MEDIUM AND HIGHER MOLECULAR WEIGHT VOLATILE THIOLS IN AGED CHEDDAR CHEESE AND THEIR RELATION TO FLAVOR

DISSEPTION

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By

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ABSTRACT

Attention has been given to expand our knowledge in respect to the role of sulfur compounds in the flavor of aged Cheddar cheese. A new method, based on the concentration of reduced thiol compounds and their analysis by a sulfur-specific GC detector shows the presence of 39 thiol compounds in the aged Cheddar cheeses. Of these 14 were tentatively identified by Kovat’s indices and the presence of 4-mercapto-4-methyl pentan-2-one confirmed with a pure standard. These 14 thiol compounds possess an additional alcohol, aldehyde, or ketone functional group.

The results of this method were substantiated by other analyses (descriptive sensory analysis and electronic nose). Sensory analysis showed that there were four different sulfur descriptors (total sulfur, egg-like, match-like and catty) that differed the eleven cheeses. Total sulfur and catty were correlated to age of the cheese and catty and match-like were also correlated. The electronic nose, using a mass spectrometer in NCI mode, gave similar differentiation patterns to the sensory and also suggest that 4-mercapto-4-methyl pentan-2-one was associated with the differentiation of the aroma.

The three analytical approaches all confirm the importance of sulfur compounds to the flavor of aged Cheddar cheese and all help support the significance of the poly
functional thiols in Cheddar cheese flavor, especially the 4-mercapto-4methyl pentan-2-one (catty) compound.

An additional 15 medium and higher molecular weight thiols were partially characterized by Kovat’s Retention Indexes, and their molecular weights were estimated within a range of a few grams.
Dedicated to my Lord and Saviour Jesus Christ, Creator of the universe, and source of all true inspiration
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CHAPTER 1

INTRODUCTION

Cheddar cheese is the most popular aged cheese manufactured in the United States. The flavor of Cheddar cheese has been the subject of academic and industrial research in the U.S., Europe, Australia, and New Zealand for almost 100 years. While a great deal of progress has been made, the Chemical basis of the flavor of Cheddar cheese remains incompletely understood.

An association of sulfur compounds with Cheddar cheese aroma and flavor has been known for over 80 years, but there is disagreement as to the role and importance of the individual sulfur compounds, and sulfur compounds as a whole.

Historically, identification and quantification of volatile sulfur compounds are problematic. A number of classes of volatile sulfur compounds associated with food aromas or flavors are air sensitive, including hydrogen sulfide, and thiols. These compounds may react with metal, plastic or glass surfaces, and are difficult to isolate. Furthermore, sulfur compounds generally have low odor thresholds, typically from low parts per billion to low parts per trillion, and occur in minute concentrations in food products.

A number of poly-functional thiol compounds have been associated with the flavor or aroma of wine, the Box tree and Broom plant, beer, passion fruit, guava fruit,
steam distillate of onion, roasted coffee, and roasted meat. The origin of all of these compounds is not fully understood. However, several nonvolatile precursors to volatile polyfunctional thiols have been identified in cultivars of *Vitis vinifera*, a grape used in the manufacture of various French wines. It has not yet been demonstrated that these precursors exist in other plants that are sources of volatile polyfunctional thiols. Polyfunctional thiols may also form due to chemical reactions occurring within a given food matrix, as in freshly roasted coffee beans, or as a flavor defect in aged Gouda cheese.

Cheddar cheese possesses certain flavor notes that are known to be air sensitive. In order to determine if air sensitive sulfur compounds are responsible for the above flavor notes, an analytical method is needed to determine concentrations of sulfur compounds in the low parts per billion to parts per trillion concentration levels.
CHAPTER 2

LITERATURE REVIEW

On average, in 1999, Americans consumed slightly more than 10 lbs of Cheddar cheese per year per capita, and 2,816,867,000 pounds of Cheddar cheese were produced in the U.S. (International Dairy Foods Association, 2000). The leading state for production of Cheddar, Colby, Washed or Stirred Curd and Monterey Jack was Wisconsin, at 973,633,000 lbs, followed by Minnesota with 639,101,000 lbs, California with 597,048,000 lbs, Idaho with 505,102,000 lbs, and New York with 98,458,000 lbs (International Dairy Foods Association, 2000). By region, the North and South Atlantic states, including New York and Vermont, produced 152,686,000 pounds of Cheddar, The Central States, including Wisconsin, Minnesota, Ohio and Michigan, produced 1,509,917,000 pounds of Cheddar, Western states, including Idaho and California, produced 1,109,016,000 pounds of Cheddar, and the South central states produced 45,248,000 pounds of Cheddar (International Dairy Foods Association, 2000).

The specific chemical nature of Cheddar cheese flavor has never been fully understood, in spite of nearly 100 years of research to determine the specific chemical compounds responsible for the flavor of Cheddar cheese.
Many different classes of compounds are important to the flavor of Cheddar cheese. More than 200 volatile compounds have been identified in Cheddar cheese, including free fatty acids, esters, alcohols, aldehydes, ketones, lactones, biogenic amines, phenols, and sulfur compounds (Maarse et al, 1994).

A number of reviews have been published concerning the development of flavor in Cheddar and other types of cheese (Aston and Dulley, 1982; Law, 1984a,b; and Urbach, 1993, McSweeney and Sousa, 2000). These reviews suggest that the flavor of Cheddar cheese is not completely understood. The development of flavor in Cheddar cheese is the result of several processes, including the metabolism of lactose and amino acids by lactic acid bacteria, the liberation of free fatty acids, methyl ketones and lactones from milk fat, and chemical reactions such as Strecker degradation and Maillard reactions (Urbach, 1995). Some of these compounds have flavor or aroma impact in very low concentrations and are listed in Table 2.1.

More than 200 volatile compounds have been identified in Cheddar cheese, although none of these compounds individually or together are able to reconstitute an authentic or “true” Cheddar cheese flavor. Classes of volatile compounds found in Cheddar cheese include inorganic and organic sulfur compounds, hydrocarbons, alcohols, aldehydes, ketones, fatty acids, esters, lactones, amines, phenols, and furanones (Maarse et al, 1994). Individual compounds within a given class may be formed by a variety of chemical or bacterial mechanisms.

Definition of a “true” Cheddar cheese flavor is also complicated by national and regional differences in the flavor of Cheddar. In the United States, Cheddar cheese produced in the Midwest tends to have a clean, slightly acid flavor in mild Cheddar and a
medium intense flavor in aged cheese (Wendorf et al, 1998). Aged cheeses may have a slight sulfur flavor, but mild Cheddar is generally downgraded if sulfide flavor is noted (Wendorf et al, 1998). New York and Vermont cheddars generally have a more intense flavor in both mild and aged cheeses (Wendorf et al, 1998). There is a slight sulfur note in mild Cheddar and a definite sulfur note in aged cheeses (Wendorf et al, 1998).

