast sulfur metabolism and copper fining in controlling sulfur aroma compounds. This paper explores how static headspace GC analysis of volatile sulfur compounds with cool-on-column injection and SCD detection has been optimized and applied for studies requiring direct analysis of fermentation-derived sulfur aroma compounds, such as H_2S, DMS, CS_2 and methanethiol.

Introduction

Volatile sulfur compounds, which can be formed at various stages during wine production and storage, typically have very low aroma detection thresholds in the ng/L to µg/L range. Positive notes are associated with low concentrations of polyfunctional thiols. These include 4-mercapto-4-methylpentan-2-one (4-MMP) which can be described as "boxtree" and "broom"; 3-mercaptohexan-1-ol (3-MH) described as "passion fruit" or "grapefruit"; and 3-mercaptohexyl acetate

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(3-MHA) with an aroma described similarly as "grapefruit", "passion fruit" or "box tree" (*1*, *4*). Dubbed "varietal thiols", these aroma compounds are released by yeast catalysed C-S lyase reactions from cystein- and glutathione-bound precursors predominantly found in Sauvignon Blanc must (Figure 1) (*2*, *3*, *35*). "Varietal thiols" have also been identified in wines made from many other varities, such as Chardonnay, Semillon, Riesling, Scheurebe, Gewurztraminer, Cabernet Sauvignon and Merlot (*4*).

Each of the varietal thiols can contribute undesirable odors in wine when present at high concentrations, changing from attractive tropical fruit aroma to sweaty or cat urine–like as concentration increases. Other sulfur compounds such as dimethyl sulfide (DMS) and furfuryl thiol can show similar behavior, giving pleasant aromas at lower concentrations but unattractive, undesirable at high levels. In addition, there are a number of volatile sulfur compounds that are key negative contributors to wine aroma when present at concentrations above their perception threshold, as they can impart unpleasant 'reduced', 'onion', 'garlic', 'rubber', or 'burnt' aromas. Figure 2 summarizes aroma descriptors and detection thresholds for volatile sulfur compounds commonly found in wine that are generally regarded as problems or faults, especially when present at high concentrations.

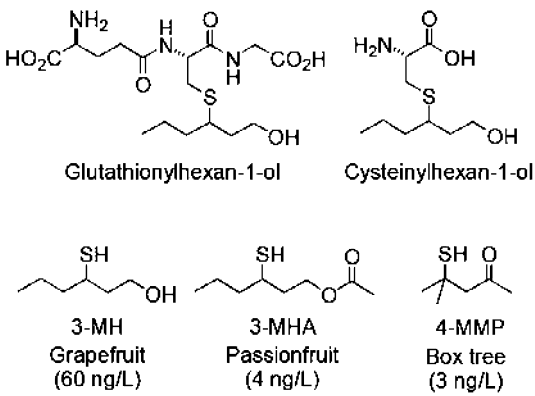

Figure 1. Varietal thiols (with detection thresholds) and non-volatile precursors of 3-MH and 3-MHA.

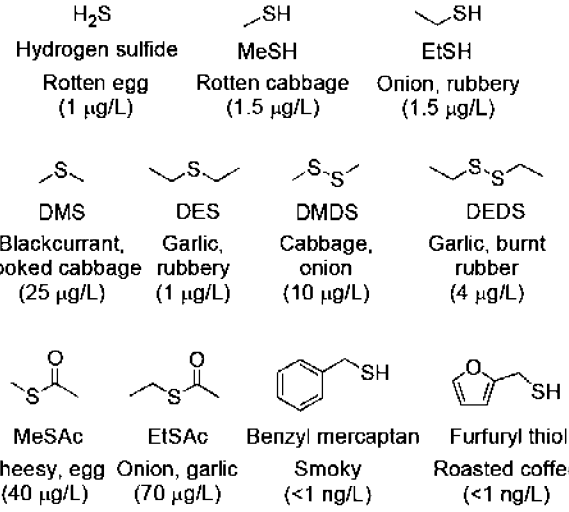

Figure 2. Undesirable volatile sulfur aroma compounds in wine (with aroma descriptors and detection thresholds).

Undesirable Volatile Sulfur Aroma Compounds in Wine

During alcoholic fermentation a large pool of volatile sulfur compounds is formed and many of these volatiles are of primary importance for wine aroma. Among these, hydrogen sulfide (H_2S) is generally considered to play a negative role, due to its characteristic aroma of rotten egg and sewage. It is well established that yeast strain and yeast nutrients such as yeast assimilable nitrogen (YAN) can be major factors in H_2S accumulation during fermentation (*5*). As such, a large amount of H_2S is produced as a biosynthetic intermediate by yeast during grape must fermentation through reduction of elemental sulfur, sulfate or sulfite. Production of excess H_2S can potentially lead to the formation of other sulfur-containing compounds, such as methanethiol (MeSH), ethanethiol (EtSH) and their acetates (Figure 2) (*6*). Although excess H_2S may be removed from wine through copper fining, this approach is not effective for sulfides, disulfides and thioacetates. Further unwanted side-effects of copper fining may be the removal of desirable varietal thiols, or formation of more stable disulfides and trisulfides which can also contribute undesirable off aromas (e.g. DMDS, DEDS, Figure 2). Finally, the excessive use of copper may require additional wine processing steps and might lead to wine instabilities.

Beyond H_2S many other volatile sulfur compounds are known to contribute distinctive off odors, such as 'putrid', 'garlic' or 'onion' aromas from MeSH and 'canned corn' or 'cooked asparagus' from DMS at higher concentrations (Figure 2). Despite these negative connotations, at lower levels DMS may contribute a pleasant 'black-currant' aroma and has been shown to enhance fruity notes in the presence of other volatile wine components (*7*). Other volatile sulfur compounds, such as carbon disulfide (CS_2), may be regarded as negative contributors to wine

aroma at high concentrations, although the contribution of CS_2 to wine aroma is unclear at the concentration typically found in commercial wine.

Analysis of Sulfur Aroma Compounds in Wine

Various methods have been reported for the measurement of varietal thiols such as 3-MH and their cysteine conjugates in grape juice or must (*8*, *37–41*). However, specialized techniques for sample preparation, efficient separation and selective detection are required due to the highly volatile nature and substantial chemical reactivity of many undesirable sulfur aroma compounds such as H_2S or MeSH. For the rapid qualitative or semiquantitative measurement of H_2S during fermentation selective detector tubes can be used (*9*), although the concomitant presence of other thiols may result in incorrect H_2S quantitation. After fermentation and bottling, the concentrations in wine of H_2S and other volatile sulfur compounds are typically too low for rapid measurement by indicator tubes and specialized gas chromatographic techniques are required.

Volatile sulfur compounds have been quantified in wine using solid-phase microextraction (SPME) followed by gas chromatography-pulsed flame photometric detection (GC-PFPD), GC-flame photometric detection (GC-FPD), GC-mass spectrometry (GC-MS) or GC-atomic emission detection (GC-AED) (*10–14*, *21*, *22*, *25*, *42–45*). Unfortunately, enrichment of volatile sulfur compounds using SPME can be complicated by matrix effects, artifact formation or sample losses upon injection. Furthermore, H_2S in particular is often missed in analyses employing SPME as the pre-concentration technique. As an alternative to SPME, methods based on static headspace or purge and trap techniques have been proposed. To address the need for a rapid, selective and accurate method to quantify commonly found volatile sulfur compounds in wine we undertook to develop an efficient multi-analyte gas chromatography method using static headspace-cool-on-column (HS-COC) injection with sulfur chemiluminescence detection (SCD) detection. Our goal was to minimize sample preparation while at the same time ensuring the robust quantification of the ten most commonly found volatile sulfur compounds, including H_2S, in wine at low µg/L concentrations. In this paper we report experiences from the method development and validation stage, and demonstrate the versatility and performance of HS-COC-GC-SCD to profile volatile sulfur compounds during wine fermentation and storage studies.

Materials and Methods

Materials

Reference standards of ethanethiol (EtSH), dimethyl sulfide (DMS), diethyl sulfide (DES), dimethyl disulfide (DMDS), diethyl disulfide (DEDS), carbon disulfide (CS_2) and ethylmethyl sulfide (EMS) were of the highest purity as supplied by Sigma-Aldrich. *S*-Methyl thioacetate (MeSAc), *S*-ethyl thioacetate (EtSAc) and propyl thioacetate (PrSAc) were of the highest purity obtainable from Lancaster Synthesis. The remaining chemicals were of analytical reagent grade quality or better. Sodium hydrosulfide hydrate (NaSH x H_2O) was supplied

by Sigma-Aldrich. Ethanol (99.5%) was redistilled in-house prior to use and water was obtained from a Milli-Q purification system (Millipore). All solvents and analytical standards were verified for purity by GC-MS and GC-AED or GC-SCD prior to use. EtSH, DMS, DES, DMDS, DEDS, CS_2, EMS, MeSAc, EtSAc and PrSAc were stored at -20 ºC to prevent degradation. Containers of $NaSHxH_2O$ and NaSMe were sparged with nitrogen and stored in a desiccator at room temperature.

Wine Samples

Commercial, bottled wines (n=68) that were thought to have 'reductive' characters or other 'sulfidic off odors' based on preliminary sensory assessments were selected. The winemaking protocols used for the fermentation trials have been described in references (*29*, *32*, *33*). For the sensory work (*34*) previously reported procedures (*36*) were followed.

Preparation of Standard Solutions

Individual stock standard solutions of EtSH, DMS, DES, DMDS, DEDS, CS_2, EMS, MeSAc, EtSAc and PrSAc were prepared by injecting 100 μL of neat standard into 50.0 mL of ethanol contained in a 125 mL 'Sure-Seal' bottle (Sigma-Aldrich) that had been crimp-capped and sparged with nitrogen. The density for each reference standard was used to calculate the actual concentration (approximately 2 g/L) and the solutions were stored at -18 ºC for up to 24 months except for EtSH, which was only stored for six months.

Stock Standard Solution of NaSH (for H_2S) and NaSMe (for MeSH)

Due to the difficulties of working with gaseous H_2S and MeSH, a suitable alternative employed the sodium salts of these analytes, which were dissolved in water and used immediately. Individual stock solutions of known concentration (approximately 300 mg/L) were prepared in amber volumetric flasks. The concentrations of NaSH and MeSH were calculated using the purity reported in their respective certificates of analysis. Individual dilute standard solutions of known concentration containing NaSH or NaSMe (approximately 7.5 mg/L) were prepared in water in amber volumetric flasks and used immediately.

Internal Standard Mix

An internal standard solution containing known concentrations of EMS (approximately 20 mg/L) and PrSAc (approximately 50 mg/L) was prepared in an amber volumetric flask by diluting the respective stock standard solutions with ethanol. The internal standard solution was stored at 4 ºC for three months.

Preparation of Model Wine

Aqueous ethanol (12 % v/v) was saturated with potassium hydrogen tartrate and the pH was adjusted to 3.2 with tartaric acid solution (40% w/v). Fermentation derived volatiles (ethyl esters, acetates, alcohols and fatty acids) were added to approximate the concentrations commonly found in wine.

Sample Preparation

Wine and fermentation samples were cooled to 4 ºC in their original containers prior to opening and all sample handling was completed in a temperature controlled room at 4 °C. An aliquot of wine (10 mL) was added to a 20 mL amber glass headspace vial containing 2 g of NaCl, 10 mg of disodium EDTA and a magnetic stir bar. Internal standard solution (25 µL) was added to give known final concentrations of EMS (approximately 50 µg/L) and PrSAc (approximately 125 µg/L). Acetaldehyde (4 µL) was added to each white wine sample vial. The vial was tightly sealed with a white PTFE/blue silicone lined screw cap.

Instrumentation

Gas Chromatography

The samples were analyzed using an Agilent 6890 gas chromatograph equipped with a Gerstel multipurpose sampler and coupled to either an SCD or AED. Instrument control and data analysis was performed with Agilent GC ChemStation software, and Maestro software integrated version 1.3.3.51/3.3. The gas chromatograph was fitted with a 15 m × 0.25 mm FactorFour VFWAXms fused silica capillary column, 0.50 µm film thickness (Varian) connected to a 60 m × 0.25 mm VICI ValcoBond VB-5 fused silica capillary column, 0.50 µm film thickness with a 2 m × 0.53 mm retention gap. Helium (Ultra High Purity), linear velocity 37 cm/s, flow-rate 2.7 mL/min in constant flow mode, was used as the carrier gas. The initial oven temperature was held at 5 °C for 5 min, increased to 150 °C at 5 °C/min, and held at this temperature for 5 min. The cool-on-column (COC) inlet (Agilent), was held at 30 °C for 10 min and ramped at the same rate as the oven. The oven and COC inlet were cryogenically-cooled with liquid nitrogen.

Sulfur Chemiluminescence Detection

An Agilent 355 SCD sulfur chemiluminescence detector coupled to the GC was used with the default SCD parameters recommended by Agilent and sulfur trap gas purifiers on all gas lines (Agilent). The detector base temperature was held at 200 °C and the Dual Plasma Controller at 800 °C. The reagent gases were air (instrument grade), 60.0 sccm; hydrogen (ultra-high purity), 45.0 sccm; and ozone, generated in-situ from air.

Atomic Emission Detection

An Agilent G2350A atomic emission detector coupled to the GC was used with AED parameters optimized for sulfur sensitivity. The AED cavity block and the transfer line were held at 250 °C. Helium was used for the microwave induced plasma at a flow rate of 25 mL/min, measured at the cavity vent. Oxygen and hydrogen were used as the reagent gases. Sulfur (181 nm) and carbon (193 nm) emission lines were monitored.

Peak Identification

Analytes were identified by comparison of their retention times with those of the corresponding pure reference compounds.

Headspace (HS) Equipment and Conditions

The refrigerated sample vials were placed into a Gerstel peltier cooled sample tray at 4 °C. The vial and its contents were heated to 45 °C for 30 min with stirring at 400 rpm. A 1.0 mL HS syringe was fitted with a custom made dual gauge cone-tip needle (0.47 mm/0.63 mm, SGE, Australia) and the syringe heating block was held at 60 °C. A 100 µL static HS sample was injected into the COC inlet at 10 µL/s. The syringe was purged in the inlet with nitrogen (ultra-high purity) for 3 minutes after injection.

Validation

Precision and calibration linearity were validated by a series of standard addition experiments to model, white and red wine matrices. Method linearity was determined for nine calibration levels, in duplicate, over the concentration ranges of 0.2 to 100 µg/L for H_2S, MeSH, EtSH, CS_2, DES, DMDS and DEDS, and 1.0 to 400 µg/L for DMS, MeSAc and EtSAc. Method precision was determined in all matrices using seven replicate samples spiked at low and high concentrations (5 and 20 µg/L for all analytes except DMS, MeSAc and EtSAc, which were 50 and 200 µg/L). For quantifying the analytes in batches of unknown samples, duplicate standards (0 and 50 µg/L for all analytes except DMS, MeSAc and EtSAc, which were 0 and 200 µg/L) were prepared using model wine and analyzed with every set of samples. To check the accuracy of the analysis, duplicate control samples, spiked with 10 µg/L for all analytes except DMS, MeSAc and EtSAc, which were spiked at 50 µg/L, were included with every set of samples to be quantified, along with blanks. Control samples were prepared in red or white wine with known low levels of the analytes. The results reported for validation of the method were derived from the average of duplicate measurements for each concentration of the analyte (seven replicates for repeatability samples).

Results and Discussion

Development of a Multi-Analyte Static Headspace-Cool-on-Column GC-SCD Method for the Quantitative Analysis of Volatile Sulfur Compounds in Wine

As initial instrumentation set-up we utilized a COC inlet and cryogenically-cooled GC oven, to overcome the challenges faced when analyzing inherently volatile, reactive and thermally-labile sulfur compounds, in combination with an AED to selectively detect sulfur (181 nm) and carbon (193 nm) containing volatile organic compounds. The method was then optimized for selectivity, sensitivity, accuracy, precision and faster sample throughput to cope with demand. Validation of the method for the quantitation of ten volatile sulfur compounds commonly found in wine was completed using an SCD after incorporating all optimized parameters.

Optimization of Headspace Sampling

The optimal incubation time was identified by comparing identical spiked bag-in-box white wine samples, after varying the incubation time (10, 20, 30, 40, 50 and 60 min) at both 35 and 45 °C immediately prior to analysis. We found that equilibrium of the analytes between the headspace and wine was reached reproducibly by stirring samples for 30 min at 45 °C prior to injection.

For static HS injection into a COC inlet cooled with liquid N_2 we chose a wide-bore retention gap, and set injection speed to 10 µL/s for a 100 µL injection. This ensured that no analytes were lost through the inlet purge vent; higher injection volumes (150, 200 and 250 µL) were also investigated but the chromatography deteriorated for the early eluting compounds. Peak broadening was evident for H_2S, MeSH, EtSH, CS_2, DMS and EMS, and worsened with each increase in injection volume, resulting in complete loss of resolution between CS_2 and DMS.

To minimize degradation of sulfur compounds prior to analysis we employed a cooled sample tray that maintained the samples at 4 °C while awaiting incubation. To investigate and rule out any artifactual formation of disulfides from thiols, or the breakdown of thioacetates, we injected individual reference standards and observed only the single peaks expected for the analytes.

Optimising the Gas Chromatographic Separation

To achieve the separation of the ten volatile sulfur compounds shown in Figure 3, a column combination similar to Hill and Smith (*11*) was chosen with 15 m of a polar FactorFour wax column connected to 60 m of a nonpolar column (VB-5). This allowed the temperature program to begin at 5 °C and resulted in much better peak shape for MeSH and adequate resolution between EtSH, DMS and CS_2.

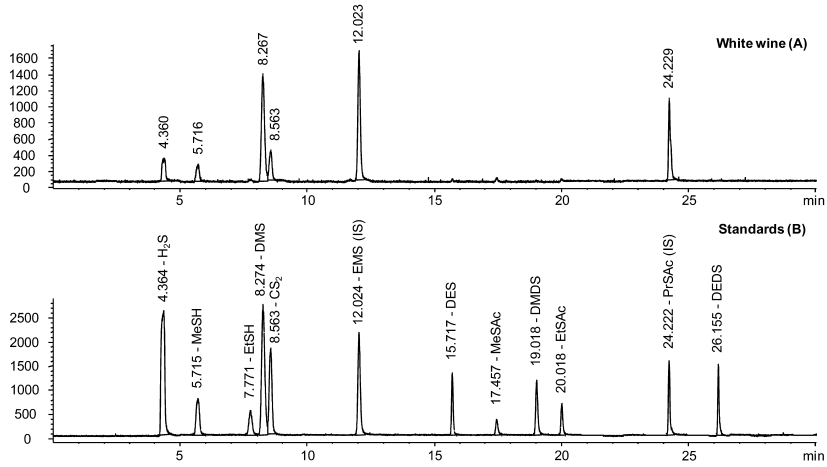

Figure 3. Typical HS-COC-GC-SCD chromatograms of (A) a commercial white wine and (B) reference standards in model wine (10). IS refers to internal standards ethylmethyl sulfide (EMS) and propyl thioacetate (PrSAc).

Validation of the Multi-Analyte HS-COC-GC-SCD Method

Internal Standards, Acetaldehyde Addition and Stock Solutions

For accurate quantitation of volatile and reactive analytes at low µg/L concentrations internal standards labeled with stable isotopes, which are ideal for GC-MS, were an obvious choice to investigate. However, preliminary work showed that we could not achieve sufficient chromatographic resolution for GC-SCD between the analytes and their labeled analogues. In accordance with previous studies we confirmed that EMS and PrSAc were suitable internal standards for the determination of sulfur compounds in wine (*10*). Repeated analysis of stock solutions over many months verified the stability of the sulfur compounds prepared in 'Sure Seal' bottles and stored under nitrogen at -18 °C. Interference from sulfur dioxide (SO_2) was sometimes observed when white wines were analyzed. Invariably these were young white wines with higher free SO_2 levels and when present, the SO_2 peak co-eluted with DES and MeSAc. To overcome any potential interferences in white wine samples, acetaldehyde was routinely added prior to analysis, to bind free SO_2 without impacting on the other analytes.

Validation and Method Performance

For method validation red and white wine, and model wine was used; the model wine was spiked with typical fermentation volatiles such as alcohols, acids and esters. In our experience this mimicked the headspace of a true wine matrix better than a simple buffered water-ethanol mixture and the resulting

calibration functions had similar slopes and good coefficients of determination (R^2) for model wine, white wine and red wine. The repeatability of the analysis was better than 10% RSD (relative standard deviation) for all compounds at the concentrations investigated. Blank runs, recoveries and negative controls were repeated regularly to evaluate method performance. Table 1 summarizes the key performance indicators including repeatability, limits of detection (LOD) and quantitation (LOQ).

Application HS-COC-GC-SCD To Quantify Sulfur Compounds in Wine

To characterize the concentrations and relevance of undesirable volatile sulfur compounds we selected 68 commercial wines which were characterized by wine judges with sensory descriptors such as 'reduced', 'struck flint' or 'off-odor' that typically indicate the presence of objectionable sulfur related aromas. The samples included white wine from seven grape varieties, one white wine blend, six red wine varieties and four different red wine blends from numerous wine regions of Australia with vintages ranging from 2004 through to 2008. The results from this comprehensive profiling study are summarized in Table 2.

Table 1. Performance of the HS-COC-GC-SCD method for the analysis of volatile sulfur compounds in wine (*10*)

		RSD[a]				
Analyte	***R²***	***5 µg/L***	***50 µg/L***	***LOD***[b]	***LOQ***[c]	***Range***
H_2S	0.9975	3.3	4.2	0.2	0.5	0.2-100
MeSH	0.9882	6.6	5.6	0.2	0.5	0.2-100
EtSH	0.9952	5.9	4.6	0.2	0.5	0.2-100
DMS*	0.9973	3.9	2.6	1.0	2.0	1.0-400
CS_2	0.9915	9.4	4.0	0.2	0.5	0.2-100
DES	0.9974	5.4	3.8	0.2	0.5	0.2-100
MeSAc*	0.9983	4.2	4.1	1.0	2.0	1.0-400
DMDS	0.9983	2.8	5.0	0.2	0.5	0.2-100
EtSAc*	0.9993	5.6	3.7	1.0	2.0	1.0-400
DEDS	0.9972	3.6	6.2	0.2	0.5	0.2-100

[a] Relative standard deviation RSD% (n=7) as measure for repeatability. [b] LOD, limit of detection (µg/L). [c] LOQ, limit of quantitation (µg/L). * Repeatability at 20 µg/L and 200 µg/L.

Table 2. Concentration of volatile sulfur compounds in commercial Australian wines from 2004-2008 with noted 'reductive' characters (*10*)

Variety	Number of Wines	Concentration (μg/L)									
		H_2S	MeSH	EtSH	DMS	CS_2	DES	MeSAc	DMDS	EtSAc	DEDS
Chardonnay	4	1.5 - 5.0	3.0 - 8.0	nd[a] - 0.5	20.0 - 185.0	0.5 - 5.0	nd	nd - 7.0	nd	nd	nd
Pinot Gris	1	2.0	3.0	nd	11.0	0.5	nd	nd	nd	nd	nd
Riesling	10	0.5 - 35.0	nd - 3.0	nd	11.0 - 37.1	nd - 21.1	nd - 0.4	nd	nd	nd	nd
Sauvignon Blanc	6	0.8 - 4.0	1.7 - 6.0	nd	25.0 - 118.2	1.0 13.5	nd - 0.4	nd	nd	nd	nd
Sauvignon Blanc/Semillon	4	2.0 - 13.0	1.0 - 4.0	nd - 1.0	25.0 - 76.0	0.5 - 14.8	nd - 0.4	nd - 2.1	nd	nd	nd
Semillon	1	2.0	3.0	nd	13.5	2.0	nd	nd	nd	nd	nd
Verdelho	1	1.0	1.6	nd	47.7	18.6	0.4	nd	nd	nd	nd
Viognier	1	0.5	3.0	nd	78.0	6.0	nd	nd	nd	nd	nd
Cabernet Merlot	2	0.5 - 0.8	0.4 - 1.0	nd	102.5 -106.0	3.5 -15.6	nd - 0.4	nd	nd - 1.5	nd	nd
Cabernet Sauvignon	5	nd - 1.6	nd - 1.5	nd	88.0 - 379.5	3.0 - 20.0	nd - 0.4	nd - 10.0	nd	nd	nd
Durif	1	2.0	2.0	nd	61.0	1.0	nd	18.0	nd	nd	nd
Grenache/Shiraz/Merlot	1	0.7	0.7	nd	111.0	18.0	0.4	nd	nd	nd	nd
Merlot	3	0.5 - 1.2	nd - 1.6	nd	48.0 - 235.0	8.0 - 17.0	nd - 0.4	3.0 - 8.0	nd	nd	nd
Red Wine Blend	1	1.0	0.2	nd	195.0	14.5	0.4	4.7	nd	nd	nd
Sangiovese	1	nd	nd	nd	68.0	4.0	nd	nd	nd	nd	nd
Shiraz	22	nd - 8.7	nd - 5.0	nd - 0.7	28.0 - 765.0[b]	2.0 - 45.1	nd - 0.5	nd - 12.5	nd - 1.5	nd	nd
Shiraz/Cabernet Sauvignon	2	0.5 - 1.0	1.0 - 1.2	nd	85.0 - 228.4	4.0 - 17.4	nd - 0.4	4.1 - 7.5	nd	nd	nd
Shiraz/Viognier	2	nd - 1.0	1.0	nd	57.0 - 112.0	2.0 - 6.0	nd	nd - 6.0	nd	nd	nd

Hydrogen Sulfide

H_2S was detected in every white wine analysed, with the highest level of 35.0 μg/L for a Riesling wine (Table 2). For the red wines, H_2S was detected in 33 out of 40 red wine samples, with a Shiraz wine containing the highest concentration of 8.7 μg/L. While H_2S may add complexity to wine aroma at low levels, undesirable 'rotten egg' or 'sewage-like' odors may arise when higher concentrations remain after fermentation. As the available literature gives vastly differing aroma thresholds we established aroma detection thresholds for H_2S at 1.1 μg/L and 1.6 μg/L in red and white wine, respectively (*15*). Consequently, in most of the 68 wines which had been characterized as 'reduced' by sensory evaluation, H_2S may contribute to the reductive aromas, although we could not detect any H_2S in some red wines despite their objectionable aroma profiles. This observation is in agreement with previously published data by Rauhut et al., who dismissed H_2S as the sole cause of 'sulfurous off-flavour' in wine (*14*) and results from a study by Fang and Qian who quantified H_2S in commercial wine at similar concentrations to our work although no apparent "off-flavor" was noted (*12*).

Methanethiol

All but one white wine and 30 of the 40 red wine samples contained detectable levels of MeSH. A Chardonnay wine had the highest overall concentration of MeSH at 8.0 μg/L, while the highest level in the red wines was a Shiraz wine with 5.0 μg/L (Table 2). We recently determined the aroma detection thresholds for MeSH in white and red wine to be 3.1 and 1.8 μg/L, respectively (*16*). Given that MeSH was measured in 57 of the 68 wines, frequently at levels at or above

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its detection threshold, this suggested that MeSH might have contributed to the 'reductive' characters of many of the red and white wines analysed.

Ethanethiol

EtSH was only detected in one red wine and two white wines, with all concentrations being below the reported white wine aroma threshold of 1.1 µg/L (*17*). Given the performance of the HS-COC-GC-SCD method (Table 1) it is unlikely that 'false negative' results have been recorded for EtSH and the contribution of EtSH on sulfidic 'off odors' appears to be negligible, at least for this sample set.

Dimethyl Sulfide

DMS had the largest overall concentration range of the volatile sulfur compounds examined, with up to 185.0 µg/L in the white wines and up to 765.0 µg/L in the red wines (Table 2). DMS is an interesting sulfur aroma compound as it may be beneficial to wine aroma at low levels with suggestions that up to 100 µg/L DMS might increase the perceived fruitiness (*7*, *18*). Without doubt DMS at higher concentrations imparts undesirable 'canned corn', 'cooked cabbage' and 'vegetal' type aromas (*17*, *19*, *20*) and DMS in some of the wines analysed can be expected to have a negative aroma impact.

Carbon Disulfide

CS_2 was detected in all samples apart from two Riesling wines, with concentrations for white and red wines up to 21.1 and 45.1 µg/L, respectively (Table 2). CS_2 was first identified in wines by Leppänen et al. (*21*), and appears to be a ubiquitous volatile sulfur compound in wine. Higher concentrations may be associated with 'reduced' wines (*22*), but addition of CS_2 to a white wine at almost 38 µg/L reportedly had no effect on the wine aroma (*23*). The formation of CS_2 and its impact on wine aroma is not well understood. Furthermore it has been suggested that negative descriptors from CS_2 samples may be due to impurities present in commercially available material (*24*). Further research is required to corroborate a potential role for CS_2 as a contributor to 'reductive' characters in wine.

Diethyl Sulfide

DES was detected in 24 wines at concentrations less than 0.5 µg/L (Table 2), below its white wine aroma threshold of 0.93 µg/L (*17*). The values we encountered were comparable to those reported for fault-free wines from North

America (*12*) and it appeared that 'reductive' characters were not related to the amounts of DES present in the wines we analyzed.

Methyl and Ethyl Thioacetates

MeSAc was detected at concentrations below 20 µg/L in 29 of the 40 red wines and in only two of the white wines, where it was below 10.0 µg/L (Table 2). Our results for MeSAc are comparable to concentrations reported by others for fault-free wines from North America (*12*), Spain (*25*) and Europe (*21*), and EtSAc was not detected in any of the 68 wines we analyzed. While the concentrations recorded for MeSAc in our study were well below the aroma detection threshold of 50 µg/L determined in beer (*26*), we propose that MeSAc may play an indirect, but important, role in the formation of 'reduced' aromas during storage. It might serve as a precursor for the much more potent thiol MeSH in some wines where, as example, chemical hydrolysis of 10 µg/L of MeSAc could yield up to 5.3 µg/L of MeSH. Clearly, the interplay between MeSH, which can be removed by copper fining, and MeSAc, which is most likely not affected by copper additions but can release MeSH with time, requires further investigation.

Dimethyl and Diethyl Disulfides

DEDS was not detected in any of the wines we analyzed and DMDS was only present in five red wines at levels well below its white wine aroma detection threshold of 29 µg/L (*17*). These results are in agreement with concentrations reported from other studies, where traces up to a few µg/L have been observed in wines that presented no sulfur-related off odors (*12*, *21*). As noted for other volatile sulfur compounds, Spanish red and white wine varieties tended to have greater amounts of DMDS or DEDS (up to 5.2 µg/L) (*13*, *25*) even though no sensory faults were evident. While we will continue to monitor these disulfides as they can be formed from their more common thiol counterparts through oxidative reactions, in the case of the 68 wines analyzed it appeared unlikely that these disulfides contributed to any off odor.

Profiles of Volative Sulfur Compounds in Wine Selected Based on Off Odor Related Sensory Descriptors

In summary, we observed no consistent profile for the ten volatile sulfur compounds measured. Not all wines that showed 'reductive' off odors had H_2S present, some wines had above-threshold concentrations of MeSH but no H_2S or MeSAc, while in other wines various combinations of two or more volatile sulfur compounds were quantified well above their detection thresholds.

Based on established aroma detection thresholds, the volatile sulfur compounds implicated as contributors to 'reduced' and other undesirable 'sulfidic' aroma descriptors were H_2S, MeSH and DMS, with CS_2 playing an uncertain

role. MeSAc, while not present at the time of analysis above its aroma detection threshold, could act as a precursor to MeSH, yielding perceivable amounts of the more potent thiol during storage.

Yeast Nutrients and Formation of Volatile Sulfur Compounds

Among the many yeast nutrients present in grape juice, yeast assimilable nitrogen (YAN) is of primary importance for yeast metabolism, formation of H_2S during fermentation and fermentation kinetics (*5*, *9*, *29*, *32*). In white winemaking it is now widely accepted that the risk of incomplete fermentation of a highly clarified grape juice with a sugar concentration of around 200 g/L is minimized when the YAN concentration exceeds 140 mg/L (*27*). Several studies have shown that supplementing grape must with YAN in the form of diammonium phosphate (DAP) lowered the risk of slow and stuck fermentation and decreased the formation of unwanted sulfur volatiles by yeast (*5*, *28*). These observations have led to the generalized practice of supplementing grape must with DAP, in some cases without the knowledge of initial YAN content (*29*).

To study the effects of nitrogen supplementation for red wine fermentation which, as opposed to white wine making, typically involves maceration of skins, presence of grape solids and aeration of the fermenting must, we investigated the effects of DAP additions (to achieve 250 and 400 mg/L YAN) to a low YAN (103 mg/L) Shiraz must which was subsequently fermented with *Saccharomyces cerevisiae* AWRI 796 (*29*). While DAP-supplemented fermentations showed the expected improvements in fermentation kinetics and were complete 6 to 8 days ahead of the low YAN control must, no major differences were observed for many aroma compounds including the volatile sulfur compounds. Irrespective of the must YAN status, all finished red wines contained 4 µg/L H_2S, 18 µg/L DMS and 4 µg/L CS_2. While it was interesting to note that DAP additions did not result in any further reduction of the low concentration of H_2S, DAP supplementation also resulted in increased concentrations of DMS after model aging. Storage of the finished red wine at 30 °C for six weeks led to the complete removal of H_2S, and CS_2 was reduced to approximately 2 µg/L in all samples. DMS increased to 88 µg/L (in the low YAN control and 250 mg/L YAN ferment) and 112 µg/L in the wine made from 400 mg/L YAN must; such formation of DMS during aging has been observed previously (*30*, *31*). The contribution of DMS to wine aroma, particularly in aged wines, has been described as enhancing the strawberry/raspberry character, adding developed notes, and DMS reportedly plays an important role in the sensory profile of Shiraz wines (*7*). Given the dual role of DMS as an enhancer of red wine fruitiness and as an off flavor compound which can give cooked vegetable-like off-odors, the mechanisms underlying its formation during aging and the sensory implications of these changes warrant further investigation (*29*).

In a subsequent experiment, we investigated the effects of DAP addition to another low YAN (100 mg/L) Shiraz must and compared profiles of volatile sulfur compounds formed by two different yeast strains, *S. cerevisiae D-254* and *S. bayanus AWRI 1176* (*32*). Again, the results raised questions concerning the widespread use of DAP in the management of low YAN fermentations with respect

to the formation of reductive characters in wine. For the red wine made with yeast *S. cerevisiae D-254*, addition of DAP to a final YAN of 250 or 400 mg/L resulted in an increased formation of H_2S compared to the slower control fermentations (100 mg/L YAN). For this *S. cerevisiae* yeast, DAP-supplemented fermentations also showed prolonged formation of H_2S into the later stage of fermentation, which was associated with detectable H_2S in the final wines. The *S. bayanus* strain showed a different H_2S production profile, in which less H_2S was formed at higher YAN, with no H_2S in any final wines. Interestingly, no correlation was found between total H_2S produced by either yeast during fermentation and H_2S concentration in the final wines. However, for both yeasts, DAP supplementation yielded higher concentrations of other volatile sulfur compounds in the finished wines, including sulfides, disulfides, thiols, and thioacetates (*32*).

In addition to the above studies, which utilized yeast starter cultures to establish a dominant population of the selected yeast strains from the beginning of fermentation, uninoculated fermentations, often referred to as 'natural' or 'spontaneous' fermentations by complex populations of different non-*Saccharomyces* and indigenous *Saccharomyces* yeasts, were also studied (*33*). In this study, volatile sulfur and other aroma compounds were quantified in 18 white wines made by barrel fermentation of nine different Chardonnay juices. Maximum concentrations of H_2S, DMS and CS_2 were found in different wines, and appearance of these volatile sulfur compounds was not related to YAN concentrations. The concentration of H_2S was higher in six of the uninoculated Chardonnay ferments, no H_2S was detected in one set of ferments irrespective of the yeast present, and two of the inoculated fermentations had higher concentration of H_2S compared to the 'natural' ferments, whereas no trends for DMS or CS_2 were noted. In summary, yeast effects on the concentrations of volatile sulfur compounds were relatively small when comparing wines made by inoculated or uninoculated fermentations. The concentration in wine of H_2S, DMS, CS_2, and other aroma compounds such as linalool and α-terpineol, were quite obviously reflecting differences in the composition of the particular grape juice used for the fermentations, rather than the inoculation technique (*33*).

Packaging Choices and Volatile Sulfur Compounds

The AWRI recently completed the testing of a 2007 unoaked Semillon wine, which had been bottled under carefully controlled oxygen management practices using ten different types of closures. The wines were then stored at approximately 17°C for two years using best practice conditions (*34*). The wine style evolution under the different closures was consistent with the different oxygen transmission rates (OTR) through the closures subsequent to bottling. Accordingly, bottling of a single Semillon base wine with different closure technologies generated ten wines with distinct sensory properties after 24 months. Some closure technologies, such as the reference screwcap, yielded a Semillon wine that was rated high in fruit attributes, such as 'fresh citrus', but also showed high 'reductive', 'cabbagey' and 'struck flint' aromas. Chemical analysis of volatile sulfur aroma compounds, using the HS-COC-GC-SCD method, demonstrated that the 'reductive' character was associated with elevated levels of methanethiol in this sample set.

A subset of seven Semillon wines from the above closure trial was also submitted to consumer testing after 24 months, including the wines stored under screwcap and natural cork. One hundred and eight Sydney consumers assessed each wine separately, and were asked to record their overall liking of each wine, together with purchase intent and, after the completion of the test, demographic information. An initial analysis of the consumer results showed that there were no significant differences among the wines for either overall liking or purchase intent for the total population. All wines, on average, were rated in a narrow range from 5.95 to 6.23 on the nine point liking scale used. Although no significant difference was found in the total population data, further statistical analysis demonstrated that the consumers' opinions were not uniform and three very different segments were identified (*34*). Cluster 1, which contained 27% of the consumers, was negatively influenced by oxidised and cork-taint (TCA) attributes. These consumers liked the wines with higher levels of fruit, estery, fresh citrus, and toasty attributes and were tolerant to the wines with 'reduced' attributes. In contrast, Cluster 2, with 41% of the consumers, did not respond negatively to the TCA-affected wine, but showed a low acceptance for wines that had higher levels of MeSH and reductive, struck flint or cabbagey attributes. Cluster 3, with 32% of the consumers, was not affected by the presence of oxidised, reductive or TCA attributes present and liking for this group appeared to be influenced by an absence of simple fruit characters.

It is important to note that only some wines will develop 'reductive' characteristics under low OTR closures, e.g. screwcaps. Still, the compositional and sensory differences that developed in the wines as a sole function of closure choice substantially affected consumers' liking of the product, with different market demographics having different preferences. The fact that a group of untrained consumers reacted negatively to the presence of 'reductive' odors, which presumably stemmed from the elevated concentration of MeSH in these wines, emphasises the need to better understand the formation of volatile sulfur compounds during winemaking and storage, and to develop strategies for the effective control of these undesirable aromas and their precursors.

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Chapter 15

Understanding Aroma Impact of Four Important Volatile Sulfur Compounds in Oregon Pinot Noir Wine

I-Min Tsai*,[1] and Mina R. McDaniel[2]

[1]General Mills, Inc., Riverside Technical Center, 330 University Ave. SE., Minneapolis, MN 55414, USA
[2]Department of Food Science and Technology, Oregon State University, 100 Wiegand Hall, Corvallis, OR 97331, USA
***E-mail: I-Min.Tsai@genmills.com.**

Aroma characteristics of methanethiol (MeSH), ethanethiol (EtSH), dimethyl disulfide (DMDS), and diethyl disulfide (DEDS) and their interactions in the experimental Oregon Pinot noir wine were determined via descriptive analysis to understand their impact on wine aromas. Aroma descriptions of individual sulfur compound were identified in Pinot noir wine. In the experiments targeting sulfur compound mixtures in wine, odors from the sulfur compounds were found to suppress each other. MeSH and EtSH dominated perceptions of off-odors in the wine when both MeSH and DMDS or both EtSH and DEDS were present. EtSH was more detrimental to wine aromas when both MeSH and EtSH were present; however, MeSH contributed to more off-aromas than EtSH under the influence of sub-threshold levels of DMDS and DEDS. Subject variability existed due to different subjects' sensitivity to each sulfur compound. In conclusion, MeSH and EtSH can significantly affect aroma quality of the experimental Oregon Pinot noir wine more than DMDS and DEDS. The wine aroma started to show signs of defects when MeSH was more than 3.3 ppb and/or EtSH was more than 1.1 ppb. It definitely became defective when MeSH reached 14.4 ppb and/or EtSH reached 7.5 ppb. Generally the wine lost its typical notes including: fruity, floral, spicy, and sweet and increased its overall aroma intensity,

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overall stinky, sulfur-related odors (i.e. rotten cabbage, rubbery and garlic) and nose burn while concentrations of the four volatile sulfur compounds increased. Changes of fruity and floral notes and nose burn can be utilized by winemakers to diagnose early presence of MeSH, EtSH, DMDS, and DEDS in the Oregon Pinot noir wine during winemaking, aging and storage.