The New England Cheddars also tend to have a sharp acid flavor along with slight rancid and astringent flavor notes (Wendorf et al, 1998). Cheddar cheese from the West Coast tends to have less intense flavor for mild and aged cheeses in comparison to Midwest and New England cheeses (Wendorf et al, 1998). Mild, clean flavors associated with these cheeses may be due in general to the low bacterial counts typically found in the raw milk used to manufacture the cheese (Wendorf et al, 1998).

At least two schools of thought have arisen in order to explain the flavor of and flavor differences among Cheddar cheese. One school of thought, known as the component balance theory, states that the same flavor compounds occur in all cheeses, only the concentrations vary among different cheese varieties. A second school of thought, the key component theory, states that although the various volatile and nonvolatile taste and aroma compounds impart background flavor and fullness to Cheddar cheese, there is an essential “true Cheddar” flavor compound that is responsible for the characteristic flavor of Cheddar cheese. This compound is believed to be air sensitive, have a high odor activity value, and occur in very low concentrations.
2.1. Sulfur compounds in Cheddar

The focus of this dissertation is the potential role that sulfur compounds play in the flavor of Cheddar cheese; and thus, particular emphasis will be placed on the current body of knowledge concerning sulfur compounds as related to Cheddar cheese. Sulfur compounds have been associated with the flavor of Cheddar cheese by a number of researchers (Moir 1933, Morgan 1933, Barnicoat 1937, Kristofferson and Nelson, 1955, Kristofferson and Gould, 1959, Kristofferson and Singh, 1967, Walker, 1959, Libbey and Day, 1963, Manning, 1973, Aston and Dulley, 1982, Lindsay and Rippe 1986, Urbach, 1993, Dimos et al, 1995). Individual groups of researchers that have made significant contributions to understanding the sulfur-related flavor chemistry of Cheddar cheese are reviewed below.

2.1.1. Moir, Morgan, and Barnicoat

Moir (1933) and Morgan (1933) were the first to chemically identify sulfur compounds in Cheddar, and qualitatively identified Hydrogen sulfide in Cheddar cheeses possessing a "bleached annatto" defect. Moir (1933) and Morgan (1933) may have been the first researchers to determine that volatile sulphhydryl compounds, most likely H_2S, were present in Cheddar cheese possessing the “stinker” flavor defect. The presence of free -SH groups was later seen in both high and low quality aged New Zealand Cheddar. Free SH- groups were present in high quality cheeses, and an over abundance was related to undesirable flavor (Barnicoat, 1937). The above determinations were only qualitative in nature, and were noted by a color change in lead acetate impregnated paper.
2.1.2. Day et al.

The Strecker degradation, a chemical reaction between a carbonyl compound and an amino acid that results in the formation of an aldehyde with one less carbon atom than the original amino acid, may play a role in the development of cheese flavor (Keeney and Day, 1957). Methional, a Strecker-type degradation product of the amino acid methionine, possesses a cheese-like odor at less than one ppm concentration, when present with other casein-derived Strecker aldehydes (Keeney and Day, 1957).

Methane thiol was first identified in Cheddar cheese by Libbey and Day (1963). The concentration of methane thiol in Cheddar was estimated to be between 3 and 30 parts per billion, by high vacuum, low temperature distillation of Cheddar cheese oil and reaction of volatiles with 2,4-dinitrofluorobenzene (DNFB) reagent (Libbey and Day, 1963). Due to the low odor threshold of this compound in water, 2 ppb, methane thiol was suggested to be an important volatile aroma compound in cheddar cheese (Libbey and Day, 1963).

2.1.3. Kristofferson

A quantitative increase in the concentration of H₂S and other volatile free sulphhydryls was observed to occur during the ripening of high quality American Cheddar cheese (Kristofferson and Nelson, 1955). Cheddar cheese has flavor notes that are sensitive to changes in the oxidation/reduction potential of the cheese (Kristofferson et al, 1964). Consumer packaging of cheese results in a loss of flavor quality; the flavor change is related to the oxidation/reduction stability of the cheese, and a decrease in free SH-groups in Cheddar cheese that has been exposed to air (Kristofferson et al, 1964). Loss of
flavor quality can be minimized in Cheddar cheese when it is sealed in a tightly fitting, oxygen-and-moisture-barrier pouch (Kristofferson et al, 1964).

A positive relationship between the concentrations of free non-volatile SH groups and Cheddar cheese flavor was reported by Kristofferson et al, (1964). Kristofferson and Gould (1959) suggested that most carbonyl compounds occurring in Cheddar, rather than contributing directly to flavor, may serve as precursors for the formation of specific flavor components of Cheddar cheese.

A method for accelerated development of Cheddar and other cheese flavors was developed that utilizes a homogenized mixture, known as a curd slurry, of 1 part of fresh cheddar cheese curd to 2 parts sterile 5.2% saline solution (Kristofferson et al, 1967). The flavor of seven to nine day old slurry was reported to be typical of that of a sharp, high quality Cheddar; addition of 100 ppm glutathione to a slurry was found to accelerate development of Cheddar-like flavor, addition of cysteine also resulted in increased flavor development and the formation of a distinct “sulfide” off-flavor (Kristofferson et al, 1967). These observations suggest that sulfur containing peptides or amino acids may be potential precursors to characteristic Cheddar cheese flavor.

A number of studies conducted by Kristofferson and Harper (1966) lend support to a hypothesis that higher molecular weight thiols flavor compounds in Cheddar may be formed from chemical reactions between H₂S and carbonyl compounds. S³⁵ cysteine was injected into the blood stream of a cow, and its fate was followed as the label was incorporated into proteins as cysteine S³⁵. No radioactive methionine was detected in milk taken from the cow within 24 hours. The milk was then fermented or made into cheese. The fermented milk or cheese was then purged with nitrogen gas, and volatiles
were collected in a series of chemical traps. Radioactive counts due to $^{35}\text{S}$ were found in chemical traps for $\text{H}_2\text{S}$, mercaptans, organic sulfides, and carbonyls. The presence of radioactivity in the 2,4 dinitrophenyl hydrazine carbonyl trap suggests that volatile thiols with a carbonyl functional group may exist in Cheddar cheese (Kristofferson and Harper, 1966).

2.1.4. Walker

Addition of a mixture of thioacetamide, a precursor to $\text{H}_2\text{S}$, and the methyl ketones acetone, butanone, pentan-2-one, heptan-2-one, nonan-2-one, undecan-2-one, and tridecan-2-one, to a 3-week-old cheese was observed to impart a “Cheddar-like” flavor. The cheese was also judged to be lacking in a “background flavor” over which the “Cheddar-like” flavor would be superimposed in an aged cheese (Walker, 1961).