Introduction

Oregon Pinot noir wine is considered one of the best Pinot noir wines in the world because the climate of Willamette Valley, Oregon is very similar to Burgundy, France where Pinot noir wine enjoys an ongoing success. In recent years, some commercial Oregon Pinot noir wines suffered from aroma defects. Volatile sulfur compounds are believed to be one of the major sources of these off-aromas. Many volatile sulfur compounds naturally exist in wine, and a few of them contribute to aroma and flavor complexity and varietal characteristics at very low concentrations (*1–15*). However, most of sulfur compounds have unpleasant aromas such as rotten eggs, garlic and rubbery (*8*, *9*, *12*). These compounds often have very low sensory thresholds, which are at ppb levels (*1*, *5*, *9*, *16*, *17*), especially mercaptans and disulfides. As a result, most volatile sulfur compounds are detrimental to wine quality.

The current study focuses on the impact of four important volatile sulfur compounds believed to have a negative effect on wine aroma: methanethiol (MeSH), ethanethiol (EtSH), dimethyl disulfide (DMDS), and diethyl disulfide (DEDS) in the Oregon Pinot noir wine. Aroma descriptions of MeSH are rotten cabbage, cooked cabbage, rotten eggs, burnt rubber, pungent, and putrefaction (*9*, *12*). Its odor thresholds range from 1.72 to 1.82 ppb in the Oregon Pinot noir wine (*16*). Formation of MeSH in wine is associated with degradation of methionine during yeast and malolactic (*O. Oeni*) fermentation and during storage (*2*, *18–20*). Hydrolysis of sulfur-containing pesticides such as acephate and methomyl in wine under light exposure during storage also results in MeSH production (*11*, *12*). EtSH has an aroma of onion, rubber, feces, burnt matches, earthy, leeks and garlic (*5*, *9*, *12*). Its odor threshold is 1.1 ppb in white wine (*5*) and ranges from 0.19 to 0.23 ppb in the Oregon Pinot noir wine (*16*). It is found more abundantly (3.2 ppb; above sensory threshold) in the Teroldego wines free of sulfur off-aromas and thus considered to be part of the varietal characteristics (*21*). EtSH can be formed by reactions between hydrogen sulfide and acetaldehyde and between hydrogen sulfide and ethanol or ethanal in wine (*8*, *11*, *22*).

MeSH and EtSH are highly reactive and rapidly oxidized to form DMDS and DEDS in wine (*11*, *22*, *23*). Aroma descriptions of DMDS include cabbage, cooked cabbage and onions-like (*9*, *11*, *12*). Odor threshold of DMDS ranges from 20 to 45 ppb in wine (*5*, *9*) and from 11.18 to 23.57 ppb in the Oregon Pinot noir wine (*16*). DMDS is also formed via degradation of acephate, a sulfur-containing pesticide during wine storage (*11*) and methionine degradation by *O. oeni* (*18*, *19*). DMDS can positively contribute to the "bottle age" aroma at low concentration

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levels (*11*, *15*). DEDS is described as garlic, onions, and burnt rubber (*9*, *11*). Its odor threshold ranges from 4.3 to 40 ppb in wine (*5*, *9*) and from 1.45 to 2.16 ppb in the Oregon Pinot noir wine (*16*). At wine pH and in the presence of sulfite ions, DEDS can be slowly converted back to EtSH during aging and storage (*1*, *21*). EtSH has a much lower sensory threshold than DEDS in wine. Therefore, inter-conversion between EtSH and DEDS may cause a wine with an undetectable level of DEDS to become off (beyond recognition level of EtSH) with time and thus may greatly influence wine quality.

Once the odors of MeSH, EtSH, DMDS and DEDS are recognized in wine, MeSH and EtSH can be removed by adding copper ions. DMDS and DEDS have to be reduced to MeSH and EtSH via reducing agents such as ascorbic acid and then are removed by copper treatment. Aeration treatment and tannin addition could reduce EtSH and DEDS in wine after 60 days of aging (*24*). Although aroma defects can be taken care of by these treatments, wine quality is already degraded (*15*, *22*). Therefore, it is important to understand how these four volatile sulfur compounds affect wine aroma to help winemakers diagnose the defects early during winemaking, aging and storage.

The objectives of this study were 1) to determine aroma characteristics of MeSH, EtSH, DMDS and DEDS individually in the Oregon Pinot noir wine and investigate how wine aroma changes when the concentration of individual sulfur compounds increases from sub- to supra-threshold and 2) to understand odor interactions among these four volatile sulfur compounds in the Oregon Pinot noir wine and how these interactions influence wine aroma.

Materials and Methods

This study consisted of two parts: Part 1 focused on aroma properties and impact of individual volatile sulfur compounds in the experimental Oregon Pinot noir wine, and Part 2 focused on aroma characteristics and influences of interactions among the four compounds in wine. Each part had four experiments as shown in Table I.

Winemaking

The experimental wine was made from Pinot noir grapes grown in the Willamette Valley, Oregon during the 2004 vintage and made in the experimental winery at Oregon State University (OSU). The grape must contained 24 ~ 25.5° Brix of reducing sugar and 149 ~ 189.5 mg/L of assimilable nitrogen. Then it was inoculated with the RC 212 Bourgovin wine yeast. The new wine only finished the primary fermentation. No malolactic fermentation was carried out. Then it was cold stabilized for one month at 3°C and then filtered with a pressure leaf filtration system (Velo, Italy). Finally it was stored in bulk at 3°C. Chemical properties of the experimental wine were 14% alcohol, pH= 3.6, titratible acidity= 5.7 mg/L, and free SO_2= 37 ppm. There was no off-odor in the new wine as perceived by two wine experts at OSU. Sulfide contents of the new wine were analyzed by the GC-PFPD methodology developed in the OSU Flavor Lab (*25*).

MeSH (1.025 ppb) and DMDS (21.5 ppt) were found and EtSH and DEDS were not found in the new wine.

Sample Preparation

Pure MeSH, EtSH, DMDS and DEDS were purchased from VWR (West Chester, PA) and Sigma-Aldrich (Milwaukee, WI). The sample preparation protocol is briefly described below. First 10,000 ppm (w/w) stock solutions of EtSH, DMDS and DEDS were made by mixing them individually with minus 15°C methanol under the presence of argon in a deactivated 20 mL glass vial (I-Chem Brand; Rockwood, TN) to prevent further degradation. MeSH stock solution was made by bubbling MeSH gas in cold methanol under argon; its concentration was calculated by weight. Then a series of dilutions of each stock solution with minus 15°C methanol under argon was quickly performed. No more than 0.1 mL of final dilution(s) was added to 30 mL experimental wine in a 120 mL wide-mouth glass bottle (I-Chem Brand) to make the target concentration(s). Reproducibility of the dilution and spiking procedure was verified by the GC-PFPD analysis (*25*) on the volatile sulfur contents in the wine samples. Because DMDS and DEDS were found to be stable in wine at least 24 hours, the bottles carrying DMDS and/or DEDS wine samples were not filled with argon. Those carrying MeSH and/or EtSH wine samples were pre-filled with argon prior to pouring the Pinot noir wine to prevent oxidation of MeSH and EtSH to DMDS and DEDS. While preparing the wine samples with mixtures of either two or four sulfur compounds, disulfide was added first and then mercaptan was added. EtSH is slightly more stable than MeSH and thus was added first when mixing MeSH and EtSH. The Pinot noir wine contained 1.025 MeSH and 21.5 ppt DMDS. The final concentrations of MeSH and DMDS in wine samples were the sum of original and spiked concentrations. Stability of mercaptans in sample bottles was also examined by the GC-PFPD analysis. The results showed that argon in the headspace inhibited oxidation of mercaptans to disulfides for at least 12 hours in sample bottles.

Table I. Experiments in Part 1 and Part 2 and The Sample Final Concentrations (ppb) in the Experimental Oregon Pinot Noir Wine

Part 1- Four Experiments

Concentration	*MESH*	*ETSH*	*DMDS*	*DEDS*
Base wine	1.025	0.000	0.0215	0.00
Lowest	1.082	0.021	0.27	0.11
	1.650	0.203	4.75	0.47
	3.251	1.063	13.19	1.74
	6.267	2.512	21.09	4.57
	10.914	5.017	36.85	7.37

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Table I. (Continued). Experiments in Part 1 and Part 2 and The Sample Final Concentrations (ppb) in the Experimental Oregon Pinot Noir Wine

Part 1- Four Experiments

Concentration	*MESH*	*ETSH*	*DMDS*	*DEDS*
	14.384	7.525	50.56	10.54
Highest	21.166	10.094	68.43	15.80

Part 2- Four Experiments

MeSH & DMDS

Notation	*MeSH*	*DMDS*
base	1.025	0.0215
DlMl	1.082	0.27
DlMm	3.251	0.27
DlMh	14.384	0.27
DmMl	1.082	36.85
DmMm	3.251	36.85
DmMh	14.384	36.85
DhMl	1.082	68.43
DhMm	3.251	68.43
DhMh	14.384	68.43

EtSH & DEDS

Notation	*EtSH*	*DEDS*
base	0.000	0.00
DlEl	0.021	0.11
DlEm	1.063	0.11
DlEh	7.512	0.11
DmEl	0.021	4.57
DmEm	1.063	4.57
DmEh	7.512	4.57
DhEl	0.021	10.54
DhEm	1.063	10.54
DhEh	7.512	10.54

MeSH & EtSH

Nota-tion	*MeSH*	*EtSH*
base	1.025	0.000
MlEl	1.082	0.021
MlEm	1.082	1.063
MlEh	1.082	7.512
MmEl	3.251	0.021
MmEm	3.251	1.063
MmEh	3.251	7.512
MhEl	14.384	0.021
MhEm	14.384	1.063
MhEh	14.384	7.512

MeSH, EtSH & Two Sub-thresholds of DMDS & DEDS

Notation	*MeSH*	*EtSH*	*DMDS*	*DEDS*
base	1.025	0.000	0.0215	0.00
DDMlEl	1.082	0.021	0.27	0.11
DDMlEm	1.082	1.063	0.27	0.11
DDMlEh	1.082	7.512	0.27	0.11
DDMmEl	3.251	0.021	0.27	0.11
DDMmEm	3.251	1.063	0.27	0.11
DDMmEh	3.251	7.512	0.27	0.11
DDMhEl	14.384	0.021	0.27	0.11
DDMhEm	14.384	1.063	0.27	0.11
DDMhEh	14.384	7.512	0.27	0.11

Three concentration level are denoted as l=low, m=medium, and h=high. Volatile sulfur compounds are denoted as D= DMDS or DEDS, M= MeSH and E= EtSH.

Testing Concentration Selection

The testing concentrations (Table I) employed in this study were based on the results of the previous threshold study (*16*), descriptive panel training for this study, and the unpublished data of GC-PFPD sulfur analysis on 39 commercial Pinot noir wines which were produced from nine Oregon wineries and had aroma defects associated with sulfur compounds in 2005 (*26*). These Pinot noir wines generally had recognizable MeSH and/or EtSH odors. Part 1 experiments used seven concentrations of each volatile sulfur compound ranged from sub-threshold to supra-threshold levels plus one blank experimental Pinot noir wine (noted as base wine). The lowest level was below all subjects' individual detection thresholds determined in the previous study (*16*). Three criteria were used to select the highest concentration. First, the highest level was above all subjects' individual detection thresholds. Second, every subject can firmly recognize the sulfur odor at this level in wine during panel training. Third, this level was the highest content of each compound measured from the 39 Oregon Pinot noir wines with aroma defects by the GC-PFPD analysis (*26*). Aroma characteristics and influences of individual volatile sulfur compound were examined in one experiment.

In Part 2, three levels (low, medium, and high) of each sulfur compound were chosen. The low level (l) was equal to the lowest concentration in Part 1. The high level (h) for disulfides was the highest concentration, and for mercaptans was the second highest level in Part 1. Since a wide range of individual detection threshold of the four compounds was found among subjects in the previous study (*16*), the basis of selecting the medium level (m) was to use a level that was detectable by the majority of subjects, but not at the recognition level. Concentration combinations in Part 2 experiments are also noted in Table I. Ten samples, nine of which consisted of combining three levels of two sulfur compounds plus one base wine was used. The first two experiments targeted the interactions between DMDS and MeSH and between DEDS and EtSH. The third experiment focused on the presence of MeSH and EtSH in wine because both of them were commonly found in aroma-defected wines. According to the unpublished GC-PFPD results of 39 commercial Oregon Pinot noir wines with aroma defects, various levels (from sub- to supra-thresholds) of mercaptans and only sub-threshold levels of disulfides (at ppt level) were found when all four compounds were present in the defective wines (*26*). Therefore, in the last experiment sub-threshold levels of DMDS (0.27 ppb) and DEDS (0.11 ppb) were added into the nine samples combining three levels of MeSH and EtSH to understand the impact on Pinot noir wine aromas and interactions between MeSH and EtSH. The sub-threshold effect of DMDS and DEDS was also examined.

Subjects

Thirteen out of sixteen subjects (six males and seven females) who participated in the previous odor threshold study (*16*) continued with the descriptive panel for the current study. They were named by the numbers (Panelists 1, 2, 3, 4, 5, 6, 8, 9, 10, 11, 12, 14 and 16). Panelist 2 participated

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in Part 1 but not Part 2; therefore, twelve panelists (six males and six females) stayed throughout the whole study. Eleven subjects were students or staffs in the Food Science department at Oregon State University. Six subjects either studied or worked in wine-related fields.

Sensory Evaluation

The modified Spectrum™ descriptive analysis method (*27*) combined with free-choice profiling (*28*, *29*) was used for panel training and sample evaluation. There were thirty 1-hour training sessions in total. Ten descriptors (called common terms, CT) either pre-determined from past sensory studies for Oregon Pinot noir wines (*30*, *31*) or reached by consensus from the 13 subjects. They were overall intensity, overall stinky, nose burn, fruity, floral, spicy, sweet, vegetative, earthy and papery. Nine of them were aroma descriptors for the experimental Oregon Pinot wine. Overall stinky was employed to evaluate the overall off-flavor intensity created by the volatile sulfur compounds in wine. Their definitions are shown in Table II.

Subjects were trained to understand these aroma descriptors with the corresponded standards and practiced with the evaluation procedures and the 16-point intensity scale (0=none and 15= extreme). Subjects could not reach a consensus on the descriptions of sulfur-related aromas in the samples during training. As a result, they were allowed to generate their own lists of sulfur-related descriptors (called free-choice terms, FT). The FT descriptors created by 13 subject for the four sulur compounds in wine are shown in Table III and Table IV. 35, 39, 18, and 27 FT descriptors were generated for MeSH, EtSH, DMDS and DEDS, respectively. Latex, rubber stopper, caramel, rotten cabbage, old cabbage and skunky were used by more than 4 subjects to describe MeSH. The most frequently used descriptors for EtSH were durian, natural gas, rubber stopper, garlic, latex and caramel. Caramel, rotten cabbage, latex, ballon, old cabbage, rubber stopper, car snoke, old eggs and burnt match were used by the majority of subjects to describe DMDS. Sweaty, tire, skunky, caramel and pine were the most frequently used FT terms for DEDS. Several standards for FT descriptors were introduced and provided during training. Subjects were asked to write down the definitions of the FT terms for which no standard was provided, or where the meaning was different from the standards. As a result, each subject evaluated the wine samples by rating intensities of 10 CT descriptors plus their own FT terms. Definitions and standards of the CT and FT descriptors are summarized in Tsai (*16*).

Twenty-four test sessions, twelve for Part 1 and twelve for Part 2 experiments were performed immediately after the panel training. All wine samples were coded with three-digit random numbers and served in random order. Each sample was replicated three times. Two additional base wine samples and all aroma standards were provided. The subjects were told to smell wine samples no more than three times and to evaluate them as quickly as possible to minimize aroma changes once the sample bottle was opened. Swirling the sample bottles was not allowed because it helped release too much pungency or noseburn sensation, which interfered with the perception of other aromas and increased olfactory fatigue. Subjects could

always re-sniff the aroma standards and the two base wine bottles if necessary. However, to avoid fatigue they were instructed not to go back to the standards and base wine too frequently. Breaks were also allowed between samples.

Table II. Definition of the Ten Common Aroma Descriptors (CT)

Descriptor	*Definition*
Overall intensity	Intensity of total aromas perceived in wine
Nose burn	Aromatics associated with nose burning or pungency
Fruity	Aromatics associated with fruits including: berry, cherry, blackcurrant, grape and plum
Floral	Aromatics associated with flowers including: rose, violet and geranium
Vegetative	Aromatics associated with vegetables including: bell pepper and cabbage
Spicy	Aromatics associated with spices including: cinnamon, black pepper and clove
Sweet	Aromatics associated with sweet substances including: honey and molasses
Earthy	Aromatics associated with mushroom and wet soils
Papery	Aromatics associated with wet paper towel
Overall stinky	Total off-flavor intensity created by the volatile sulfur compounds in wine

Table III. Free-Choice (FT) Terms Generated by Thirteen Subjects to Decribe MeSH and EtSH in the Experimental Oregon Pinot Noir Wine and Their Usage Frequency (Indicated by No. of Subjects)

MeSH		*EtSH*	
FT Descripor	*No. of Subject*	*FT Descripor*	*No. of Subject*
Latex	6	Durian	8
Rubber stopper	6	Natural gas	6
Caramel	5	Rubber stopper	5
Rotten cabbage	4	Garlic	5
Old cabbage	4	Latex	4
Skunky	4	Caramel	4
Car smoke	3	Balloon	3

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Table III. (Continued). Free-Choice (FT) Terms Generated by Thirteen Subjects to Decribe MeSH and EtSH in the Experimental Oregon Pinot Noir Wine and Their Usage Frequency (Indicated by No. of Subjects)

MeSH		*EtSH*	
FT Descripor	*No. of Subject*	*FT Descripor*	*No. of Subject*
Sweaty	3	Green onion	3
Cooked vegetables	3	Old cabbage	2
Cabbage	3	Skunky	2
Flatulence	3	Sweaty	2
Balloon	2	Tire	2
Old eggs	2	Sauerkraut	2
Tire	2	Ginger	2
Natural gas	2	Rotten cabbage	1
Fecal	2	Cooked vegetables	1
Slop	2	Old eggs	1
Burnt match	1	Fecal	1
Sulfury	1	Burnt match	1
Rotten egg	1	Sulfury	1
Solvent	1	Rotten egg	1
Animal urine/ feces	1	Solvent	1
Caramel sweet	1	Pine	1
Green	1	Cigarette smoke	1
Green grass/cut	1	Burnt rubber	1
Hot rubber belt (car)	1	Cantaloupe seed	1
Metallic	1	Cheesy	1
Old tire	1	Durian/Tropical fruit	1
Pumpkin	1	Fermented sour	1
Rotten milk	1	Medicinal bitter	1
Rotten vegetables	1	Old/burnt butter smoky	1
Seaweed	1	Ranch dressing	1
Slop/animal	1	Raw rotten egg	1

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Table III. (Continued). Free-Choice (FT) Terms Generated by Thirteen Subjects to Decribe MeSH and EtSH in the Experimental Oregon Pinot Noir Wine and Their Usage Frequency (Indicated by No. of Subjects)

MeSH		*EtSH*	
FT Descripor	*No. of Subject*	*FT Descripor*	*No. of Subject*
Sour milk	1	Roasted garlic	1
Urine/ammonia	1	Sulfur	1
		Tropical fruit	1
		Musty/cantaloupe seed	1
		Smoked wood	1
		Spoiled sauerkraut	1

Table IV. Free-Choice (FT) Terms Generated by Thirteen Subjects to Decribe DMDS and DEDS in the Experimental Oregon Pinot Noir Wine and Their Usage Frequency (Indicated by No. of Subjects)

DMDS		*DEDS*	
FT Descripor	*No. of Subject*	*FT Descripor*	*No. of Subject*
Caramel	12	Sweaty	11
Rotten cabbage	11	Tire	11
Latex	10	Skunky	10
Balloon	10	Caramel	5
Old cabbage	10	Pine	5
Rubber stopper	9	Old cabbage	3
Car smoke	9	Rotten cabbage	2
Old eggs	8	Latex	2
Burnt match	8	Balloon	2
Sweaty	1	Balloon/latex	2
Cigarette smoke	1	Cooked vegetables	2
Cabbage	1	Car smoke	1
Balloon/latex	1	Cigarette smoke	1
Fermented sweet	1	Cabbage	1
Fingernail polish	1	Sauerkraut	1
Overripened fruity	1	Sulfury	1

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Table IV. (Continued). Free-Choice (FT) Terms Generated by Thirteen Subjects to Decribe DMDS and DEDS in the Experimental Oregon Pinot Noir Wine and Their Usage Frequency (Indicated by No. of Subjects)

DMDS		*DEDS*	
FT Descripor	*No. of Subject*	*FT Descripor*	*No. of Subject*
Special sweet	1	Black licorice sweet	1
Sugar cane sweet	1	Black pepper	1
		Burnt tire	1
		Candy/anise	1
		Copper	1
		Fresh spice	1
		Match	1
		Meaty	1
		Mushroom	1
		Rubbery cabbage	1
		Wood alcohol	1

Statistical Analysis

Data collected from each experiment were analyzed separately. Data obtained from the last two experiments in Part 2 were combined and analyzed together. Subject repeatability of each experiment was first examined via the Generalized Procrustes analysis (GPA). GPA has been successfully used to monitor discrepancies between replications and between subjects (*29*, *32–34*). The GPA results showed that subjects were able to repeat their own evaluations (results are not shown). The outcome of the subject repeatability analysis for each experiment is summarized in Tsai (*16*).

Intensity scores for each descriptor rated by each subject were averaged across three replications. The averaged scores were analyzed by GPA in each experiment. GPA translates each subject's configuration to a common centroid, shrinks or stretches panelists' configurations to a similar size (isotopic scaling), and finally adjusts these configurations to fit together by rotation or reflection (*32*). Before performing GPA, one-way analysis of variance (ANOVA) was conducted on each descriptor for every subject to examine whether the descriptor could be used to differentiate wine samples by each particular person. The level of significance was 0.05. The descriptors which cannot be utilized to significantly differentiate the wine samples ($p > 0.05$) were excluded from the GPA to reduce noise. The minimum of 0.5 of descriptor loading coefficient was chosen as a selection criterion with the exception that 0.4 was chosen for the MeSH & DMDS mixture experiment to acquire a sufficient number of FT descriptors. The sample

loading scores across subjects in each dimension were averaged and plotted to provide the consensus configuration. One-way ANOVA followed by Tukey-HSD multiple comparisons were performed on the sample loading scores to examine the sample difference in each dimension.

The GPA loading scores of wine samples and aroma descriptors from individual subjects were also plotted to show subject variability. Because three replications were averaged before performing GPA, one-way ANOVA and Tukey-HSD test cannot be performed on the individual GPA results of the subjects. As a result, the individual GPA results can only provide a qualitative indication to subject variability.

Results

Aroma Characteristics of Individual Volatile Sulfur Compounds in the Experimental Oregon Pinot Noir Wine

MeSH

Figure 1 shows the GPA results from concensus data that demonstrate the aroma characteristics and differences of the seven wine samples with MeSH as well as base wine. 75.29% of total variance was extracted from GPA and dimension 1 accounted for 68.46% of total variance. Therefore, only the results in dimension 1 are summarized. For better illustration, the aroma descriptors shared similar characteristics were grouped and renamed. "Stale vegetables" covered cabbage, old cabbage, rotten cabbage, cooked vegetables and rotten vegetables. Rotten egg and old egg were grouped and renamed "stale eggs." "Rubbery" represented rubber stopper, hot rubber belt and latex. Fecal, flatulence/fart, sweaty, skunky, urine were called "animal." The descriptors which could not be grouped and were used by more than three subjects are also listed in Figure 1. The samples are labeled as their MeSH levels in ppb and base wine is noted as "base." Wine samples bearing different superscripts are significantly different at $p < 0.05$ by one-way ANOVA and Tukey-HSD multiple comparisons.

In dimension 1, the samples with the three highest MeSH levels (10.914, 14.348 and 21.166 ppb) were rated to have significantly stronger non-wine aromas (stale vegetables, stale eggs, animal, rubbery, slop and natural gas), overall intensity, overall stinky and nose burn and less intense wine-related aromas (fruity, floral and sweet) than the other four samples with lower MeSH levels and base wine. Base wine and the samples with 1.082 and 1.650 ppb MeSH were rated to have significantly less non-wine aromas, overall intensity, overall stinky, and nose burn and more fruity, floral, and sweet notes than the one with medium level of MeSH, 6.267 ppb. As a result, while MeSH concentration increased in the Pinot noir wine, the typical wine aromas such as fruity, floral and sweet diminished and overall aroma in the wine samples became more intense, driven by overall stinky, nose burn and off-aromas. Aroma characteristics of MeSH found in this study are consistent with those that have been summarized in the literatures (*9*, *11*, *12*). Small subject variability was observed in the individual GPA results. Overall,

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subjects' individual GPA configurations showed trends similar to the consensus configuration with minor variations (results are not shown).

EtSH

Aroma characteristics and differences of the seven EtSH wine samples and base wine are shown in Figure 2. 88.79% of total variance was extracted from GPA. 82.84% of total variance was explained by dimension 1 and thus only the results in dimension 1 are demonstrated below. The descriptors having similar aroma characteristics were grouped together and renamed. Sweaty, skunky, and fecal were grouped and renamed "animal." "Rubbery" represented balloon, latex, rubber stopper, burnt rubber, and tire. Old egg, raw rotten egg, and rotten egg were renamed "stale eggs." Rotten cabbage, old cabbage, sauerkraut, green onion and cooked vegetables were called "stale vegetables." Garlic and roasted garlic were renamed "garlic." Sulfur" covered sulfur, sulfury, burnt match and smoked wood. The descriptors which could not be grouped and were used by more than three subjects are also shown in Figure 2. The samples are labeled as their EtSH concentrations in ppb and base wine is noted as "base." Wine samples bearing different superscripts are significantly different at $p < 0.05$ by one-way ANOVA and Tukey-HSD multiple comparisons.

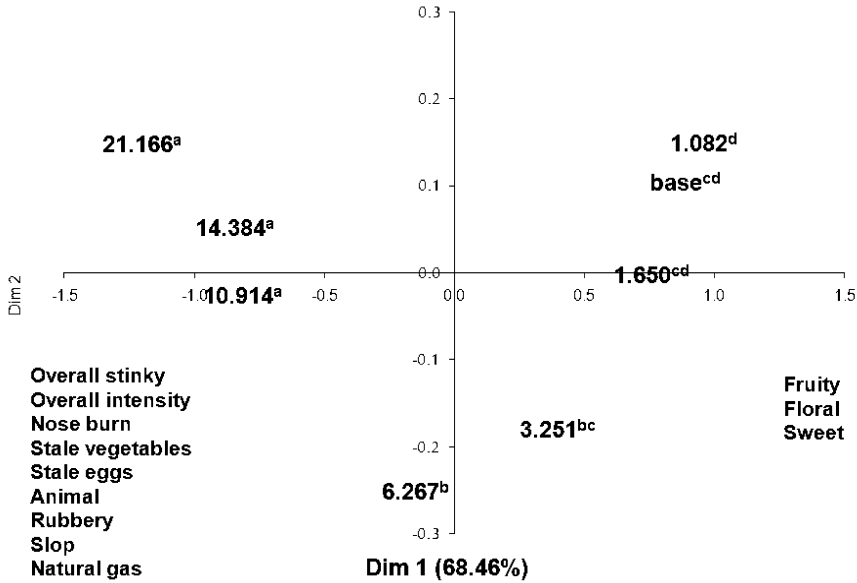

Figure 1. Consensus Configuration Plot of the Seven MeSH Wine Samples and Base Wine from GPA. Only Dimension 1 Is Demonstrated. The samples are labeled as their MeSH levels in ppb and base wine is noted as "base." Samples bearing different superscripts are significantly different at 95% level by 1-way ANOVA and Tukey-HSD multiple comparisons.

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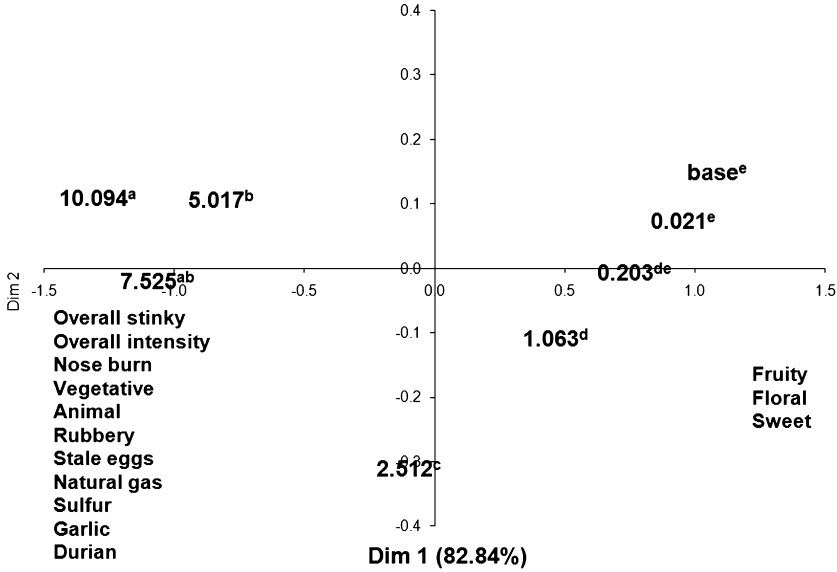

Figure 2. Consensus Configuration Plot of the Seven EtSH Wine Samples and Base Wine from GPA. Only Dimension 1 Is Demonstrated. The samples are labeled as their EtSH levels in ppb and base wine is noted as "base." Samples bearing different superscripts are significantly different at 95% level by 1-way ANOVA and Tukey-HSD multiple comparisons.

The three highest EtSH concentrations (5.017, 7.525 and 10.094 ppb) in the Pinot noir wine were found to have significantly the highest overall stinky, overall intensity, nose burn, vegetative, animal, rubbery, stale eggs, stale vegetables, garlic, sulfur, natural gas and durian and the least fruity, floral and sweet among the eight samples. Base wine and the sample with 0.021 ppb EtSH had more wine-related aromas and less non-wine aromas, overall stinky, overall intensity and nose burn than the samples with 1.063 and 2.512 ppb EtSH. The wine with 0.203 ppb EtSH was rated in between and not significantly different from the ones with 0.203 and 2.512 ppb EtSH. Pinot noir wine gradually lost its typical aromas including fruity, floral and sweet and it became more intense and stinky overall and had more nose burn and non-wine off-aromas when EtSH was increasing from sub- to supra-threshold levels.

The most common FT descriptor chosen by the majority of the subjects was durian. Durian is a large, green thorny fruit grown in Southeast Asia. It is known to have a very potent odor resulting from thiols and thioesters (*35*). The other FT descriptors created in this study are similar to those reported in the literature (*5*, *9*); furthermore, durian was incorporated the first time for describing EtSH aroma in wine. Subject variability was very minimal after examining the individual GPA results and there was no opposite or dissimilar evaluation behavior found among subjects (results are not shown).

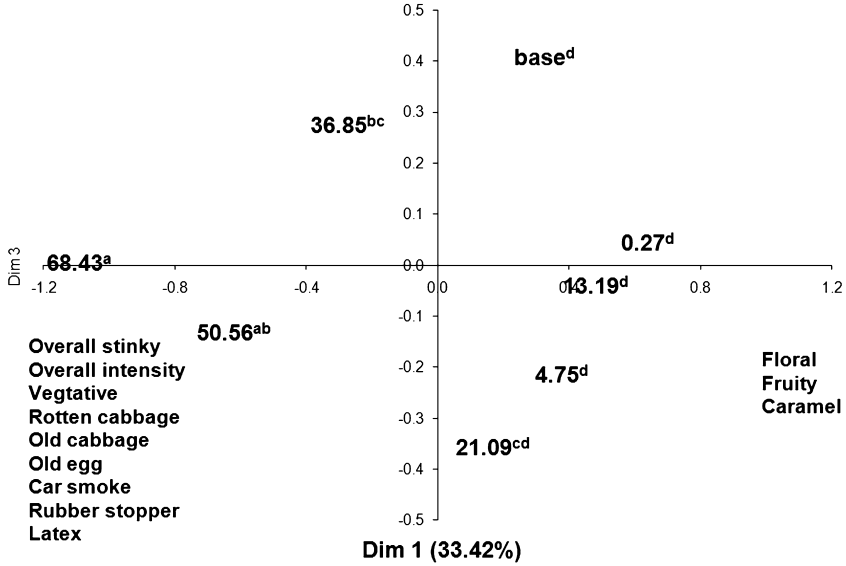

Figure 3. Consensus Configuration Plot of the Seven DMDS Wine Samples and Base Wine from GPA and Dimensions 1 Is Demonstrated. The samples are labeled as their DMDS levels in ppb and base wine is noted as "base." Samples bearing different superscripts are significantly different at 95% level by 1-way ANOVA and Tukey-HSD multiple comparisons.

DMDS

The GPA results from concensus data which indicated the aroma characteristics and differences among the eight samples consisted of seven DMDS concentrations and base wine are shown in Figure 3 and Figure 4. 58.94% of total variance was extracted, and dimension 1 and dimension 2 accounted for 33.42% and 7.70% of the total variances respectively. The aroma descriptors which were used by more than three subjects are shown in dimension 1 (Figure 3), and the descriptors used by more than two subjects are shown in dimension 2 (Figure 4). The samples are labeled as their DMDS concentrations in ppb and base wine is noted as "base." The samples bearing different superscripts are significantly different at $p < 0.05$ by one-way ANOVA and Tukey-HSD multiple comparisons.

In dimension 1 (Figure 3), the wine samples with 50.56 and 68.43 ppb of DMDS were rated significantly higher for overall stinky, overall intensity, vegetative, and the non-wine aromas including: rotten cabbage, old cabbage, old egg, car smoke, rubber stopper and latex; and were also rated lower in floral, fruity and caramel than the samples with 21.09, 13.19, 4.75, and 0.27 ppb of DMDS and base wine. The sample with 36.85 ppb DMDS was rated in between 50.56 and 21.09 ppb of DMDS and not significant different from them. Rotten cabbage and old cabbage were utilized by the majority of subjects to separate the

wine samples in this dimension. They are associated with the aroma description of DMDS, cabbage and cooked cabbage, reported by the literature (*9*, *12*, *36*). It is clear that as DMDS increased in the Pinot noir wine, the wine tended to lose its typical characteristics (fruity and floral) and acquired more non-wine aromas such as rotten and old cabbage.

The GPA results also revealed that not all subjects' FT descriptors were extracted to dimension 1. Dimension 2 consisted of the aroma descriptors mainly used by Panelists 1, 2, 9, and 12 (Figure 4). The wine sample with 36.85 ppb DMDS was rated to have significantly higher latex, balloon, rotten cabbage, and caramel but lower overall intensity and less rubber stopper aroma than the other samples. The two samples with 4.75 and 21.09 ppb DMDS had higher latex, balloon, rotten cabbage, and caramel but lower overall intensity and less rubber stopper than the wines with 62.43, 50.56, and 13.19 ppb DMDS and base wine.

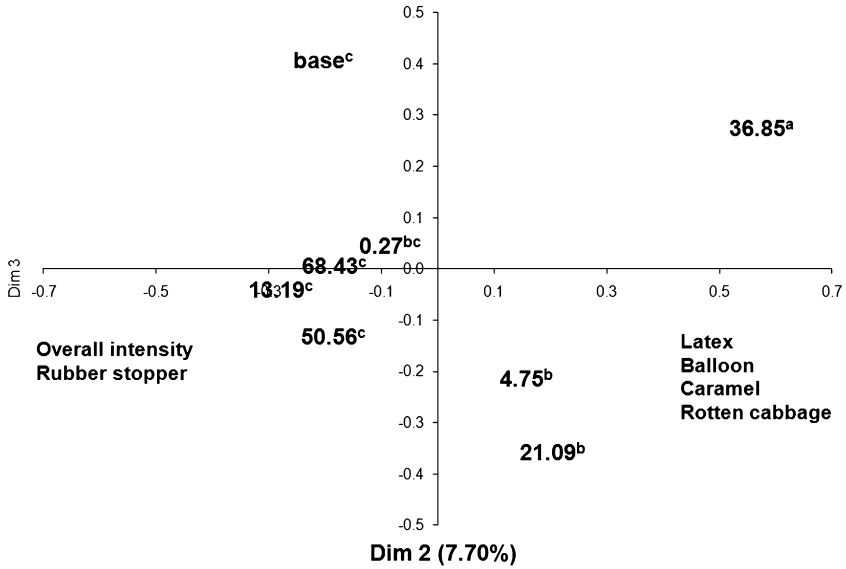

Figure 4. Consensus Configuration Plot of the Seven DMDS Wine Samples and Base Wine from GPA and Dimensions 2 Is Demonstrated. The samples are labeled as their DMDS levels in ppb and base wine is noted as "base." Samples bearing different superscripts are significantly different at 95% level by 1-way ANOVA and Tukey-HSD multiple comparisons.

From the concensus GPA results, it is clear that subject variability existed. Subject variability was observed in the individual GPA results. Ten subjects' individual results showed similar trends to the concensus configuration in dimension 1 (Figure 3) with minor variations. Panelists 2, 8 and 9 behaved differently. Panelist 2 rated the sample with 36.85 ppb of DMDS as the highest and the ones with 50.56 and 68.43 ppb of DMDS as the lowest for overall stinky, rotten cabbage, rubber stopper, balloon and sugarcane sweet. The other samples were rated in between for these attributes. Panelist 9 rated 21.09 and 13.19 ppb as the highest and base wine as the lowest for overall stinky and old cabbage. Panelist 8 almost had an opposite evaluation behavior to the majority of subjects. This person rated base wine as the strongest for overall stinky, rotten cabbage and car smoke and the wine with 0.27 ppb DMDS as the strongest for rubber stopper. The four highest concentrations of DMDS were perceived as low intensity for these aromas (results are not shown).

DEDS

The consensus GPA results for the eight wine samples in the first two dimensions are shown in Figure 5 and Figure 6. 59.21% of the total variance was extracted from GPA. Dimension 1 accounted for 39.05% and dimension 2 accounted for 7.95% of the total variance. The aroma descriptors used by more than two subjects were shown in Figure 5 and Figure 6. The samples are labeled as their DEDS concentrations in ppb and base wine is noted as "base." The samples bearing different superscripts are significantly different at $p < 0.05$ by one-way ANOVA and Tukey-HSD multiple comparisons. In dimension 1 (Figure 5), four samples with the highest DEDS concentrations, 4.57, 7.37, 10.54, and 15.80 ppb, were rated to have significantly stronger overall stinky, overall intensity, nose burn, spicy and the FT aromas including old cabbage, skunky, sweaty, pine and tire and weaker fruity and floral notes than the two samples with the lowest DEDS concentrations (0.11 and 0.47 ppb) and base wine. The wine with 1.74 ppb DEDS was rated in between for these attributes and not significantly different from the ones with 7.37 and 0.11 ppb of DEDS. Dimension 2 was composed of the aroma descriptors mainly contributed by Panelists 8 and 12 (Figure 6). The samples with the lowest (0.11 ppb) and the highest (15.8 ppb) levels of DEDS were rated as having significantly stronger fruity and floral and lower overall intensity, skunky, sweaty and tire than the other six wine samples. The wine with 10.54 ppb DEDS was significantly fruitier, more floral, less intense overall and had significantly weaker skunky, sweaty, and tire aromas than the sample with 4.57 ppb DEDS. Base wine and the three samples with 0.47, 1.74, and 7.37 ppb of DEDS were rated in between and not significantly different for these attributes. The FT aromas were close to the aroma descriptions of DEDS reported in the literature, such as onion, garlic and burnt rubber (*9*, *11*).

Overall, the DEDS results shared a similar pattern observed in the findings for MeSH, EtSH, and DMDS. The experimental Oregon Pinot noir wine gradually lost its own aroma characteristics and non-wine aromas became apparent and finally dominated the perceived wine aromas.

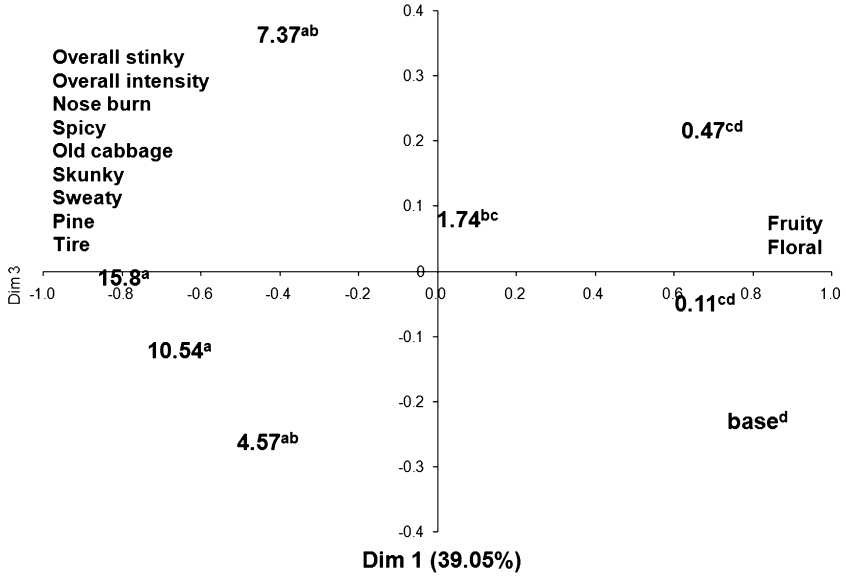

Figure 5. Consensus Configuration Plot of the Wine Samples with Seven Levels of DEDS and Base Wine from GPA and Dimensions 1 Is Demonstrated. The samples are labeled as their DEDS levels in ppb and base wine is noted as "base." Samples bearing different superscripts are significantly different at 95% level by 1-way ANOVA and Tukey-HSD multiple comparisons.