2.1.5. Manning et al

Selective removal of sulfur compounds from cheese headspace with mercuric chloride or mercuric cyanide trapping salts resulted in destruction of characteristic Cheddar aroma (Manning and Price, 1977). Removal of sulfur compounds, $\text{H}_2\text{S}$, thiols, and dimethyl sulfide produced an aroma that was considered to be “butter-like” (Manning and Price, 1977). Selective removal of thiols from the aroma of Cheddar cheese resulted in the destruction of typical Cheddar aroma, and it was suggested that a thiol, possibly $\text{CH}_3\text{SH}$, is essential to the aroma of Cheddar (Manning and Price, 1977). Manning and Price (1977) also suggested that other undetected thiols with extremely low thresholds might be present in Cheddar cheese.
2.1.6. Aston

Aston and Douglas (1983) studied the use of elevated ripening temperatures and mutant starter cultures to accelerate ripening of Cheddar cheese. They found little correlation of H$_2$S, dimethyl sulfide, and methane thiol with total flavor and mature flavor scores of Cheddar cheeses (Aston and Douglas, 1983).

2.1.7. Samples and Richter

Addition of reduced glutathione to Cheddar cheese slurries did not significantly alter the oxidation-reduction potential of the system, however, an elevation in hydrogen sulfide, methane thiol, carbonyl sulfide and dimethyl sulfide was observed; addition of cysteine to slurries had a similar effect upon the above volatiles (Samples and Richter, 1985). Addition of methionine to the slurries did not significantly increase production of hydrogen sulfide or methane thiol (Samples, 1985). Milk heat treatments had a significant effect upon hydrogen sulfide and methane thiol production; milk treated at 75°C for 15 minutes failed to develop hydrogen sulfide (Samples, 1985).

2.1.8. Lindsay

Lindsay and Rippe (1986) studied the role of methane thiol in the flavor of Cheddar cheese. Methionine and a methionase enzyme obtained from *Pseudomonas putida* were added or encapsulated in butter fat capsules and then added to cheese milk at the time of renneting.

Addition of encapsulated enzyme and substrate as well as addition of free enzyme and substrate resulted in increased production of methane thiol. Methane thiol by itself, in young Cheddar cheese, did not impart a Cheddar flavor to the cheese (Lindsay and Rippe, 1986).
Addition of methional to a bland cheese base caused an unclean, harsh, and dulling flavor sensation (Dunn and Lindsay, 1985).

2.1.9. Urbach et al

Urbach (1993) reviewed the results of five Cheddar cheese ripening studies sponsored by the CSIRO of Australia, as well as information on volatile sulfur compounds previously published in peer-reviewed journals, and concluded the following:

None of the volatile sulfur compounds in the headspace of Australian Cheddar cheese, H₂S, methane thiol, dimethyl sulfide, dimethyl disulfide, carbonyl sulfide, or carbon disulfide, has an odor reminiscent of Cheddar. Dimethyl sulfide plays no part in the odor of Australian Cheddar. Dimethyl disulfide is an oxidation product of methane thiol, and possesses a “cabbage-like” odor; this odor description is not associated with the aroma of a quality, mature Cheddar cheese. COS and CS₂ or methane thiol may be breakdown products of a volatile, unstable Cheddar flavor compound. COS and CS₂ may form in Cheddar as a result of chemical reactions; cysteine may be the source of sulfur for both compounds. Dimos et al (1996), found a straight line relationship between actual flavor and fitted flavor based on the log of the concentration of H₂S, 2-heptanone, butanone, γ-decalactone and 2-propanol. Also, the presence of methane thiol is undoubtedly a necessary but not sufficient condition for Cheddar flavor.

2.1.10. Reineccius et al

Christensen and Reineccius (1995) and Milo and Reineccius (1997) applied aroma extraction dilution analysis (AEDA) to Cheddar cheese, in order to determine the most potent odorants in full fat and reduced fat samples. Methional is a potent odorant in Cheddar cheese (Christensen and Reineccius, 1995). A static headspace HRGC/O (high
resolution gas chromatography/olfactometry) analysis of Cheddar cheese was utilized to demonstrate that methane thiol and dimethyl trisulfide were among the most potent odorants in the headspace of Cheddar cheese, followed by dimethyl sulfide and finally methional in terms of their odor potency (Milo and Reineccius, 1997). Methional is the most potent odorant associated with the meaty/brothy flavor defect of reduced fat Cheddar cheese (Milo and Reineccius, 1997).

2.1.11. Weimer et al

Weimer et al (1999) recently published an extensive review of the sulfur flavor chemistry of Cheddar cheese as well as the metabolism of sulfur amino acids in cheese ripening bacteria. *Brevibacterium linens* was added to a 50 percent reduced fat Cheddar cheese as a flavor adjunct, and was found to improve the flavor (Broadbent et al, 1997).

Dias and Weimer (1998a) evaluated the ability of several strains of *Lactococcus*, *Lactobacillus*, and *Brevibacterium* to produce methane thiol. Whole cells of *Brevibacterium* had the highest methane thiol producing capacity compared to *Lactococcus* and *Lactobacillus*. Dias and Weimer (1998b) purified and characterized L-methionine gamma lyase from *Brevibacterium linens*, and suggested that this enzyme could have activity under salt and pH conditions found in ripening Cheddar cheese.

2.1.12. Summary of research relevant to this study

Several studies in Cheddar cheese flavor have been conducted over the span of the last 70 years that laid the intellectual groundwork for this current study. Barnicoat (1937) determined that free thiol groups were present in high quality Cheddar, and that an over abundance of these groups cause flavor defects. Kristofferson and Gould (1959) suggested that carbonyl compounds may serve as precursors of Cheddar cheese flavor,
and Walker (1961) demonstrated that the addition of hydrogen sulfide precursor and ketones found in ripening Cheddar cheese to Cheddar curd resulted in formation of aged Cheddar-like flavor notes. The later study is of particular importance, in that it demonstrates that chemical reactions occurring between carbonyls and H₂S within the cheese matrix may be important to formation of characteristic Cheddar cheese flavor. Manning and Price (1977) determined that thiols are essential components of Cheddar cheese aroma; furthermore, they suggested that as yet unidentified thiols with very low odor thresholds may be present in Cheddar cheese.

Kristofferson et al (1964) demonstrated that oxidation/reduction potential is important in Cheddar cheese flavor development. If Cheddar cheeses do not reach a low enough oxidation/reduction potential, they do not develop a characteristic Cheddar-like flavor. Furthermore, if this value is too low, the cheese develops sulfide off-flavors.