Subject variability was found in the individual GPA results. Individual results from the majority of subjects were similar to the consensus results shown in Figure 5 with small variations. Panelists 2 and 12 had different behaviors. Panelist 2 rated base wine and the wine with 0.47 ppb DEDS as having stronger overall intensity and sweaty than the other six samples. Panelist 12 rated the three samples with 0.11, 10.54 and 15.80 ppb DEDS as having stronger fruity and floral and weaker overall intensity, overall stinky, nose burn, vegetative, spicy, sweet and the FT aromas including tire, fresh spice, sweaty and skunky than base wine and the wines with 0.47, 1.74, 4.57 and 7.37 ppb DEDS (results are not shown).

Aroma Characteristics and Effects of Volatile Sulfur Compounds' Interactions in the Experimental Oregon Pinot Noir Wine

MeSH and DMDS Mixtures

Aroma characteristics and differences among the nine samples mixing three levels of DMDS (low=0.27, medium=36.85 and high=68.43 ppb) and three levels of MeSH (low=1.082, medium=3.251 and high=14.384 ppb) and base wine from GPA are shown in Figures 7 and 8. 67.00% of the total data variance extracted and dimension 1 and dimension 2 accounted for 45.87% and 7.03% of the total

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variance respectively. No new aroma descriptor was generated for these wine samples. For illustration purposes, the FT descriptors used by more than two subjects were included and shown. The nine mixture samples are labeled by the following abbreviations: D=DMDS, M=MeSH, l=low, m= medium and h= high. For example, the wine sample containing 0.27 ppb MeSH and 36.85 ppb DMDS is labeled as "DlMm." Base wine is noted as "base." Wine samples bearing different superscripts are significantly different at $p < 0.05$ by one-way ANOVA and Tukey-HSD multiple comparisons.

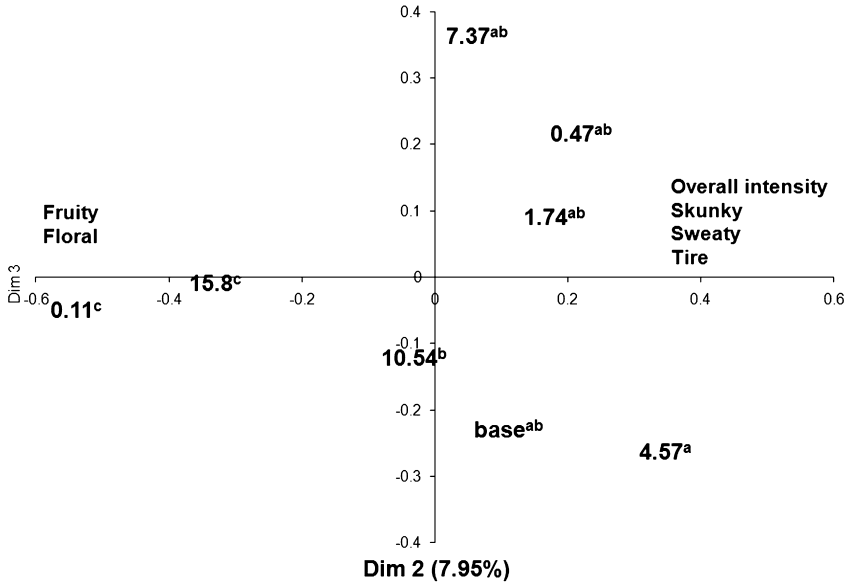

Figure 6. Consensus Configuration Plot of the Wine Samples with Seven Levels of DEDS and Base Wine from GPA and Dimensions 2 Is Demonstrated. The samples are labeled as their DEDS levels in ppb and base wine is noted as "base." Samples bearing different superscripts are significantly different at 95% level by 1-way ANOVA and Tukey-HSD multiple comparisons.

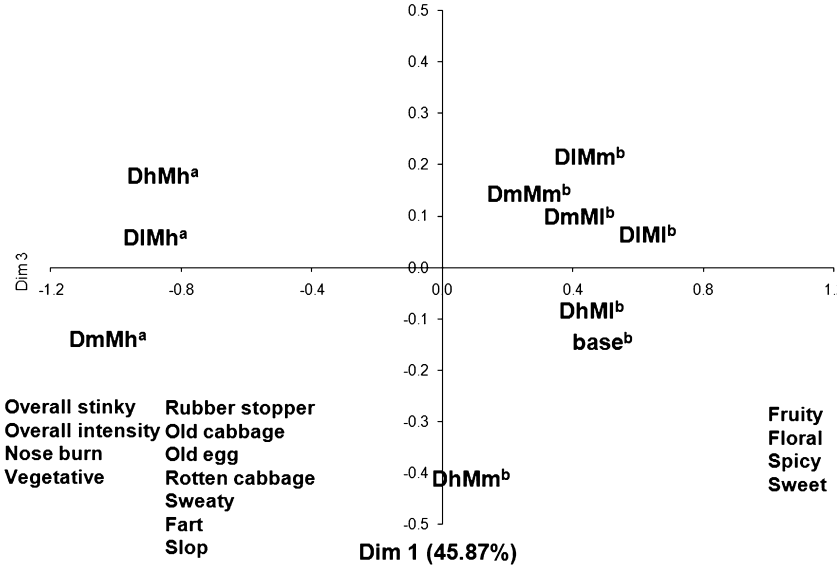

Figure 7. GPA Consensus Plot for the Nine MeSH and DMDS Mixture Samples and Base Wine. Dimensions 1 Is Demonstrated. Abbreviations: D=DMDS, M=MeSH, l=low, m= medium, h= high and base= base wine. Samples bearing different superscripts are significantly different at 95% level by 1-way ANOVA and Tukey-HSD multiple comparisons.

From the GPA results, the high level of MeSH was separated from medium and low levels of MeSH regardless of the DMDS levels across dimension 1 (Figure 7). The three mixture samples with 14.384 ppb MeSH (noted as DhMh, DmMh, and DlMh) were rated as having significantly higher overall stinky, overall intensity, nose burn, vegetative and the FT aromas including rubber stopper, old cabbage, old egg, rotten cabbage, sweaty, flatulence, and slop than the six samples with 3.521 or 1.082 ppb MeSH (noted as DhMm, DmMm, DlMm, DhMl DmMl, and DlMl) and base wine. DhMh, DmMh, and DlMh were also rated to have significantly less wine-related aromas such as fruity, floral, spicy and sweet than the other samples. DhMm, DhMl, DmMm, DmMl, DlMm, DlMl, and base wine were not significantly different for the aroma attributes described in dimension 1. In spite of the insignificance of aroma characteristics among these seven wine samples, the three samples with medium level of MeSH, DhMm, DmMm, and DlMm, were slightly separated from the other four samples. They were slightly less intense for fruity, floral, spicy and sweet characters and more intense for non-wine (FT) aromas, overall stinky, overall intensity nose burn, and vegetative than DhMl, DmMl, DlMl, and base wine.

Dimension 2 accounted for 7.03% of the total variances and was composed of the aroma descriptors mainly utilized by Panelists 3, 4, 14 and 16. Only the aroma terms which were used by more than two subjects are shown in Figure 8. In this dimension, the mixture samples with high level of DMDS (DhMh, DhMm and DhMl) were rated as having significantly stronger overall stinky, overall intensity

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and the non-wine aromas such as latex, old egg, car smoke and sweaty and less sweet than the two samples with low level of DMDS, DlMl and DlMh, and base wine primarily by Panelists 3, 4, 14, and 16. Aroma intensity of the two samples with medium level of DMDS (DmMl and DmMh) was rated in between the three samples containing high level of DMDS and two samples containing low level of DMDS. However, the samples containing medium level of MeSH and low and medium levels of DMDS (DmMm and DlMm) were not significantly different from DhMh, DhMm and DhMl. As the results, Panelists 3, 4, 14, and 16 can differentiate high and low DMDS levels in the mixture samples under the influence of MeSH. The reasons that only four out of 13 subjects can identify different DMDS levels may relate to the wide range of detection thresholds of DMDS determined from the 13 subjects, reported from the previous study (*16*), and subject variability observed in the DMDS experiment in Part 1.

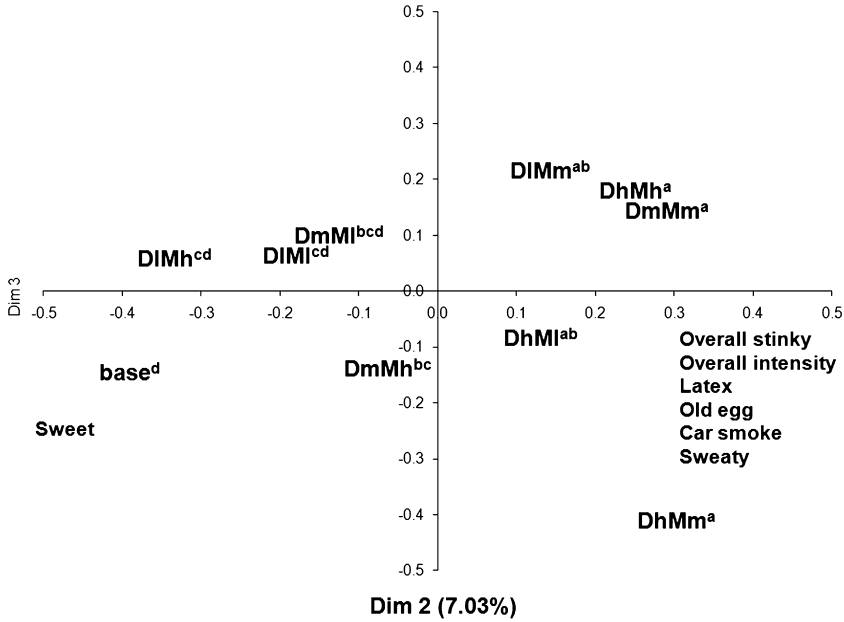

Figure 8. GPA Consensus Plot for the Nine MeSH and DMDS Mixture Samples and Base Wine. Dimensions 2 Is Demonstrated. Abbreviations: D=DMDS, M=MeSH, l=low, m= medium, h= high and base= base wine. Samples bearing different superscripts are significantly different at 95% level by 1-way ANOVA and Tukey-HSD multiple comparisons.

From the individual GPA results, the majority of subjects can discriminate high level of MeSH regardless of the DMDS levels. Panelists 4, 14, and 16 can differentiate not only high level of MeSH but also high level of DMDS. Panelist 3 can discriminate high level of DMDS. This person also rated the three wine samples with medium level of MeSH slightly higher for overall stinky, overall intensity, nose burn, spicy, earthy, and the FT aromas including old cabbage and rotten cabbage than those with low and high MeSH levels except DhMh. Panelist 3's behavior for the MeSH evaluation in this experiment was similar to his behavior in the MeSH experiment in Part 1. The medium level of MeSH was perceived as higher for overall stinky, overall intensity, nose burn and the FT aromas than the highest and the lowest levels (results are not shown).

The overall pattern found in the consensus GPA results shows that most subjects perceived that the three wines with high levels of MeSH lost typical experimental wine aromas including: fruity, floral, spicy and sweet, while gaining non-wine aroma characteristics. The non-wine aromas were very close to the typical MeSH aromas: flatulence, slop and sweaty. Although four subjects showed their discrimination ability to high and low levels of DMDS from wine samples, perceiving DMDS in the Pinot noir wine was suppressed by MeSH in general. DhMm, DhMl, DmMm, DmMl, DlMm and DlMl were not significantly different from base wine for the aroma descriptors listed in dimension 1 (Figure 7). However, when examining results from the DMDS experiment in Part 1, a significant aroma difference existed between base wine and the two samples with 36.85 and 68.43 ppb of DMDS in dimension 1 (Figure 3). These two samples were rated significantly higher for non-wine aromas but lower for fruity, floral and sweet than base wine. As a result, low and medium levels of MeSH can suppress high and medium levels of DMDS in the wine. In sum, when both MeSH and DMDS are present in the experimental Pinot noir wine, wine aromas can be significantly affected by high level of MeSH. The medium level (between detection and recognition thresholds for the majority of subjects) of both sulfur compounds may or may not affect wine aromas dependent on subjects' individual sensitivity to DMDS. The sub-threshold level of MeSH may play an important role for suppressing non-wine aromas resulted from the medium and high levels of DMDS.

EtSH and DEDS Mixtures

The GPA consensus results for the wine samples mixing three levels of DEDS (low=0.11, medium=4.57 and high=10.54 ppb) with three EtSH levels (low=0.021, medium=1.063 and high=7.512 ppb), plus base wine are shown in Figure 9. A total data variance of 82.54% was extracted from the statistical analysis. Because dimension 1 (72.41% of total variance) accounted for over 85% of the extracted variance, only the results in this dimension are summarized. There were no new aroma descriptions generated for evaluating the wine samples. The FT descriptors sharing similar characteristics were grouped together and renamed. Sweaty, skunky, meaty and fecal were renamed "animal." Balloon, latex, rubber stopper, burnt tire, and tire were grouped and called "rubbery." Old

egg, raw rotten egg, and rotten egg were called "stale eggs." Rotten cabbage, old cabbage, rubbery cabbage, sauerkraut, green onion and cooked vegetables were called "stale vegetables." Garlic and roasted garlic were called "garlic." The other descriptors which could not be grouped and were used by more than three subjects were also included. The nine mixture samples are labeled by the following abbreviations: D=DEDS, E=EtSH, l=low, m= medium and h= high. For instance, the wine sample containing 1.063 ppb EtSH and 10.54 ppb DEDS is labeled as "DhEm." Base wine is noted as "base." Wine samples bearing different superscripts are significantly different at $p < 0.05$ by one-way ANOVA and Tukey-HSD multiple comparisons.

On the left side of dimension 1 (Figure 9), three wine samples with high level of EtSH (DhEh, DmEh, and DlEh) were rated to have significantly stronger overall stinky, overall intensity, nose burn and the FT aromas including: animal, rubbery, stale eggs, stale vegetables, natural gas, garlic and durian and less Pinot noir wine-related aromas such as fruity, floral, spicy, sweet and earthy than the six samples with low or medium levels of EtSH and base wine. The two samples with high level of DEDS (DhEm and DhEl) had significantly more non-wine aromas, overall stinky, overall intensity and nose burn and less typical experimental wine aromas than DlEl and base wine. Aroma characteristics of the four samples with medium and low levels of EtSH and DEDS (DmEm, DlEm, DmEl, and DlEl) were not significantly different from the base wine. From the results, it is obvious that the wine samples with high level of EtSH were separated from those with medium and low concentrations regardless of the DEDS levels. DhEm was separated from DmEm and DlEm, and DhEl was separated from DlEl. Therefore, subjects were able to differentiate high level of EtSH as well as high level of DEDS in the experimental Pinot noir wine. High EtSH level can affect the Pinot noir wine aromas more than high DEDS level by reducing the typical wine aromas (fruity, floral, spicy, sweet and earthy) and increasing non-wine aromas when both EtSH and DEDS are present. Minimal subject variability was observed from the individual GPA results. Subjects showed similar evaluation trends to the consensus configuration in dimension 1 with very little variation (results are not shown).

Aroma interactions between three levels of EtSH and DEDS resuled in different perceptions of EtSH and DEDS in the wine and thus they affected the Pinot noir wine aroma differently. High EtSH level can disturb perception of high and medium levels of DEDS in the wine since aroma characteristics of DhEh, DmEh and DlEh were not significantly different in dimension 1. Subjects mentioned they perceived EtSH aromas (i.e. "durian") more clearly than DEDS odors (i.e. "tire") in these three samples. Medium level of EtSH and high level of DEDS may slightly suppress each other in DhEm since aroma intensity of this sample was rated the second highest for the FT aromas, nose burn, overall intensity and overall stinky. Subjects also mentioned they perceived DEDS odors were more intensely than EtSH odors in DhEm. Low level of EtSH may suppress perception of high level of DEDS because DhEl was not significantly different from DmEm, DmEl and DlEm. Low and medium levels of EtSH (0.021 and 1.063 ppb) and DEDS (0.11 and 4.57 ppb) may suppress each other in the wine and may not significantly affect the Pinot noir wine aroma since aroma characteristics of

DmEm, DlEm, DmEl, and DlEl were not significantly different from base wine in dimension 1. In addition, results from the Part 1 experiments for individual EtSH and DEDS showed that the wine sample with 1.063 EtSH and the one with 4.57 ppb DEDS had signficiantly different aroma profiles from base wine's (Figures 2 and 5).

MeSH and EtSH Mixtures

In addition to examining interactions between mercaptans and disulfides, aroma influences of the two mercaptans upon each other and on the Pinot noir wine were determined. Aroma characteristics and differences among the nine samples mixing with three levels of MeSH (low=1.082, medium=3.251 and high=14.384 ppb) and EtSH (low=0.021, medium=1.063 and high=7.512 ppb) and base wine after GPA are shown in Figure 10. 81.97% of total variance was extracted, and dimension 1 (70.79%) accounted for more than 85% of the extracted data variance. No new aroma descriptors were generated for these samples. Because so many FT descriptors are extracted to dimension 1, some of them were grouped together based on their aroma similarities for better illustration. Cabbage, old cabbage, rotten cabbage, cooked vegetables, rotten vegetables, sauerkraut, and green onion were grouped and named "stale vegetables." Old egg, rotten egg and raw rotten egg were combined and called "stale eggs." "Animal" represented skunky, sweaty, fecal, flatulence, and feces. Latex, balloon, rubber stopper, burn rubber, hot rubber belt, and tire were called "rubbery." Burnt match, car smoke, smoked wood, sulfury, and sulfur were grouped and renamed "sulfur." "Garlic" represented garlic and roasted garlic. The other aroma descriptors which could not be grouped and were used by more than three subjects were included. The nine mixture samples are labeled by the following abbreviations: M=MeSH, E=EtSH, l=low, m= medium and h= high. Base wine is noted as "base." Wine samples bearing different superscripts are significantly different at $p < 0.05$ by one-way ANOVA and Tukey-HSD multiple comparisons.

The consensus GPA results (Figure 10) revealed that subjects were able to discriminate high levels of EtSH and MeSH respectively across dimension 1. The three samples with high level of EtSH (MhEh, MmEh, and MlEh) were rated as significantly the highest for overall stinky, overall intensity, nose burn, and the FT aromas which included stale eggs, stale vegetables, durian, garlic, animal, rubbery, sulfur and natural gas; but were rated as significantly the lowest for wine-related aromas such as fruity, floral, sweet, earthy and papery among the ten wine samples. The two samples containing high level of MeSH, MhEm and MhEl, had significantly stronger non-wine aromas, overall stinky, overall intensity and nose burn and less wine-related aromas than MmEl, MlEm, MlEl and base wine. Aroma characteristics of the four samples combining low and medium levels of MeSH and EtSH were not significantly different from base wine. From the results, high level of EtSH can affect the Pinot noir wine aromas more significantly than high level of MeSH. Minimal subject variability was found and the individual GPA results were similar to the consensus configuration (results are not shown).

Aroma interactions between EtSH and MeSH at different concentration levels affected subjects' perception of MeSH and EtSH in the wine. High level of EtSH interfered with subjects' perception of high and medium levels of MeSH in the wine since aroma characteristics of MhEh, MmEh, and MlEh were not significantly different in dimension 1. Subjects indicated that both EtSH (i.e. durian) and MeSH (i.e. flatulence) odors were perceived in MhEh but EtSH odors were more potent, and perceiving MeSH odors was difficult in MmEh and MlEh. Perceiving high level of MeSH may be suppressed by low and medium concentrations of EtSH since aroma characteristics of MhEm and MhEl were not significantly different from MmEm. Odors of MeSH were perceived as more intense than EtSH aromas in MhEm and MhEl. Low and medium levels of EtSH and MeSH may suppress each other in the wine and may not significantly influence the Pinot noir wine aroma since aroma characteristics of MmEm, MlEm, MmEl, and MlEl were not significantly different from base wine in dimension 1.