Synthesis of the above findings suggests then that there may be essential thiols with low odor thresholds present in Cheddar, these compounds may be formed by reactions between carbonyls and H₂S, and in order for these compounds to form in ripening Cheddar cheese, the oxidation reduction potential must be within a certain range. Sulfur compounds identified in Cheddar cheese, odor descriptions, and odor thresholds of these compounds are listed in Table 2.1. Based on the odor descriptions of these compounds, it can be seen that none of these compounds possess a “Cheddar-like” aroma.
<table>
<thead>
<tr>
<th>compound</th>
<th>Odor threshold</th>
<th>Odor description</th>
</tr>
</thead>
<tbody>
<tr>
<td>H$_2$S</td>
<td>10 ppb, water$^1$</td>
<td>Sulfury, eggy$^1$</td>
</tr>
<tr>
<td>CH$_3$SH</td>
<td>0.2; 0.02 ppb, water$^1$</td>
<td>Sulfury, fecal$^1$</td>
</tr>
<tr>
<td>CH$_3$SCH$_3$</td>
<td>0.3; 1.0 ppb, water$^1$</td>
<td>Sulfury, canned corn-like$^1$</td>
</tr>
<tr>
<td>CH$_3$SSCH$_3$</td>
<td>0.16; 12 ppb, water$^1$</td>
<td>Old cabbage-like$^1$</td>
</tr>
<tr>
<td>methionol</td>
<td>0.2; 1.8 ppb, water$^1$</td>
<td>Potato - like$^1$</td>
</tr>
<tr>
<td>methionol</td>
<td>5 ppb, water$^1$</td>
<td>Potato - like$^1$</td>
</tr>
<tr>
<td>COS</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>CS$_2$</td>
<td>0.2 ppb, air$^2$</td>
<td>N/A</td>
</tr>
</tbody>
</table>

Table 2.1 Volatile Sulfur Compounds in Cheddar Cheese

1) Compilation of Odor Thresholds, Odor Qualities, and Retention Indexes of Key Food Odorants. 1998. Rychlik, M.; Schieberle, P.; Grosch, W. Deutsche Forschungsanstalt fur Lebensmittelchemie ans Institut fur Lebensmittlechemie der Technischen Universitat Munchen
2.2. Volatile sulfur compounds in other cheese varieties

A number of sulfur compounds occur in other major varieties of commercially produced cheeses, including Blue; surface ripened cheeses such as Brick, Brie and Camembert; Swiss; Dutch-type cheeses such as Edam and Gouda; hard Italian cheeses such as Parmesan, and Mozzarella, and brine ripened cheese such as Feta. *Micrococcus*, coryneform bacteria, and *Brevibacterium linens*, a type of coryneform bacteria, are all associated with the surface ripening of Brie and Camembert cheeses. Strains of the above organisms isolated from traditional Camembert cheese are capable of producing methane thiol from the amino acid methionine, and hydrogen sulfide from cysteine (Bloes-Breton and Bergere, 1997). A number of *Micrococcus*, and coryneform bacteria as well as some *Leuconostoc* and *Lactococcus lactis* are capable of synthesizing thioesters (Bloes-Breton and Bergere, 1997, Lamberet et al, 1997.).

These microorganisms synthesize esters from alcohols and free fatty acids as a detoxification strategy for the elimination of toxic compounds from growth media (Molimard and Spinnler, 1996). Microorganisms involved in surface ripening of cheese may employ this strategy to detoxify methane thiol, which would account for the abundance of methyl thio-esters found in Camembert cheese. Other sulfur compounds isolated from cultures of *Micrococcus* and coryneform bacteria include 2-propanethiol, 2-butanethiol, 2-methyl-1-pentanethiol, and benzylthiazole (Lamberet et al, 1997).

*Geotrichum candidum* and *Debaromyces hansenii* are two species of yeast used as starter cultures for the maturation of certain European surface ripened cheeses; these organisms are capable of synthesizing methane thiol, dimethyl sulfide, dimethyl disulfide, dimethyl trisulfide, and S-methylthioacetate from L-methionine (Spinnler et al,
(Gallios et al 1990) detected methane thiol, hydrogen sulfide, and dimethyl sulfide in French blue cheeses.

4-mercapto-4-methyl pentan-2-one, a compound with a very potent “catty” aroma, has been identified in Gouda cheeses that possessed an intense off flavor (Badings, 1967). This compound is suggested to form by condensation of 2 acetone molecules to form mesityl oxide, followed by addition of H$_2$S across the carbon-carbon double bond (Badings, 1967). Recently, it has been suggested that "catty" is a descriptive term that can be used to describe a sulfur note occurring in Cheddar cheese (Drake et al, 2001).

### 2.3. Volatile sulfur compounds in other foods

Several volatile sulfur compounds occur in fresh and fermented Brassica (sp). Kimchi is a food that is popular in Korea, consisting of fermented cabbage, and may contain other vegetables such as onion, garlic, and mustard that contain precursors to volatile sulfur compounds. A number of sulfur containing volatiles have been isolated from Kimchi, including H$_2$S, Methane thiol, dimethyl disulfide, dimethyl trisulfide, (methylthio)-1-propene, and methyl-2-propenyl trisulfide (Kim and Sohn, 2001). Methane thiol, dimethyl disulfide, and dimethyl trisulfide are formed during the disruption of the tissue of fresh cabbage by the action of cysteine sulfoxide lyase on S-methyl-L-cysteine sulfoxide (Chin and Lindsay, 1994).

### 2.4 Polyfunctional thiols

“Polyfunctional thiol” is a term used to describe organic molecules that contain a thiol and additional functional group. Over 40 polyfunctional thiols have been identified as flavor components in a number of foods including pork, chicken, tuna fish, cheese,
olive oil, syrup, durian fruit, passion fruit, grape fruit, grape, black currant, asparagus, onion, scallion, sesame, popcorn, bread, yeast extract, buchu, hops, wine, beer, coffee, and tea (Vermuelen et al, 2001). Recently, a number of desirable polyfunctional thiol compounds, containing a thiol group and either a ketone, alcohol or acetate functional group, have been detected in wine and are believed to be liberated from precursor molecules during fermentation (Tominaga et al, 1998).

The recovery of these compounds from the sample matrix, wine, was facilitated by use of a thiol specific trapping reagent, p-hydroxymercuribenzoic acid. The polyfunctional thiol compounds are believed to exist as non-volatile S-cysteine conjugates that are released by yeast degradation during alcoholic fermentation (Tominaga et al, 1998). A cysteine conjugate B-lyase (E.C. 4.4.1.13) which is contained in a cell free extract of yeast is capable of liberating these thiols from the conjugate molecule (Tominaga et al, 1998). In heated foods, polyfunctional thiols may arise from chemical reactions between reducing sugars or aldehydes and cysteine or free H₂S. In many cases, the reaction mechanisms are unclear.

4-mercapto-4-methyl pentanone, a compound with a very potent “catty” aroma, has been identified in Gouda cheeses that possessed an intense off flavor (Badings, 1969).

Table 2.2 presents the odor description, origin and Kovat’s retention index for a large number of polyfunctional thiols, many of which have been synthesized by combinatorial chemistry (Vermeulen et al. 2001, 2002). It is of interest to note that these compounds can give a wide range of different odor descriptors, which include cheese,
<table>
<thead>
<tr>
<th>Compound</th>
<th>Odor description</th>
<th>Kovat’s Retention Index</th>
<th>GC column</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-mercaptopentan-2-one</td>
<td>Greenery, potato, black currant</td>
<td>884</td>
<td>pdms</td>
<td>11</td>
</tr>
<tr>
<td>4-mercapto-4-methylpentan-2-one</td>
<td>Catty, meaty black currant, broom, vinaigrette, citrus fruit</td>
<td>937, 938/915 / 1377</td>
<td>5DP/pdms/PEG</td>
<td>2, 4, 10, 11</td>
</tr>
<tr>
<td>4-mercapto-3-methylpentan-2-one</td>
<td>Sweat, cooked milk</td>
<td>959, 967 (2 isomers)</td>
<td>pdms</td>
<td>11</td>
</tr>
<tr>
<td>5-mercaptohexan-3-one</td>
<td>Box tree, fresh, empyreumatic</td>
<td>984</td>
<td>pdms</td>
<td>11</td>
</tr>
<tr>
<td>5-methyl-4-mercapto-2-hexanone</td>
<td>exotic fruit, sweet, sulfury</td>
<td>1069</td>
<td>pdms</td>
<td>3, 11</td>
</tr>
<tr>
<td>4-mercapto-3-methylpentan-2-ol</td>
<td>Onion, leek, sweat, soup</td>
<td>1037</td>
<td>pdms</td>
<td>11</td>
</tr>
<tr>
<td>3-methyl-4-mercaptohexan-2-ol</td>
<td>Rhubarb, lemon, spicy, peppery, meaty</td>
<td>1097, 1107 (2 isomers)</td>
<td>pdms</td>
<td>11</td>
</tr>
<tr>
<td>4-mercapto-4-methylpentan-2-ol</td>
<td>Broom, black currant, solvent, fresh, sweet</td>
<td>1459</td>
<td>PEG</td>
<td>11</td>
</tr>
<tr>
<td>5-mercaptohexan-3-ol</td>
<td>Sweat, meat broth, citrus fruit, cooked milk</td>
<td>1012, 1023 (2 isomers)</td>
<td>pdms</td>
<td>11</td>
</tr>
<tr>
<td>4-mercaptopentan-2-ol</td>
<td>Broom, black currant, catty, Raw onion</td>
<td>914 / 926</td>
<td>pdms</td>
<td>11</td>
</tr>
<tr>
<td>3-mercapto propanal</td>
<td>Rotten potatoes, broth</td>
<td>756 / 1382</td>
<td>Pdms/PEG</td>
<td>13</td>
</tr>
<tr>
<td>3-mercapto butanal</td>
<td>Broth, cheese, pungent</td>
<td>803 / 1372</td>
<td>Pdms/PEG</td>
<td>13</td>
</tr>
<tr>
<td>3-mercapto-3-methylbutanal</td>
<td>Broth, cheese, pungent</td>
<td>842 / 1353</td>
<td>Pdms/PEG</td>
<td>13</td>
</tr>
<tr>
<td>3-mercapto-2-methylpropanal</td>
<td>Meat, broth, raw bread paste</td>
<td>826 / 1400</td>
<td>Pdms/PEG</td>
<td>13</td>
</tr>
<tr>
<td>3-mercapto-2-methylbutanal</td>
<td>Broth, onion, meat, cheese</td>
<td>892 / 1424</td>
<td>Pdms/PEG</td>
<td>13</td>
</tr>
<tr>
<td>3-mercapto-pentanal</td>
<td>Broth, raw onion, flowery</td>
<td>910 / 1476</td>
<td>Pdms/PEG</td>
<td>13</td>
</tr>
<tr>
<td>3-mercapto-2-ethylpropanal</td>
<td>Broth, rotten potatoes, plastic, ground nut</td>
<td>926 / 1481</td>
<td>Pdms/PEG</td>
<td>13</td>
</tr>
<tr>
<td>3-mercapto-2-methylpropanal</td>
<td>Broth, meat, onion, pepper</td>
<td>991 / 1508</td>
<td>Pdms/PEG</td>
<td>13</td>
</tr>
<tr>
<td>3-mercaptopentanal</td>
<td>Citrus fruit peel, fresh</td>
<td>1004 / 1560</td>
<td>Pdms/PEG</td>
<td>13</td>
</tr>
<tr>
<td>3-mercaptodecanal</td>
<td>Flowery, citrus peel</td>
<td>1108 / 1659</td>
<td>Pdms/PEG</td>
<td>13</td>
</tr>
<tr>
<td>3-mercapto-2-butyllpropanal</td>
<td>Plastic, rhubarb, pungent</td>
<td>1133 / 1667</td>
<td>Pdms/PEG</td>
<td>13</td>
</tr>
<tr>
<td>3-mercaptooctanal</td>
<td>Citrus fruit peel, grapefruit, greenery, fresh</td>
<td>1214 / 1764</td>
<td>Pdms/PEG</td>
<td>13</td>
</tr>
<tr>
<td>3-mercaptopnonanal</td>
<td>Stale odor, greenery</td>
<td>1320 / 1873</td>
<td>Pdms/PEG</td>
<td>13</td>
</tr>
</tbody>
</table>