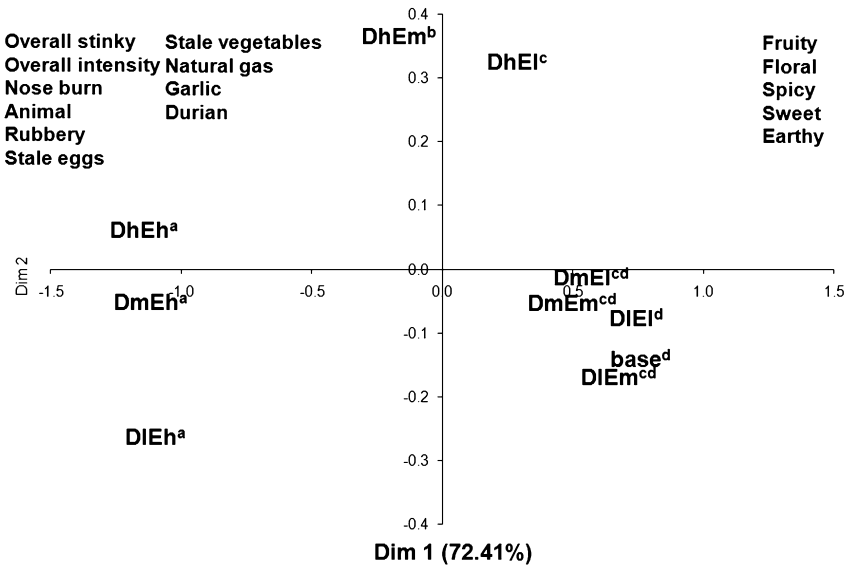

Figure 9. GPA Consensus Configuration Plot for the EtSH and DEDS Mixture Samples and Base Wine. Only Dimension 1 Is Demonstrated. Abbreviations: D=DEDS, E=EtSH, l=low, m= medium, h= high and base= base wine. Samples bearing different superscripts are significantly different at 95% level by 1-way ANOVA and Tukey-HSD multiple comparisons.

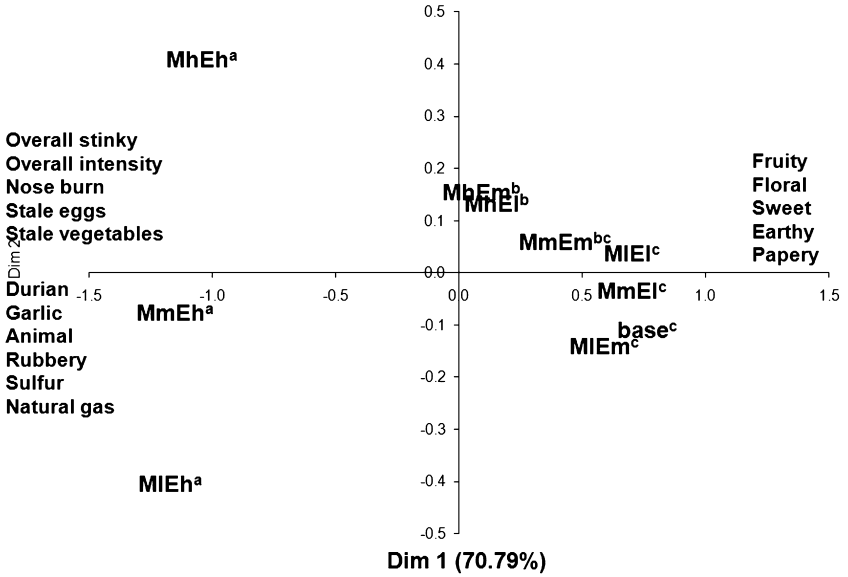

Figure 10. GPA Consensus Configuration Plot for the MeSH and EtSH Mixture Samples and Base Wine. Only Dimension 1 Is Demonstrated. Abbreviations: M=MeSH, E=EtSH, l=low, m= medium, h= high and base= base wine. Samples bearing different superscripts are significantly different at 95% level by 1-way ANOVA and Tukey-HSD multiple comparisons.

MeSH and EtSH Mixtures under the Influence of Sub-Threshold Levels of DMDS and DEDS

The low (sub-threshold) levels of DMDS (0.27 ppb) and DEDS (0.11 ppb) were added into the nine wine samples combining three levels of MeSH (low=1.082, medium=3.251 and high=14.384 ppb) and three levels of EtSH (low=0.021, medium=1.063 and high=7.512 ppb) to examine the impact on the Pinot noir wine aromas and aroma interactions between MeSH and EtSH under the influence of sub-threshold levels of the two disulfides. The consensus configuration from the GPA is shown in Figure 11. A total data variance of 76.08% was extracted from the GPA. Over 75% of the extracted data variance was described by dimension 1 (57.04%), and thus only the GPA results from dimension 1 are summarized. No new aroma descriptors were generated for the samples. The FT descriptors were categorized based on aroma similarity. Cooked vegetables, rotten vegetables, rotten cabbage, old cabbage, cabbage, rubbery cabbage, ginger and green onion were grouped together and called "stale vegetables." Latex, balloon, burnt rubber, rubber stopper, tire and burnt tire were included and named "rubbery." "Stale eggs" covered old egg, rotten egg, and raw rotten egg. "Animal" was created to represent skunky, sweaty, meaty, flatulence and fecal. Burnt match and car smoke were called "sulfur." "Garlic" covered

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garlic and roasted garlic. "Spoiled milk" represented sour milk and rotten milk. The other descriptors which could not be grouped and were used by more than three subjects were included and are shown in Figure 11. The nine mixture samples are labeled by the following abbreviations: DD= DMDS and DEDS, M=MeSH, E=EtSH, l=low, m= medium and h= high. Base wine is noted as "base." Wine samples bearing different superscripts are significantly different at $p < 0.05$ by one-way ANOVA and Tukey-HSD multiple comparisons.

In dimension 1 (Figure 11), subjects were able to differentiate high level of MeSH as well as high level of EtSH under the presence of subthreshold levels of DMDS and DEDS in the experimental Pinot noir wine. High level of MeSH can affect the Pinot noir wine aromas more significantly than high level of EtSH while sub-threshold levels of DMDS and DEDS were present. The three samples with high MeSH level (DDMhEh, DDMhEm, and DDMhEl) were not significantly different for their aroma characteristics in dimension 1. They were rated to have significantly the highest overall intensity, overall stinky, nose burn and the FT aromas (stale vegetables, stale eggs, rubbery, animal, garlic, sulfur, spoiled milk, durian and natural gas) but the lowest typical Pinot noir wine aromas (floral, fruity, vegetative, spicy, sweet, and earthy). MeSH and EtSH odors were perceived in DDMhEh and DDMhEm and subjects indicated that MeSH odors were more potent than EtSH odors. High level of MeSH may interfere with perceiving high and medium levels of EtSH in the wine. DDMmEh and DDMlEh had significantly stronger overall intensity, overall stinky, nose burn and the FT aromas and less wine-related aromas than DDMlEm, DDMmEl, DDMlEl and base wine. Subjects mentioned they perceived EtSH aromas more intensely than MeSH aromas in DDMmEh and DDMlEh. Perceiving high level of EtSH may be disturbed by low and medium concentrations of MeSH since aroma characteristics of DDMmEh and DDMlEh were not significantly different from DDMmEm. Aroma characteristics of the four wine samples with low and medium levels of MeSH and EtSH, DDMmEm, DDMmEl, DDMlEm, and DDMlEl, were not significantly different from base wine in dimension 1. These five samples were rated significantly the highest for wine-related aromas and the lowest for overall intensity, overall stinky, nose burn and the non-wine aromas. Low and medium levels of EtSH and MeSH may suppress each other in the wine and may not significantly influence the Pinot noir wine aroma. Minimal subject variability was observed and the individual GPA results were similar to the consensus GPA results (results are not shown).

With or without the influence of added sub-threshold levels of disulfides in the Pinot noir wine (Figures 10 and 11), subjects can distinguish high levels of MeSH and EtSH. Mutual suppression between MeSH and EtSH at low and medium levels was observed. However, there is one major difference between the outcomes from the two experiments. High level of EtSH was more influential in suppressing experimental wine aromas and increasing overall stinky, overall intensity, nose burn and FT aromas than high level of MeSH when sub-threshold levels of the disulfides were not added (Figure 10). On the contrary, high level of MeSH was more effective upon these aroma changes than high concentration of EtSH when 0.27 ppb DMDS and 0.11 ppb DEDS were present in the wine (Figure 11). In order to examine sub-threshold effects from the two disulfides,

data from the MeSH and EtSH mixtures and those from the MeSH, EtSH, DMDS, and DEDS mixtures were combined and re-analyzed by GPA. The FT terms which did not appear in both experiments within a subject were excluded. These excluded descriptors solely described either DMDS or DEDS, or both. Most of the excluded FT terms (> 80%) cannot be utilized to significantly differentiate wine samples within a subject in the data from the last four-sulfur-compound mixture experiment after performing one-way ANOVA ($p > 0.05$). Therefore, excluding these FT terms had a minimal effect on losing information from the data obtained in the last mixture experiment. Therefore, the two datasets from the last two experiments can be successfully combined. The intensity ratings given to two base wine samples within a subject were averaged in the combined data. Statistical analysis of the combined data followed the same procedures as described in the section of Materials and Methods. The minimum of 0.5 of loading coefficient was chosen as a selection criterion.

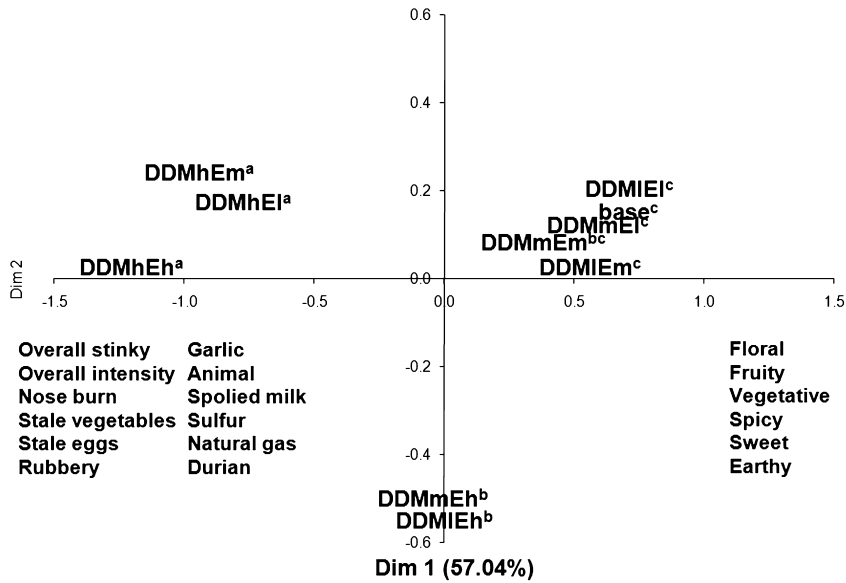

Figure 11. GPA Consensus Configuration Plot for the MeSH and EtSH Mixture Samples with Sub-threshold Levels of DMDS and DEDS and Base Wine. Only Dimension 1 Is Demonstrated. Abbreviations: DD= DMDS and DEDS, M=MeSH, E=EtSH, l=low, m= medium, h= high and base= base wine. Samples bearing different superscripts are significantly different at 95% level by 1-way ANOVA and Tukey-HSD multiple comparisons.

The consensus configuration of the combined data from the GPA shows in Figure 12. Only findings across dimension 1 are summarized because this dimension (59.49%) accounted for 81% of the total extracted data variance (73.14%). The FT descriptors extracted to this dimension were very similar to those which appeared in dimension 1 in the MeSH and EtSH mixture experiment. Therefore, grouping FT descriptors was the same as the one performed in the MeSH and EtSH mixture experiment with the exception that an extra term, "spoiled milk," was added to represent sour milk and rotten milk. The other descriptors which could not be grouped and were employed by more than three subjects were included and are shown in Figure 12. On the right side of dimension 1, aroma characteristics of the eight samples which combined low and medium levels of MeSH and EtSH with or without the two added disulfides were not significantly different from base wine. Although aroma characteristics were not significantly different among these eight samples, intensity of the FT aromas, overall intensity, overall stinky and nose burn was slightly lower, and the Pinot noir wine aromas were slightly higher in the samples containing sub-threshold levels of two disulfides (i.e. DDMlEm) than in those which were at the same MeSH and EtSH combination without adding the two disulfides (i.e. MlEm). Directionally sub-threshold levels of the two disulfides could slightly reduce intensity of non-wine aromas, overall intensity, overall stinky and nose burn in the samples combining low and mid-levels of MeSH and EtSH.

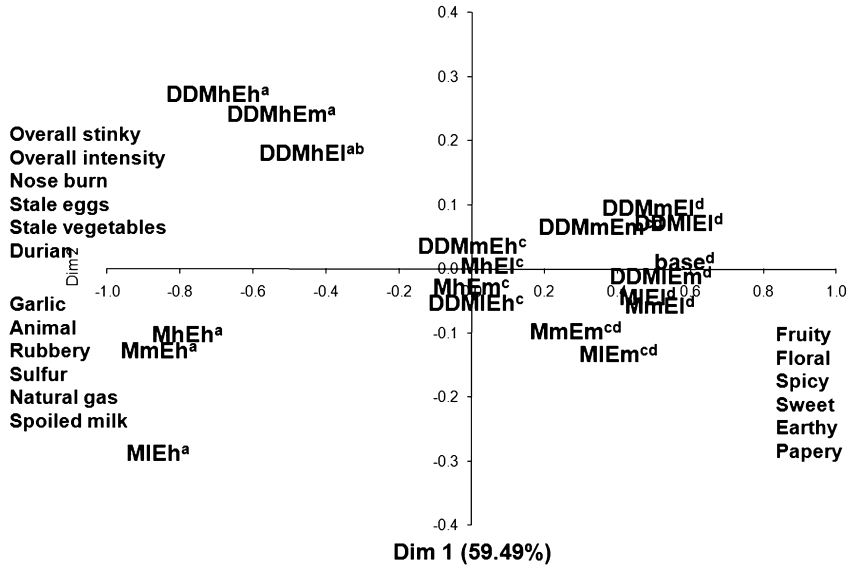

Figure 12. Consensus Configuration Plot for the Combined Data Which the Wine Samples Were Obtained from the Last Two Mixture Experiments. Only Dimension 1 Is Demonstrated. Abbreviations: DD= DMDS and DEDS, M=MeSH, E=EtSH, l=low, m= medium, h= high and base= base wine. Samples bearing different superscripts are significantly different at 95% level by 1-way ANOVA and Tukey-HSD multiple comparisons.

In the middle and on the left side of dimension 1 (Figure 12), although aroma characteristics of DDMhEh and MhEh were not significantly different, directionally sub-threshold levels of the two disulfides could slightly decrease intensity of non-wine aromas, overall intensity, overall stinky and nose burn in the sample containing high levels of MeSH and EtSH. DDMmEh and DDMlEh were rated as having significantly less overall stinky, overall intensity, nose burn and the FT aromas (stale eggs, stale vegetables, durian, garlic, animal, rubbery, sulfur, natural gas and spoiled milk), but more of the Pinot noir wine aromas (fruity, flora, spicy, sweet, earthy, and papery) than MmEh and MlEh. DDMhEm and DDMhEl were significantly more intense for overall stinky, overall intensity, nose burn and the FT aromas, but less intense for the Pinot noir wine aromas than MhEm and MhEl. As a result, the presence of sub-threshold levels of the two disulfides in the Pinot noir wine may strongly diminish the influence of high EtSH level on the wine and non-wine aromas; however, it may reduce the suppression effect of perceiving high level of MeSH by low and medium levels of EtSH in the wine.

Discussion

From the results of the four experiments in Part 1, subjects showed different abilities to discern the wine samples with different levels of sulfur compounds. They showed more ability to separate the wine samples with mercaptans than disulfides. Similarly, Goniak and Noble (*5*) used a trained panel to differentiate three concentration levels of DMS (500, 575 abd 650 ppb) and EtSH (5.00, 5.75 and 6.50 ppb) in white wine, and they found that subjects could discriminate EtSH more easily than DMS. Subjects' discernment ability for individual sulfur compounds in the Pinot noir wine influenced their behavior for evaluating sulfur compound mixtures. Mercaptans dominated perceptions of off-odors in the Pinot noir wine when both MeSH and DMDS or both EtSH and DEDS were present. As a result, MeSH and EtSH can significantly affect the experimental Oregon Pinot noir wine aromas more than disulfides.

Subject variability on sample discrimination was mainly observed in the experiments associated with disulfides. Two reasons could explain subject difference in the DMDS and DEDS evaluations. First, from the previous threshold study (*16*), a wide range of individual detection thresholds among the 13 subjects was found for DMDS (0.29~47.22 ppb) and DEDS (0.14~4.06 ppb) in the experimental Pinot noir wine. Subjects who had lower detection thresholds might behave differently than those who had higher thresholds. For example, Panelist 12 had the lowest (0.3~0.5 ppb) and Panelist 14 had the highest (45~47 ppb) threshold of DMDS among the 13 subjects, and they behaved differently on the DMDS evaluation. However, this may not be always applicable to the subjects. In addition to detection threshold, subejcts' ability of recognizing the volatile sulfur compounds in wine is also an important factor. Some subjects indicated that recognizing DMDS in the experimental wine at low concentration levels was difficult. The aroma of DMDS in wine was mainly associated with cabbage. The experimental Pinot noir wine itself also had a vegetative aroma which was

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related to cabbage and bell pepper (Table II). The similarity between aromas of low levels of DMDS and vegetable aromas in the Pinot noir wine may result in recognition difficulties. Aroma characteristics of MeSH (i.e. flatulence and slop) and EtSH (i.e. durian, fecal and natural gas) were more foreign in the Pinot noir wine. Therefore, recognizing their presence in wine was relatively easier.

The GPA results revealed that the experimental Pinot noir wine gradually lost its aroma characteristics, mainly including fruity and floral, and increased overall intensity, overall stinky, nose burn and non-wine aroma components while concentrations of the four volatile sulfur compounds increased. These echoed the findings from the previous threshold study, in which changes of the Pinot noir wine aromas (loss of overall intensity, fruity and floral and increasing pungency) were observed when the concentrations of the four volatile sulfur compounds reached the peri-threshold levels (*16*). As a result, a hypothesis of aroma change in the Pinot noir wine could be summarized as follows. Overall intensity, fruity and floral perceived in the experimental Pinot noir wine are gradually reduced, and pungency/nose burn increases while concentration of a volatile sulfur compound is increasing from zero (or the lowest possible) to the detection threshold. There are no non-wine aromas perceived at this time. After the concentration of a sulfur compound is above its detection threshold and keeps increasing, fruity and floral continue declining. Nose burn/pungency continues increasing, and overall intensity starts to increase. Non-wine aromas appear in the wine and then are intensified. Therefore, the perceptual aroma change of fruity, floral and nose burn/pungency can be utilized as an index to diagnose early presence of sulfur compounds before these compounds result in significant aroma damage to Oregon Pinot noir wine during winemaking, aging and storage.

Results from the four experiments for sulfur compound mixtures revealed that odor intensity suppression was observed. Suppression is the most common phenomenon in odor mixtures (*37*). Low levels of sulfur compounds were found to suppress medium levels (sometimes high levels) of their counterparts in the first three experiments in Part 2. Sub-threshold levels of the two disulfides were also found directionally to have a suppression effect on most MeSH and EtSH mixtures with the exception of DDMhEm, MhEm, DDMhEl, and MhEl (Figure 12). These phenomena were similar to the results found by Laing (*38*). This researcher reported that lower intensity levels of odorants tended to suppress odorants with higher intensity in binary mixtures. Overall, odor suppression was found in sulfur compound mixtures in the present study. The same level (medium or high level) of a sulfur compound in a single component wine system can impact the experimental wine aromas more than in a binary system with the presence of another compound at low or medium levels. Therefore, odor suppression occurring in a more complex wine medium may lower the risk of aroma defects caused by medium or even high levels of volatile sulfur compounds.

The Part 2 results revealed that medium concentrations of disulfides may not result in aroma defects while sub-threshold levels of mercaptans are found in the Pinot noir wine. However, the inter-conversion between mercaptans and disulfides during storage could significantly impact Pinot noir wine aromas under these circumstances. If a Pinot noir wine contains about 4 to 5 ppb DEDS, and a sub-threshold level of EtSH, it might not be perceived as an aroma-defected wine

in the beginning. During storage, DEDS can be converted to supra-threshold levels of EtSH, because EtSH can be detected at 0.3 ppb and recognized at 2.5 ppb or higher in Pinot noir wine. As a result, the wine aroma becomes off. No research has demonstrated the inter-conversion between MeSH and DMDS in wine during storage. If this phenomenon existed, a Pinot noir wine containing 37~68 ppb of DMDS, and a sub-threshold level of MeSH (i.e. 1 ppb) might not have off-aromas initially, but wine aroma could become stinky later during storage.