Table 2.2 Polyfuctional Thiols in foods

Continued
<table>
<thead>
<tr>
<th>Compound</th>
<th>Odor Notes</th>
<th>Retention Time</th>
<th>Solvent System</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-furfuryl thiol</td>
<td>Roasted, coffee-like</td>
<td>912/983/1431</td>
<td>5DP/CPP+MP/PEG</td>
<td>1, 8</td>
</tr>
<tr>
<td>2-methyl-3-furantoil</td>
<td>Meat-like</td>
<td>848/869/1300</td>
<td>pdms/5DP/PEG</td>
<td>1</td>
</tr>
<tr>
<td>3-mercapto-2-pentanone</td>
<td>Meat-like</td>
<td>907/1347</td>
<td>5DP/PEG</td>
<td>1</td>
</tr>
<tr>
<td>1-mercapto-2-propanone</td>
<td></td>
<td>743</td>
<td>pdms</td>
<td>1, 12</td>
</tr>
<tr>
<td>2-thiethyl mercaptan</td>
<td></td>
<td>1092/1682</td>
<td>5DP/PEG</td>
<td>1</td>
</tr>
<tr>
<td>Trans-8-mercapto-p-menthan-3-one</td>
<td>catty</td>
<td></td>
<td></td>
<td>2</td>
</tr>
<tr>
<td>cis-8-mercapto-p-menthan-3-one</td>
<td>Buchu leaf oil</td>
<td></td>
<td></td>
<td>2</td>
</tr>
<tr>
<td>3-mercapto-3-methyl-2-pentanone</td>
<td>catty</td>
<td></td>
<td></td>
<td>2</td>
</tr>
<tr>
<td>Tert-amyl mercaptan</td>
<td>catty</td>
<td></td>
<td></td>
<td>2</td>
</tr>
<tr>
<td>p-1-menthene-8-thiol</td>
<td>Grapefruit-like</td>
<td>1598/1283</td>
<td>PEG/5DP</td>
<td>4</td>
</tr>
<tr>
<td>4-methoxy-2-methyl-2-butanethiol</td>
<td>Meaty/Black currant/catty</td>
<td>917/1207</td>
<td>5DP/PEG</td>
<td>5</td>
</tr>
<tr>
<td>3-mercaptohexyl acetate</td>
<td>Box tree, grapefruit</td>
<td>1650</td>
<td>PEG</td>
<td>10</td>
</tr>
<tr>
<td>3-mercapto-hexan-1-ol</td>
<td></td>
<td>1808</td>
<td>PEG</td>
<td>10</td>
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<tr>
<td>3-mercapto-3-methylbutan-1-ol</td>
<td></td>
<td>1620</td>
<td>PEG</td>
<td>10</td>
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<tr>
<td>2-mercaptoethyl acetate</td>
<td></td>
<td></td>
<td></td>
<td>10</td>
</tr>
<tr>
<td>3-mercaptopropylacetate</td>
<td></td>
<td></td>
<td></td>
<td>10</td>
</tr>
<tr>
<td>Furfurylmercaptan</td>
<td>na</td>
<td></td>
<td></td>
<td>7</td>
</tr>
<tr>
<td>(5-methylfurfuryl) mercaptan</td>
<td>na</td>
<td></td>
<td></td>
<td>7</td>
</tr>
<tr>
<td>4-methoxy-2-methyl-2-butanethiol</td>
<td>Black currant-like</td>
<td></td>
<td></td>
<td>9</td>
</tr>
<tr>
<td>3-mercaptopropylacetate</td>
<td>na</td>
<td></td>
<td></td>
<td>6,</td>
</tr>
<tr>
<td>2-(1-mercaptoethyl)furan</td>
<td>na</td>
<td></td>
<td></td>
<td>6</td>
</tr>
<tr>
<td>2-tetrahydrothiophenethiol</td>
<td>Onion, roasty, tropical fruit, meaty, sulfurous</td>
<td></td>
<td></td>
<td>6</td>
</tr>
<tr>
<td>2-methyl-2-tetrahydrothiophenethiol</td>
<td>Tropical fruit, sulfurous, buccu, meaty, black currant</td>
<td></td>
<td></td>
<td>6</td>
</tr>
<tr>
<td>2-methyl-4,5-dihydro-3-furanthol</td>
<td></td>
<td></td>
<td></td>
<td>6</td>
</tr>
<tr>
<td>2-methyl-3-thiophenethiol</td>
<td></td>
<td>1059/1559</td>
<td>5DP/PEG</td>
<td></td>
</tr>
<tr>
<td>3-thiophenethiol</td>
<td>na</td>
<td></td>
<td></td>
<td>6</td>
</tr>
<tr>
<td>3-mercapto-2-methylpropanol</td>
<td></td>
<td></td>
<td></td>
<td>10</td>
</tr>
<tr>
<td>Mercapto-propanone</td>
<td>Sulfurous, meaty, pickled</td>
<td></td>
<td></td>
<td>6</td>
</tr>
<tr>
<td>Furfurylthiol</td>
<td></td>
<td></td>
<td></td>
<td>6</td>
</tr>
<tr>
<td>3-mercapto-2-butanone</td>
<td></td>
<td>780/820/1282</td>
<td>pdms/5DP/PEG</td>
<td>1, 12</td>
</tr>
</tbody>
</table>

Table 2.2 Polyfunctional Thiols in Foods

na – data not available
5) Kumazawa, K. and Masuda, H. 1999
6) Gunert, M., Bruning, J., Emeberger, R., Kopsel, M., Kuhn, W., Thielman, T. and Werkhoff, P. 1990.
7) Tressl, R. and Silwar, R. 1981
8) Schieberle, P. Primary Odorants in Popcorn.
9) Reiners, J. and Grosch, W. 1999.
11) Vermeulen, Pellaud, Gijs, and Collin
12) Zhang and Ho

Pdms - 100% polydimethyl siloxane
5dp - 5% diphenyl, 95% polydimethyl siloxane
PEG - polyethylene glycol
CPP+MP - 14%cyanopropylphenyl 86%methyl polysiloxane
2.5. Precursors to volatile sulfur compounds in Cheddar cheese

2.5.1. Free amino acids in milk

There are two known amino acid precursors to volatile sulfur compounds in bovine milk. These include the free amino acids cysteine and S-methyl methioninesulfonium salt. A precursor to dimethyl sulfide, S-methyl methioninesulfonium salt, has been identified in milk by thin layer chromatography (Keenan and Lindsay, 1968). This compound is believed to be heat labile and undergo degradation in heated milk to form dimethyl sulfide (Keenan and Lindsay, 1968).

The concentration of free cysteine in fresh bovine milk, 0.9 μmole/100ml, was measured by Meihiaia and Kanhal (1992), Csapo et al (1995) reported 11 μg/L. Several studies related to free amino acids in milk have failed to detect the presence of free methionine (Thomas and Mills, 1981).

2.5.2. Peptides in milk

2.5.2.1. Methionine containing peptides

Methionine containing peptides are present in the non-protein nitrogen (NPN) fraction of bovine milk at approximately 3.7 μg/ml (Mehaia and Al-Kanhal, 1992).

2.5.2.2. Glutathione

Glutathione is a tripeptide consisting of the amino acids glycine, cysteine and glutamic acid. This peptide has been associated with the accelerated development of Cheddar cheese flavor in model systems. In slurry consisting of 1 part fresh Cheddar curd and 2 parts salt water, addition of the tripeptide glutathione has been demonstrated to accelerate bacterial growth, increase the concentration of C4 and higher free fatty acids
and soluble protein, and result in the development of a more intense Cheddar-like flavor (Kristofferson et al, 1967).

Addition of cysteine also resulted in accelerated flavor development. However, the flavor was not as desirable as that of glutathione supplemented slurries, and was described as having a distinct sulfide flavor (Kristofferson et al, 1967). Furthermore, addition of glutathione to Cheddar cheese slurries stimulated esterase activity, and was potentially involved in cheese ripening associated feedback mechanisms (Harper and Kristofferson, 1970). Addition of glutathione or cysteine to Cheddar cheese slurries was also observed to elevate the headspace concentration of H₂S and methanethiol (Samples, 1985).

Although glutathione is known to occur naturally in milk, the concentration ranges of this peptide in bovine milk have not been reported in a peer-reviewed journal or other publication.

2.5.3. Milk Proteins

2.5.3.1. Caseins

Milk proteins contain potential precursors to volatile sulfur compounds in cheese, the amino acids methionine and cysteine. Urbach (1995) noted that αs₁ casein, B-casein, and γ-caseins do not contain cysteine, and could not serve as precursors to volatile sulfur compounds. Furthermore, the αs₂ casein and K-kappa-casein contain only a limited number of cysteine residues, and she concluded that casein proteins are probably not a significant source of the amino acid precursor to H₂S (Urbach, 1995). However, given the large amount of casein-derived protein in Cheddar cheese, the above conclusions of Urbach (1995) are open to challenge.
2.5.3.2. Whey proteins

The majority of whey protein is expelled from the cheese curd during the various steps of Cheddar cheese manufacture, and constitutes approximately 1% of the protein in a traditional cheese (O’Keefe et al, 1978). The whey proteins α-lactalbumin, β-lactoglobulin and serum albumin in their native conformation are completely resistant to proteolysis by rennet, plasmin, and starter culture proteases (O’Keefe et al, 1978). Free amino acids in cheese then are not likely to arise from whey proteins.

2.6. Microorganisms and their sulfur amino acid degrading enzymes associated with the formation of volatile sulfur compounds in Cheddar cheese

Lactic acid bacteria play a number of roles in the development of flavor in Cheddar cheese. This subject has been covered in depth in a number of lengthy reviews (Urbach, 1993, 1995, Olson, 1990, Crow et al, 1995, Crow et al, 1993, Law, 2001, McSweeny and Fox, 1993). The relationship between microorganisms and their associated enzymes in the development of sulfur flavor compounds is reviewed below.

2.6.1. Adjunct addition

In addition to acid producing starter cultures, adjunct starter cultures may be added during cheese making to enhance flavor development. Adjunct starters are usually strains of *Lactobacillus caseii*, *Lactobacillus plantarum*, or *Lactobacillus helveticus* (Law, 1999). Strains of lactobacilli isolated from New Zealand Cheddar cheese were added back to Cheddar cheese as adjunct starters and resulted in the formation of “sulfide” off flavors, establishing the importance of lactic acid bacteria in the formation of volatile sulfur compounds in Cheddar cheese (Sherwood, 1939).
Kristofferson and Nelson (1955) isolated from Cheddar cheeses strains of *Lactobacillus casei* that were capable of producing H$_2$S on tomato juice agar. These strains were present in greatest numbers in Cheddar cheeses with the highest flavor scores. It was reported that a direct relationship did not exist between concentrations of free H$_2$S and other sulphhydryl compounds in Cheddar cheese (Kristofferson and Nelson, 1955). Furthermore, a “true Cheddar flavor” required within the ripening cheese the presence of H$_2$S and a correct mixture of other products of bacterial metabolism (Kristofferson and Nelson, 1955).

2.6.2. Enzyme encapsulation

Encapsulation of methionine with methionase enzyme obtained from *Pseudomonas putida* in milk fat capsules and incorporation of enzyme/substrate capsules into cheese resulted in accelerated development of methane thiol; methane thiol alone did not cause formation of a true Cheddar-like flavor (Lindsay and Rippe, 1986).

2.6.3. Cysteine and methionine degrading enzymes in lactic acid bacteria

Enzymes in lactic acid bacteria that degrade cysteine, methionine, and other small sulfur containing, biologically important molecules have been identified (Dobric et al, 1998). Cystathionine gamma-lyase (EC 4.4.1.1) was purified from *Lactococcus lactis* subsp. cremoris SK11 Dobric et al, 1998). This enzyme liberates methane thiol from methionine, and H$_2$S from cysteine. Furthermore, it is active under conditions that simulate cheese ripening, namely pH 5.0-5.4 and 5% NaCl (Dobric et al, 1998). A cystathionine gamma lyase has also been purified from *Lactobacillus fermentum* DT41. Cysteine was the best substrate for this enzyme, and it produced a free thiol group, a
keto-acid, and ammonia from several sulfur containing amino acids and amino acid derivatives (Smacchi and Gobetti, 1998).

2.7. Sulfur containing amino acids and peptides in stress responses in lactic acid bacteria

Glutathione is imported from growth media or synthesized by lactic acid bacteria in response to oxidative stresses in the environment. Stress inducing compounds may include molecular oxygen or hydrogen peroxide (Sherrill and Fahey, 1998). Hydrogen peroxide is a metabolite of certain lactic acid bacteria (Condon, 1987). Pebay et al (1995) recently identified a gene, gor, in Streptococcus thermophilus encoding for the enzyme glutathione reductase, and expression of this gene appears to be regulated by oxygenation of the cell growth medium. This finding is significant, because glutathione reductase activity is associated with oxidative stress responses in other species of bacteria, and suggests that glutathione reductase is part of the oxygen stress response in some lactic acid bacteria. This enzyme uses NADH to reduce glutathione disulfide to 2 equivalents of glutathione (Pebay et al, 1995).

Turner et al (1999) identified an extra cellular protein, BspA that is required for glucose dependent L-cystine uptake. When cells were incubated in the presence of cystine and glucose, large amounts of an unidentified -SH containing compound were exported from cells, which were believed to play a role in protecting the cells from oxidative stress. Furthermore, the intracellular concentration of this compound appeared to be tightly regulated (Turner et al, 1999). It was suggested that the enzyme cystathionine γ-lyase is involved in production of the unidentified thiol. Compared to the parental strain, mutants deficient in this enzyme grew more slowly in the presence of
oxygen and were more sensitive to paraquat, a superoxide generating chemical (Turner et al, 1999).

Shay et al (1988) isolated from vacuum packaged beef a strain of *Lactobacillus sake* possessing a high affinity cysteine transport system and cysteine desulphhydrase activity. This trait was plasmid associated, and could be lost by curing of the cells (Shay et al, 1988).

Several Lactic acid bacteria, including specific strains of *Streptococcus agalactiae, Streptococcus thermophilus,* and *Enterococcus faecalis* possess the ability to synthesize glutathione, whereas other strains of *Streptococcus mutans,* *Enterococcus faecalis,* and *Enterococcus faecium* accumulated glutathione from the growth medium (Newton et al, 1996). Also, the ability of Lactic acid bacteria to import from growth medium and/or synthesize glutathione may be species or even strain dependent (Sherrill and Fahey, 1998). *Streptococcus mutans* was observed to import by a glucose-requiring process glutathione, S-methylglutathione and several peptides containing a disulfide bond and at least one glutathione or γ-GluCys moiety (Sherrill and Fahey, 1998). The above organism efficiently imported glutathione and maintained a relatively steady intracellular concentration of glutathione; intracellular glutathione was also observed to protect cells against a thiol oxidant, diamide, and it was suggested that glutathione accumulation is a protective strategy against oxidative damage (Sherrill and Fahey, 1998). However, glutathione accumulation by *S. mutans* had no effect on growth rate (Sherrill and Fahey, 1998).