Most mixture samples from the current study imitate the practical sulfur contents of the 39 aroma-defected commercial Oregon Pinot noir wines (*26*). These samples are DlMl, DlMm, and DlMh from the DMDS and MeSH mixture experiment, DlEl, DlEm, and DlEh from the DEDS and EtSH mixture experiment, and all samples from the last two experiments. The unpublished results demonstrated that the off-aroma descripotions for these 39 wines were sulfurous, mercaptan-like, rotten cabbage, rotten egg, garbage, burnt match and rubber. The concentration of MeSH and EtSH in the 39 wines ranged from 3.31 to 17.90 ppb, and from not detected to 4.96 ppb respectively (*26*). The current study has demonstrated that aroma characteristics of DlMl, DlMm, DlEl, and DlEm were close to the experimental wine as well as the wine samples combining low and medium levels of MeSH and EtSH (MlEl, MlEm, MmEl, and MmEm). Therefore, an assumption is created from results of the current study: Oregon Pinot noir wines may be starting to become aroma-defected when the level of MeSH is more than 3.3 ppb and/or EtSH is more than 1.1 ppb. They become definitely aroma-defected when MeSH levels reaches 14.4 ppb and/or EtSH level reaches 7.5 ppb. This assumption not only matches the unpublished results but also provides a complete sensory description and supports aroma character explanations and predictions for the sulfur-defected Oregon Pinot noir wines.

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Chapter 16

Antimicrobial Activity of Volatile Sulfur Compounds in Foods

Kyu Hang Kyung*

Department of Food Science, Sejong University, Seoul 143-747, Korea
***E-mail: kyungkh@sejong.ac.kr.**

Common foods which show appreciable antimicrobial activities through volatile sulfur compounds are mostly vegetables belonging to *Allium* and *Brassica*. *Allium* contains *S*-alk(en)yl-L-cysteine sulfoxides (sulfoxides), represented by alliin in garlic, as the precursors of antimicrobial alk(en)yl alk(a/e)nethiosulfinates (thiosulfinates), represented by allicin in garlic (*Allium sativum* L.). Sulfoxides are hydrolyzed to antimicrobial thiosulfinates as the tissue of fresh *Allium* is disturbed. *Brassica* contains alk(en)yl glucosinolates (glucosinolates), represented by sinigrin in cabbage (*Brassica oleracea*), as the precursors of antimicrobial alk(en)yl isothiocyanates (isothiocyanates), represented by allyl isothiosulfinate in cabbage. *Brassica* vegetables contain *S*-methyl-L-cysteine sulfoxide which is activated to methyl methanethiosulfinate in the same way as are sulfoxides in *Allium*. Thiosulfinates formed in *Allium* are degraded to various polysulfides and ajoenes which also exhibit different degrees of antimicrobial activity. Horseradish and mustard contain large amounts of glucosinolates to inhibit microorganisms, while other common brassicas do not contain them in such large amounts. Thiosulfinates, isothiocyanates and transformation products of thiosulfinates all inhibit microorganisms by reacting with sulfhydryl groups of cellular protein(s) of microorganisms to disturb cellular metabolism. The volatile sulfur compounds show more potent inhibitory effects towards fungi than bacteria.

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Plants synthesize various natural antimicrobial substances, most of which are phenols and their oxygen-substituted derivatives and most of which are secondary metabolites. They include phenolics, polyphenols, terpenoids, essential oils, alkaloids, lectins and polypeptides (*1*). In many cases these substances serve as defense mechanisms against predators including microorganisms, insects and other herbivores. These substances appear in nature as they are and most of them are not volatile and do not contain sulfur in their molecules.

A great number of volatile sulfur compounds appear in heated foods including meats, bread, popcorn, roasted coffee and cooked milk as well as fruits and dairy products, and vegetables such as cruciferous vegetables and alliums (*2*). The volatile sulfur compounds in heated foods are not natural constituents of food plants. They are formed through thermal degradation of non-volatile sulfur compounds including sulfur-containing amino acids, peptides or proteins. Most of the volatile sulfur compounds are present in foods in extremely minute quantities and are valuable in those foods as characteristic flavor contributors, but not as antimicrobial substances.

The two most interested groups of antimicrobial volatile sulfur compounds found in common foods are isothiocyanates derived from glucosinolates in *Brassica* and thiosulfinates derived from sulfoxides in both *Brassica* and *Allium,* respectively. Isothiocyanates and thiosulfinates do not appear in plants as they are, instead formed upon enzymatic activation from otherwise innocuous substrates, glucosinolates and sulfoxides, respectively. Sulfur compounds appearing in foods in minute quantities and exhibiting antimicrobial activity only at exaggerated concentrations are not addressed in this chapter. Those volatile sulfur compounds which exert appreciable antimicrobial activity in common food items such as thiosulfinates in *Allium* and isothiocyanates in *Brassica* are dealt with.

Antimicrobial Activity of Volatile Sulfur Compounds in *Allium*

Thiosulfinates Represented by Allicin

Alk(en)yl alk(a/e)nethiosulfinates (thiosulfinates) are produced in *Allium* by enzymatic conversion of corresponding *S*-alk(en)yl-L-cysteine sulfoxides (sulfoxides) by alliinase (L-cysteine sulfoxide lyase, EC 4.4.1.4) to form ammonia, pyruvate and an alk(en)ylsulfenic (sulfenic) acid. Two sulfenic acids combine to form a thiosulfinate and water (Figure I). Alliinase acts on all three sulfoxides with different R groups, being methyl-, allyl-, and 1-propenyl-, in garlic, and methyl-, and 1-propenyl-, in onion. Therefore, eight different kinds of thiosulfinates will be formed in garlic by the combination of three different sulfenic acids. They are methyl methanethiosulfinate, allyl methanethiosulfinate, methyl-2-propenethiosulfinate, *trans*-1-propenyl methanethiosulfinate, methyl *trans*-1-propenethiosulfinate, allyl 2-propenethiosulfinate (allicin), allyl *trans*-1-propenethiosulfinate, and *trans*-1-propenyl 2-propenethiosulfinate. By analogy, four different thiosulfinates can be expected in onion.

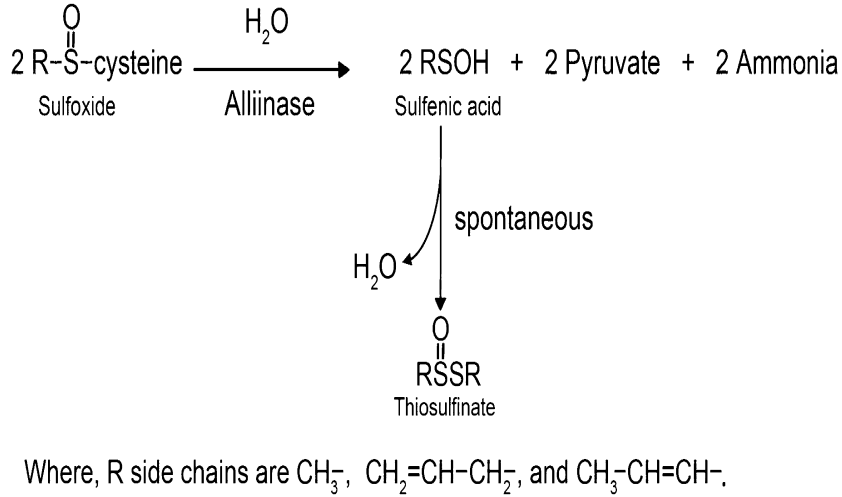

Figure I. Enzymatic cleavage of a S-alk(en)yl-L-cysteine sulfoxide by alliinase to an alk(en)yl alk(a/e)nethiosulfinate.

Some are unmixed thiosulfinates [RS(O)SR] such as methyl methanethiosulfinate solely from *S*-methyl-L-cysteine sulfoxide (methiin), allyl 2-propenethiosulfinate (allicin) solely from *S*-allyl-L-cysteine sulfoxide (alliin), and *trans*-1-propenyl 1-propenethiosulfinate (isoallicin) solely from *S-trans*-1-propenyl-L-cysteine sulfoxide (isoalliin). There are five mixed thiosulfinates [R′S(O)SR] formed by combinations of three sulfenic acids. Allicin exists in the most amounts (70%), thus most of the antimicrobial activity of garlic should be attributed to allicin. Other minor thiosulfinates in quantity should also contribute appreciable antimicrobial activity to garlic.

Thiosulfinates are highly reactive oxidants selectively toward thiols (*3*). The reaction of thiosulfinate [RS(O)SR] with a thiol (R-SH) results in a mixed disulfide (RSSR′) and a sulfenic acid (R-SOH). The latter reacts with a second thiol equivalent (R′SH) to form a mixed disulfide (RSSR′) and water. Depending on the relative redox potentials and concentrations of RSSR′ and RSH, the reaction may further progress. As an example, one allicin molecule can oxidize four reduced glutathione (GSH) molecules to generate two GSSG and two allylmercaptan molecules. Microorganisms seem to be readily inhibited by thiosulfinates such as allicin, while mammalian cells are protected against thiosulfinates by GSH in their body fluid.

Allicin exhibits potent antimicrobial activities, and the minimum inhibitory concentrations (MICs) for *Escherichia coli, Staphylococcus aureus, Streptococcus pyogenes,* and *Pseudomonas* sp., were 15 μg/mL or lower (*4*). Even lower MIC values have been obtained for fungi such as *Candida albicans*, *C. neoformans*, *C. tropicalis*, *Torulopsis glabrata*, *Cryptococcus neoformans* and *Aspergillus fumigatus*. Different thiosulfinates have different degree of inhibition, and allicin (allyl-S-S(O)-allyl) was twice as potent as the allyl methyl thiosulfinates (allyl-S-S(O)-methyl) (*5*). The antimicrobial activity of aqueous extract of fresh

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garlic was completely eliminated when thiosulfinates were removed with an organic solvent.

Cavallito and Bailey (*6*) isolated an antimicrobial active compound from crushed garlic and named it allicin. The naming of thiosulfinates is not simple because common names and systemic names coexist. The IUPAC name of allicin is 2-propenyl 1-propenethiosulfinate. Diallyl thiosulfinate is another common name for allicin. IUPAC name for thiosulfinates will be in the text throughout except for allicin, because it is commonly used in scientific literature. When we mention allicin in garlic, allicin represents the whole thiosulfinates formed in garlic, where allicin actually occupies the most (70%) of total thiosulfinates in garlic homogenate. Allicin is unstable, somewhat soluble in water (2.5% at 10℃) and unstable in alkaline condition (*6*). Other thiosulfinates are unstable also. Autoclaving garlic extract abolishes antimicrobial activity (*7*). Antimicrobial activity was lost gradually when garlic extract was kept for several days at room temperature. Antimicrobial activity of garlic is also lost at refrigerated temperature, MIC decreasing from 1:128 to 1:16 in a two week period (*8*). Garlic antimicrobial activity is not totally stable even at -60℃.

The antimicrobial effectiveness of garlic extract against *E. coli* B34 remained stable for 3 days when both the pH and the storage temperature were kept below 6.0 and 20℃, respectively (*9*). When garlic extract was stored at 40℃ and above, most or all of garlic antimicrobial activity disappeared within 24 hr regardless of pH.

The antimicrobial activity of garlic is due to allicin and other minor thiosulfinates. The -S(O)S- (thiosulfinate) structure seems to play an important role, because upon reduction of allicin to diallyl disulfide, the antimicrobial activity is greatly reduced. Inhibition of certain SH-containing enzymes in the microbial cells by the reaction of thiosulfinates is regarded as the action mechanism of the antimicrobial activity of allicin (*6*, *7*, *10*). Antimicrobial activity of aqueous garlic extract was drastically lowered by the addition of cysteine or glutathione. Allicin was reported to inhibit various SH enzymes important in cellular metabolisms.

S-Alk(en)yl-L-cysteine Sulfoxides: The Precursors for Thiosulfinates

The precursor compounds including alliin and the other sulfoxides have been shown to have no antimicrobial activity (*5*, *11*, *12*). Alliin is a common name of 2-propenyl-L-cysteine sulfoxide. Stoll and Seebeck (*13*) isolated a crystalline form of the precursor of allicin. IUPAC names for the sulfoxides will also be used throughout the text except allicin in most of the cases. Sulfoxides are non-protein sulfur-containing amino acids normally found in *Allium* vegetables, including garlic (*Allium sativum* L.), onion (*Allium cepa*), elephant garlic, leek, scallion, shallot, chive, Chinese chive, wild onion, wild garlic. Methyl, 1-propenyl and 2-propenyl derivatives of L-cysteine sulfoxide are found in garlic, elephant garlic, wild garlic while *S*-2-propenyl derivative (alliin) is missing in onion, leek, scallion, shallot (*14*). Among various *Allium* vegetables most research interest has been focused on garlic. The general structure of sulfoxides is shown in Figure I. Typically three sulfoxides differing in R-side group have been commonly

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reported. Three R-groups, methyl, 1-propenyl and 2-propenyl, are found in garlic, while methyl and 1-propenyl groups are found in onion. Still another R-group, propyl, has been reported in onion (*15*), leek, scallion, shallot in very small quantities (*14*). The sulfoxide with the allyl group (alliin) is most abundant (85%) in garlic, followed by 1-propenyl (isoalliin; 5%) and methyl group (10%) (*16*). The sulfoxide with 1-propenyl group (85%) is abundant in onion, followed by the methyl (15%) group (*17*).

A small amount of cycloalliin is present in fresh garlic, which, however, is excluded because it is not transformed into antimicrobial thiosulfinate. The alliin contents in garlic show significant variation, ranging from less than 1 to 14 mg/g fresh weight basis. Typical garlic contains alliin in the range between 5 and 14 mg/g. There are great variations in the amounts of individual sulfoxides as well as the total contents of sulfoxides in garlic and onion (*17*, *18*). The total contents of sulfoxides in onion were 0.59-1.55mg/g fresh weight basis, which is about one-tenth those of garlic. From our experience, methyl- and 1-propenyl- derivatives of *S*-alk(en)yl-L-cysteine sulfoxides in garlic are in approximately equal quantities with a little more of methyl derivative.

Antimicrobial Activity of Fresh Garlic and Onion

Fresh garlic shows a broad antimicrobial spectrum against bacteria, yeasts and molds (*7*, *19*, *20*). The antimicrobial activity of fresh garlic extract has been recognized for many years. It has been reported that 1-2% garlic extract showed inhibitory effect toward microbial growth, and higher concentrations are germicidal. Garlic has been shown to be more inhibitory against fungi than bacteria. Garlic inhibited aflatoxin production by molds. Much interest was placed on the anti-*Helicobacter* activity of garlic. Garlic inhibited *Helicobacter pylori in vitro*, but not *in vivo* (*21*). Alliinase enzyme inhibition by low pH of the stomach and possible complex formation of allicin with proteins (and free amino acids) in foods may play a role in abolishing garlic effect *in vivo*. Walker and Stahmann (*22*) pointed out that onion had antifungal activity. Garlic extract shows greater antimicrobial activity as compared to onion extract. In addition to the fact that garlic contains total sulfoxides about ten times that of onion, much of sulfoxides in onion is used to make lachrymatory factor, propanethial *S*-oxide ($C_2H_5CH{=}SO$), when the tissue is injured. Homogenized garlic has an antimicrobial activity 10 to 20 times higher than onion (*15*). Onion, as a homogenate in a 10 to 15-fold dilution, inhibited the growth of *Staphylococcus* for 24 hr. The lachrymatory factor has not been reported to be antimicrobial, it sure is useful as an additional defense mechanism of onion just like allicin in garlic, because it irritates onion-eating creatures.

Antimicrobial Activity of Heated Garlic and Onion

Antimicrobial activity has been known to be generated only when fresh garlic is injured to make alliinase enzyme contact its substrate alliin. Therefore prolonged heating at high temperatures causes a loss of antimicrobial activity of garlic and onion (*16*) because alliinase enzyme is inactivated by heating. It has

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been recently shown that garlic heated at 121℃ showed a potent antimicrobial activity against yeasts (*23*). Diallyl trisulfide formed by thermal degradation of alliin was thought to be the causative agent of antimicrobial activity. Follow-up studies revealed that the actual principal antimicrobial compound of autoclaved garlic was allyl alcohol generated from alliin by thermal degradation (*24*). Allyl alcohol was more potent against yeasts than against bacteria. Diallyl sulfides including diallyl trisulfide were believed to be the secondary inhibitory compounds and inhibit microorganisms synergistically with allyl alcohol (*25*). Allyl alcohol is devoid of sulfur atom in its molecule. This is the only example of volatile antimicrobial compound derived from garlic, but without sulfur in its structure. The extraordinarily potent anti-yeast activity of allyl alcohol was explained by the fact that the unsaturated alcohol is oxidized to corresponding aldehyde by cellular alcohol dehydrogenase in yeasts.

Lowly volatile heterocyclic sulfides also contributed a significant part of antimicrobial activity of heated garlic. The sulfides identified by HPLC, GC/MS, and NMR were heterocyclic compounds with 3 to 5 sulfur atoms in their molecules. They were 4-methyl-1,2,3-trithiolane, 5-methyl-1,2,3,4-tetrathiane and 6-methyl-1,2,3,4,5-pentathiepane, with 3, 4, and 5 sulfur atoms, respectively (Figure II; (*26*)). More heterocyclic polysulfides were formed as the pH was lowered to 2. Antimicrobial activities of 5-methyl-1,2,3,4-tetrathiane and 6-methyl-1,2,3,4,5-pentathiepane were more potent compared with diallyl trisulfide. Antimicrobial activity was inactivated by cysteine as with other sulfur compounds derived from *Allium* and *Brassica*. Heterocyclic sulfur compounds were more inhibitory against yeasts than against bacteria. Cooked onions may have a certain antimicrobial activity, even though alliinase enzyme has been inactivated during cooking, because dialk(en)yl sulfides with antimicrobial activity are formed (*15*).

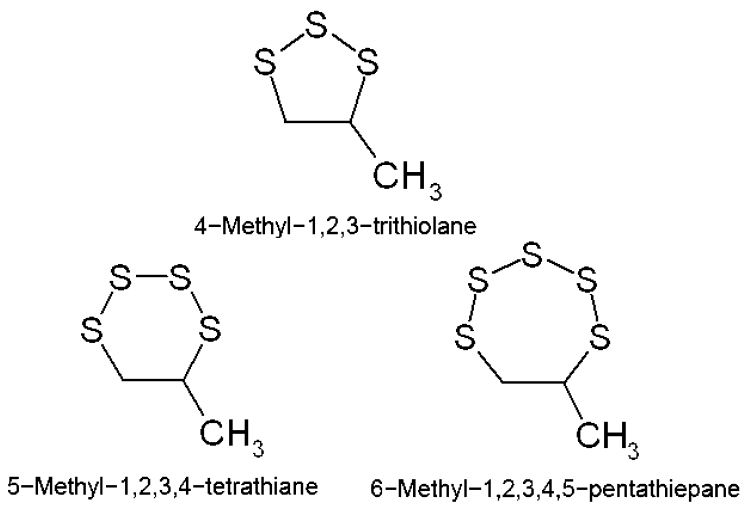

Figure II. Heterocyclic sulfur compounds formed in heated garlic (26).

Antimicrobial Activity of Garlic Oil and Ajoene

Although allicin is the most active antimicrobial compound, diallyl sulfides are also inhibitory to microbial growth. Garlic oil is produced by boiling crushed garlic and collecting the vapor as a distillate. During the heating process, allicin is converted to various diallyl sulfides. The composition of garlic oil differs due to variation in raw material, preparation and process (Table I). Garlic oil is composed of dialk(en)yl sulfides of various possible combinations of allyl, methyl and propenyl groups with a different number of sulfur atoms from one through six (*27*). The most abundant sulfide in garlic oil is diallyl disulfide (30.6-53.0%), followed by diallyl trisulfide (11.5-30.1%) (*27–29*).

Since, when allicin was discovered and its potent antimicrobial activity was known in 1944 (*6*), the authors mentioned that diallyl sulfides and diallyl polysulfides did not have antimicrobial activity, the antimicrobial activity study was very much delayed. Bacteria in general are much less sensitive to garlic oil and its individual sulfides, while yeasts are highly sensitive to both garlic oil and its sulfides (Table II; (*28*, *30*)). The antimicrobial activity of garlic oil is significant against *S. aureus* and yeasts (*28*). Garlic oil showed about four times as great as expected from the sum of the activities of its component sulfides, indicating synergistic activity among different sulfides (*16*). It was demonstrated that the addition of 1.5 mg garlic oil per gram of sausage meat prevented the formation of *Clostridium bolulinum* toxin. Even very small amounts of garlic oil have been found to inhibit food spoilage yeasts as well as industrial yeasts (*28*).

Diallyl monosulfide exhibited MIC of about 1000 ppm, while diallyl trisulfide and diallyl tetrasulfide showed MICs ranging from 2 to 25 ppm (*28*) against many yeasts. Garlic oil was effective in suppressing the growth of *C. utilis* at 25 ppm for 16 days at 37℃. *S. aureus* is the only bacterium which is sensitive to the antimicrobial activity of individual sulfides and garlic oil among the test bacteria, with MIC being 100 ppm.

Ajoene is another antimicrobial transformation product of allicin. Three molecules of allicin combine to make two molecules of ajoene. There is a small amount of ajoene present only in oil-macerated commercial garlic products (*27*), but not in other products such as garlic oil (*31*). Ajoene is lowly volatile and exists in *trans* and *cis* forms, with *cis* being the more potent of the two. Ajoene is the most active one among allicin transformation products and is active in about half of allicin against *Staphylococcus aureus*. Yoshida et al. (*32*) observed that ajoene had a strong inhibitory activity toward *Aspergillus niger* and *C. albicans*.

They found that ajoene was even more potent in inhibiting fungal growth than allicin and that ajoene showed little antibacterial activity. Whereas Naganawa et al. (*33*) who investigated antimicrobial activity of various sulfur compounds derived from garlic found that ajoene exhibited a broad-spectrum antimicrobial activity. The growths of Gram positive and negative bacteria as well as fungal species were inhibited by ajoene. Ajoene seemed to inhibit microorganisms by reacting with sulfhydryl groups of cellular protein, since cysteine abolished the antimicrobial activity of ajoene as is the case with allicin.

Measurable antibacterial activity of dehydrated onion powder was observed at 1% and maximal death rate was obtained with 5% against *Salmonella typhimurium*

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and *Escherichia coli* (*34*). Since onion contains much less sulfoxides than garlic, it has weaker antimicrobial activity than garlic (*5*).

Table I. Composition of sulfides in garlic oil. Adapted with permission from reference (*28*)

Compound (%)	*References*		
	(28)	*(27)*	*(29)*
Diallyl monosulfide	2.82	2.0	10.6
Diallyl disulfide	30.58	25.9	53.0
Diallyl trisulfide	30.11	18.5	11.5
Diallyl tetrasulfide	14.05	8.1	4.3
Diallyl pentasulfide	3.64	2.1	1.1
Diallyl hexasulfide	1.39	0.4	0.01
Methyl allyl monosulfide	0.36	0.9	ND
Methyl allyl disulfide	2.60	12.5	4.4
Methyl allyl trisulfide	4.67	15.2	7.0
Methyl allyl tetrasulfide	2.05	6.0	2.5
Methyl allyl pentasulfide	0.87	1.7	0.6
Methyl allyl hexasulfide	1.82	0.3	0.2
Dimethyl monosulfide	ND	ND	ND
Dimethyl disulfide	0.71	1.3	ND
Dimethyl trisulfide	0.15	3.4	1.2
Dimethyl tetrasulfide	2.95	1.3	0.2
Dimethyl pentasulfide	0.47	0.4	0.2
Dimethyl hexasulfide	ND	0.1	ND

ND, not detected.

Table II. Minimum inhibitory concentrations (MIC) of diallyl disulfide (DADS), diallyl trisulfide (DATS), diallyl tetrasulfide (DATTS), dimethyl trisulfide (DMTS), and allyl isothiocyanate (AITC) found in *Allium* and *Brassica* against various bacteria and yeasts. Adapted with permission from reference (*28*)

Microorganism	*MIC (ppm)*					
	DADS	*DATS*	*DATTS*	*DMDS*	*DMTS*	*AITC*
Staphylococcus aureus B33	1000	100	ND	>1000	500	150
Escherichia coli B34	>1000	>1000	ND	>1000	>1000	100
Enterobacter aerogenes B146	>1000	>1000	ND	>1000	>1000	200
Leuconostoc mesenteroides LA10	>1000	500	ND	>1000	400	400
Pediococcus pentosaceus LA3	>1000	500	ND	>1000	500	200
Lactobacillus plantarum LA97	>1000	500	ND	>1000	300	200
Candida albicans KCTC 7121	100	8	6	800	18	3
Candida albicans KCTC 7965	120	10	8	900	20	4
Candida utilis ATCC 42416	110	7	4	700	15	4
Saccharomyces cerevisiae ATCC 4216	100	5	2	>1000	10	2
Pichia membrane-faciens Y20	80	3	2	900	5	1
Zygosaccharomyces bisporus CCM50168	80	6	5	700	10	2
Zygosaccharomyces rouxii KCCM11300	100	7	10	>1000	15	2
Zygosaccharomyces rouxii KCCM50523	140	20	25	>1000	20	5

ND, not determined.

Antimicrobial Activity of Volatile Sulfur Compounds in *Brassica*

Glucosinolate Hydrolysis Products: Isothiocyanates

Isothiocyanates (ITCs) are generated from glucosinolates by the action of myrosinase (thioglucoside glucohydrolase EC 3.2.3.1), when plant tissues are injured (Figure III). Among various ITCs, allyl ITC is most widespread in plants and present in variable amounts in *Brassica* and contributes the characteristic flavor to cabbage and other cole crops and some other vegetables including rutabaga, wasabi and turnip. Glucosinolates are β-thioglucoside *N*-hydroxysulfates with an alk(en)yl side chain and a sulfur-linked β-D-glucopyranose (*35*). Chemical diversity and distribution of glucosinolates among plants were thoroughly reviewed by Fahey et al. (*35*), who listed 120 glucosinolates appearing in numerous higher plants. Table III shows some of the glucosinolates appearing in common food plants.

Since sinigrin is most widespread and abundant in cole crops and in other vegetables including horseradish and wasabi, more part of ITC will be focused on sinigrin and allyl ITC. Cabbage contains only up to 150 ppm of sinigrin. Sinigrin is not inhibitory to the growth of microorganisms up to 1000 ppm in broth (*36*, *37*). This means that sinigrin itself is not antimicrobial and that microorganisms do not hydrolyze it into allyl ITC. Allyl ITC is known to be antimicrobial against Gram positive, Gram negative, pathogenic bacteria, lactic acid bacteria, and fungi. It has been reported that ITCs inhibit microorganisms by reacting with the sulfhydryl group of proteins, which adversely affects cellular metabolism. Tang (*38*) proposed a reaction between papain and benzyl ITC: Papain-SH + benzyl NCS --papain S-C(S)-NH-benzyl.

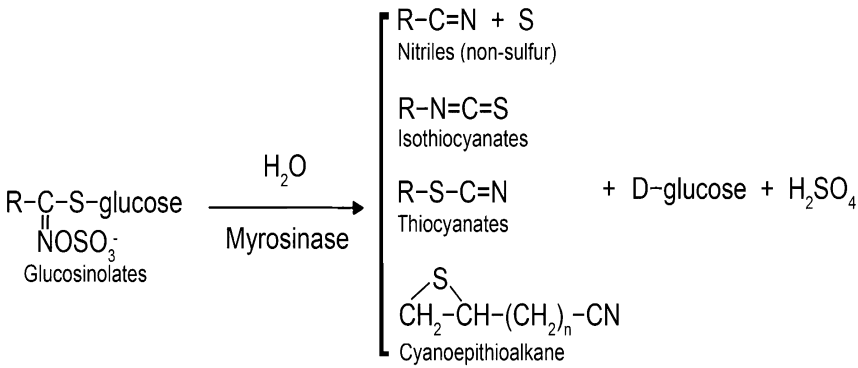

Figure III. Hydrolysis of a glucosinolate by myrosinase.

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Table III. Volatile isothiocyanate-generating glucosinolates in common food plants

Common name of glucosinolates	*Side chain*	*Plants*
Glucobrassicanapin	4-pentenyl	Cabbage, horseradish, mustard, rutabaga, wasabi
Glucobrassicin	Indol-3-ylmethyl	Broccoli, cabbage, cress, mustard
Glucocapparin	Methyl	Brussels sprouts, cabbage, horseradish, wasabi
Gluconapin	3-Butenyl	Mustard, turnip, wasabi, watercress
Gluconasturtiin	2-Phenylethyl	Horseradish, rapeseed, turnip, watercress
Glucoraphanin	4-(Methylsulfinyl) butyl	Broccoli, Brussels sprouts, cabbage, cauliflower, collards, kohlrabi, mustard, radish, turnip
Glucotropaeolin	Benzyl	Cress, horseradish, radish,
Progoitrin	2(*R*)-2-Hydroxy-3-butenyl	Brussels sprouts, cabbage, kale, rapeseed,rutabaga
Sinigrin	Allyl	Brussels sprouts, cabbage, cauliflower, horseradish, kale, mustard, turnip, wasabi
−	Phenyl	Horseradish, mustard, spinach

−, No common name available.

Glucosinolate hydrolysis products include ITCs, nitriles, and thiocyanates (Figure III), ITCs and thiocyanates, but not nitriles, contain sulfur and most of them are volatile (*39*). Sinigrin yields allyl cyanide, 1-cyano-epithiopropane and allyl cyanate in addition to allyl ITC (*37*, *40*) on hydrolysis. The formation of cyanoepithioalkane is unique only to the hydrolysis of glucosinolates which possess an unsaturated bond at the terminal of alkenyl group such as singrin and progoitrin. In this case the presence of epithiospecifier protein and ferrous ions is essential (*41*).

The kinds of sinigrin hydrolysis products depend on processing conditions. Allyl ITC is usually produced at neutral pH, and AC at pH 4 (*40*). 1-Cyano-epithiopropane is formed by the combined action of ferrous ion and epithiospecifier protein on myrosinase (*42*). Allyl ITC which has been reported to be the important aroma compound of freshly cut cabbage was not detected from cabbage juice (*43*). Instead 1-cyano-epithiopropane, one of the isomers of allyl ITC, appeared as the principal sinigrin hydrolysis product. 1-Cyano-epithiopropane was not inhibitory to the growth of bacteria and yeasts at concentrations of up to 1000 ppm (*37*), whereas the MICs of allyl ITC were 100-1000 ppm for bacteria and 4 ppm for yeasts, respectively. According to Shofran, et al. (*37*) allyl ITC was inhibitory to all the test microorganisms while allyl thiocyanate was inhibitory against only part of the test organisms.

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1-Cyano-epithiopropane and allyl cyanate were not inhibitory to any of test microorganisms at the concentration of 1000 ppm.

Allyl ITC exhibited greater antimicrobial activity at low pHs. ITCs are, in some cases, unstable and decompose rapidly to form a variety of other compounds (*44*) and lose their antimicrobial activity. Allyl ITC is decomposed quickly in water at 37℃, generating degradation products all without antimicrobial activity. Diallylthiourea was the largest component representing approximately 80% (*45*) of allyl ITC degradation products. The antimicrobial activity of horseradish vapors was greater than that of garlic, but was more quickly exhausted than that of garlic, suggesting that allyl ITC may be less stable than allicin. The effect of mustard on *S. aureus* and *E. coli* was bacteriostatic at 0.8% while that on *P. aeruginosa* was bactericidal at 0.2% when tested in nutrient broth (*46*).

There are numerous reports concerning the antimicrobial activity of allyl ITC (*15*, *47–50*), while there are not as many reports on antimicrobial activity of other glucosinolate hydrolysis products. Generally bacteria are less sensitive to ITCs than fungi and sensitivity to individual ITC is strain-specific. Gram-positive bacteria were more resistant than Gram-negatives toward ITCs. Allyl ITC and methyl ITC had no effect against some of the bacteria at concentrations that severely inhibited yeasts and molds. Aromatic ITCs were most effective against molds in the order of beta-phenylethyl ITC, benzyl ITC, and methylthio-3-butenyl ITC. Although allyl ITC was much less inhibitory than β-phenylethyl ITC, allyl ITC was the most active compound among aliphatic ITCs, followed by methyl ITC. Phenethyl ITC occurring in white mustard (*Sinapsis alba* L.) strongly inhibited the growth of *Clostridium difficile* and *C. perfringens*, *E. coli*, but did not inhibit bifidobacteria and lactobacilli (*51*). It was reported that aromatic ITCs demonstrated greater inhibitory activity against clostridia and *E. coli* than aliphatic ITCs. Purified sulforaphane [(4*R*)-(methylsulfinyl) butyl ITC], a volatile ITC formed from glucoraphanin in broccoli sprouts has shown *in vitro* antibacterial activity against *H. pylori* (*52*). Glucoraphanin is found in Brussels sprouts, cauliflower, cabbage, kohlrabi, mustard, turnip, radish and watercress.

Although there have been attempts to use allyl ITC and other allyl ITC-yielding plants (e.g., mustard, horseradish, wasabi etc.) as alternative food preservatives (*53*), few were practically applied. The inhibition efficacy of allyl ITC and wasabi (*Wasabia japonica*) against *Vibrio parahaemolyticus* was better in fatty tuna than in lean tuna. *E. coli* O157:H7 numbers were reduced in fermented sausages containing 500 ppm or more allyl ITC, and the organism did not recover beyond 40 days (*54*).

Methyl Methanethiosulfinate Generated from *S*-Methyl-L-cysteine Sulfoxide

The presence of antimicrobial activity in cabbage has been initially demonstrated by Sherman and Hodge (*55*) who found that the activity was heat-labile. Pederson and Fisher (*56*) who extensively studied the antibacterial activity of cabbage reported that bacterial growth inhibition was eradicated when inhibitory cabbage was steamed for 10 min. before juice extraction.

Since allyl ITC found in *Brassica* and other plants inhibited the growth of microorganisms (*22*, *57*), it has been postulated that allyl ITC of cabbage

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was responsible for antimicrobial activity. However, steamed cabbage juice (noninhibitory) supplemented with various glucosinolate hydrolysis products did not inhibit *Leuconostoc mesenteroides* at the concentrations much higher than concentrations reported in cabbage (*12*). Myrosinase did not restore the inhibitory activity when added to steamed cabbage juice, either. Although cabbage species contain several different glucosinolates, the antimicrobial activity of the cabbage is weak. Therefore the activity may be the combined effect of many different substances which are enzymatically activated. Therefore glucosinolate hydrolysis products were eliminated as the possible antibacterial compound(s) of cabbage.

Cabbages contain appreciable amounts of *S*-methyl-L-cysteine sulfoxide and hydrolase enzyme. Once methyl methanethiosulfinate, hydrolysis product of *S*-methyl-L-cysteine sulfoxide, is formed, a higher antimicrobial activity could be expected in cabbage homogenate (*15*). Kyung and Fleming (*12*) looked for a new explanation and concluded that methyl methanethiosulfinate (Figure IV), was responsible for the antimicrobial activity of cabbage, as allicin is responsible for the antimicrobial activity of garlic. When pH 4.0 precipitate from fresh cabbage juice (inhibitory) was added to the juice (non-inhibitory) extracted from cabbage steamed before juice extraction, the antimicrobial activity was restored. A heat-labile factor seemed to be involved in activating a non-inhibitory precursor into an inhibitory compound. Methyl methanethiosulfinate was formed in inhibitory fresh cabbage juice and in a model system consisting of *S*-methyl-L-cysteine sulfoxide and pH 4.0 precipitate of fresh cabbage. Therefore it was confirmed that methyl methanethiosulfinate is the principal antimicrobial compound in cabbage and maintained that glucosinolate hydrolysis products were not responsible for antimicrobial activity of cabbage (*12*).

S-Methyl-L-cysteine sulfoxide, a non-protein sulfur-containing amino acid, and its hydrolase enzyme are present in *Brassica*, and *S*-methyl-L-cysteine sulfoxide has common functional groups in structure to alliin (*S*-allyl-L-cysteine sulfoxide) (Figure IV), another non-protein sulfur amino acid commonly found in garlic. Garlic and onion become antimicrobial when they are crushed, which is also true with cabbage. Methyl methanethiosulfinate is less potent than allicin (*5*). Sulfoxides are suggested to be a soluble pool of organic sulfur important in sulfur metabolism.

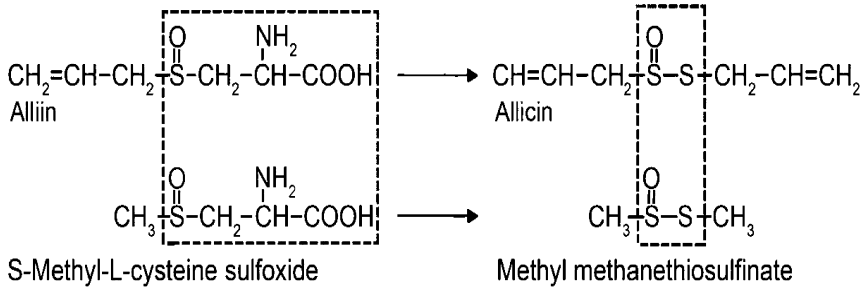

Figure IV. Structural similarities between alliin and S-methyl-L-cysteine sulfoxide and allicin and methyl methanethiosulfinate. The structural parts in dotted boxes are common.

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Conclusion

Garlic and onion become inhibitory to various microorganisms because they contain high levels of sulfoxides, while horseradish, mustard seeds and wasabi become inhibitory because they contain high levels of allyl glucosinolates. Most cole crops do not show appreciable inhibitory activity against microorganisms since the concentrations of sulfoxides and glucosinolates are not high enough. Plant foods that can generate potent antimicrobial compounds have the potential of being used as natural alternative food preservatives. However, there are few practical applications as food preservatives of such plants because thiosulfinates and isothiocyanates are unstable in aqueous matrix. In addition to the crucial technical shortage, the antimicrobial food materials are not well accepted by many people because of their strong flavor.

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Editors' Biographies

Michael C. Qian

Dr. Michael C. Qian is faculty member at Oregon State University. He has received his BS degree in Chemistry from Wuhan University of China and his MS degree in Food science from University of Illinois at Urbana-Champaign and his PhD from University of Minnesota under the guidance of Dr. Gary Reineccius. Dr. Qian's research interests at Oregon State University focus on flavor chemistry and instrumental analysis of volatile compounds. He is specialized in solventless sample preparation such as solid phase micro-extraction, solid phase dynamic extraction, stir bar sorptive extraction, fast GC, and multi-dimensional GC/GC-MS analysis of volatile aroma compounds. His current research projects involve the understanding of chemical and biochemical generation of aroma and flavor in dairy products, small fruits (blackberries, raspberry, and strawberry), wine, wine grapes, beer, and hops. He has made significant contribution in the analysis of volatile sulfur compounds in various food systems. He has published more than fifty peer-reviewed original research papers and ten book chapters in the field of flavor chemistry and analytical chemistry. He has previously co-edited two books related to flavor chemistry that were published by American Chemical Society, and is a frequent speaker at national and international meetings. Before he came to academia, Dr. Qian spent 10 years in the food industry as a research scientist. Dr. Qian is a former chair of the ACS Agricultural/Food Chemistry Division Flavor Chemistry Sub Division.

Xuetong Fan

Dr. Xuetong Fan is the Lead Scientist/Research Food Technologist with the Eastern Regional Research Center, Agricultural Research Service, U.S. Department of Agriculture in Wyndmoor, PA. His current research focuses on developing intervention technologies and treatments to enhance microbial safety of fresh produce while maintaining product quality. Research projects include analyzing and minimizing formation of undesirable chemicals and chemical by-products (furan, volatile surface compounds, etc) that are induced by processing technologies and chemical sanitization. He has published more than 100 peer review articles and book chapters as well as two books.

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Kanjana Mahattanatawee

Native to Thailand, Kanjana Mahattanatawee, received a B.Sc in Microbiology from Sri-Nakharinwirot University in 1988 and a M.Sc. in Industrial Microbiology from Chulalongkorn University in 1991, both in Bangkok, Thailand. In 1993, after an UNESCO fellowship in Biotechnology at Osaka University in Japan, Dr. Mahattanatawee returned home to be a lecturer at Department of Food Technology, Siam University, Bangkok. In 2004, a doctoral study under the tutelage of Prof. Russell Rouseff at the University of Florida resulted in the article, "Identification of new citrus norisoprenoid in orange juice using time intensity GC-O and GC-MS". After two years of postdoctoral fellowship at USDA, ARS, Winter Haven, Florida, she returned home to Siam University. Currently, she is the Dean of the Faculty of Science and is very active in the research of flavor chemistry. Dr. Mahattanatawee is a former chair of ACS AgFd Flavor Sub Division, a regular reviewer for the JAFC, and the Chair of the ACS Thai Chapter.

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