*Streptococcus mutans* appears to have at least two transport systems associated with sulphydryl uptake from the growth medium, and at least 2 reductase systems
associated with disulfide reduction (Thomas, 1984). A NADH-dependent reductase appears to be associated with the cell wall/membrane, whereas a glutathione disulfide reductase appears to be located in the cytoplasm (Thomas, 1984). Reduction of disulfides required glucose and occurred under aerobic or anaerobic conditions, release of reduced glutathione or other sulphydryl into the growth media was suggested to be a strategy for protecting the bacteria from oxidative or electrophilic chemicals found in the growth environment (Thomas, 1984).

2.8 The role of reduction/oxidation potential in Cheddar cheese flavor

Oxidation/Reduction potential is a convention used to describe the electron transfer potential of a given chemical reaction. In this convention, electromotive force (emf) is the driving force for the transfer of electrons from one molecule to another (Rawn, 1989). A negative reduction potential indicates that a substance (or system) has a lower affinity for electrons than hydrogen, the standard reference compound for measuring emf, and is thus a reducing agent (Rawn, 1989). Conversely, a positive reduction potential indicates that a substance or system has a higher affinity for electrons than hydrogen (Rawn, 1989).

Oxidation/reduction potential in a closed system such as a ripening block of Cheddar cheese is important in that it determines the oxidation state of sulfur compounds in the system (Kristofferson, 1967). In a closed system, thiol compounds exist in equilibrium with disulfides. The oxidation/reduction (red/ox) potential of the closed system will determine how far the equilibrium is shifted towards formation of thiols or disulfides, with a low potential favoring free thiols, and high potential favoring disulfides.
The reduction/oxidation potential of a free thiol group (-SH) at pH 5.5 = (-)150mV (Kristofferson 1959, 1967).

Red/ox potential in ripening Cheddar cheese can be considered to be a dynamic property. Initially, the red/ox potential in milk is above 0.0, due to diffusion of molecular oxygen from the atmosphere into the milk. After pasteurization, the red/ox potential may be elevated further, due to the inactivation of the enzyme catalase. After addition of starter cultures, pressing of the curd, and sealing the green cheese in wax or plastic bags, the red/ox potential begins to drop, as lactic acid bacteria metabolize molecular oxygen. (Kristofferson and Gould, 1959, 1960).

During Cheddar cheese ripening, a general trend in red/ox is noted to occur:

-104 mV initial
-90 mV after 1 month
-217 mV after 6 months

Deviations from the above red/ox trends generally result in the formation of flavor defects (Kristofferson and Gould, 1959, 1960). Aged Cheddar cheeses with higher red/ox potentials were observed to develop rancid, fermented, or oxidized flavor defects, whereas lower red/ox potentials were associated with unclean and sulfide flavors. (Kristofferson and Gould, 1959, 1960).

The flavor stability of Cheddar cheese after cutting and packaging is related to the poise, or oxidation/reduction capacity of the cheese. Poise has been observed to vary among cheeses, and has been determined to be a good indicator of the retention of characteristic Cheddar flavor after a cheese block is cut (Kristofferson and Gould, 1959,
1960). After cutting, a loss of flavor quality was noted to occur in cheeses that experienced a decrease in active sulphydryl groups with a concomitant increase in pyruvic acid. Coating of the cheese with an effective oxygen barrier immediately after cutting resulted in much better retention of Cheddar flavor quality (Kristofferson and Gould, 1959, 1960).

2.9. Methods of detection of volatile sulfur compounds

Low levels of sulfur compounds are not possible to detect with standard FID detectors, as the combustion of volatile sulfur species in an flame ionization detector will actually depress the flame ionization signal (Gaines et al, 1990, Shearer and Meyer, 1999); optimum detection of volatile sulfur compounds by GC requires the use a mass spectrometer, chemiluminescence, or flame photometric, pulsed flame photometric detector.

2.9.1. Chemiluminescence

When light is emitted from a molecule in an excited state that has been induced by a chemical reaction, the phenomenon is called chemiluminescence (Steadman and Fraser, 1985). Chemiluminescence is used as an analytical detection method for both gas phase and liquid phase samples (Steadman and Fraser, 1985). In gas phase chemiluminescence, the light emission is produced by the reaction of an analyte and a strongly oxidizing gas, such as ozone or fluorine (Steadman and Fraser, 1985).

Several conditions must be met in order for a given chemical reaction to be measured by chemiluminescence. First, a chemical reaction must occur rapidly between a species of interest and a reagent supplied by the analyst, and this reaction must emit a photon of light. Second, the emitted photon must have a wavelength between 200 – 800
nm if it is to be measured with a photomultiplier tube. Finally, the reaction must channel its energy into an electronically excited molecular state with an allowed transition to the ground state (Stedman and Fraser, 1985).

2.9.2. Sulfur chemiluminescence detectors

There are currently three types of gas phase chemiluminescence used in analytical applications: homogeneous reactions at room temperature, homogeneous reactions in flames, and gas/surface heterogeneous reactions (Stedman and Fraser, 1985). Homogeneous flame chemiluminescence is used for detection of sulfur; phosphorous and nitrogen, and halogenated organic compounds. A fuel rich, reduced temperature flame is used in the flame photometric detector for detection of sulfur. An emission is measured from the recombination of sulfur atoms in the flame to form \( S_2 \); the emission appears as a series of bands from 260 to 620 nm. Peaks at 384 or 394 nm are generally chosen for analytical measurement (Stedman and Fraser, 1985). This reaction is susceptible to quenching by hydrocarbons. This chemistry is the basis for flame photometric detectors (Stedman and Fraser, 1985).

Phosphorous and nitrogen compounds may chemiluminesce in an oxygen/hydrogen flame. The emission maximum for the chemiluminescence of HNO is at 690 nm. Organic halogens have been found to chemiluminesce in a hydrogen/oxygen flame, and can be detected at ppm and sub-ppm concentrations (Stedman and Fraser, 1985).

There are 4 species of volatile sulfur that can chemiluminesce: HSO, \( S_2 \), SF\(_2\), and SO\(_2\). In SO\(_2\)-based chemiluminescence, SO is combined with ozone in a vacuum chamber to produce SO\(_2\) and light. This type of chemiluminescence will detect all volatile species
of sulfur with an oxidation state of 4 or less. This reaction produces photons with wavelengths in the region of 260-480 nm (Stedman and Fraser, 1985). SO₂-based chemiluminescence generates a linear and equimolar response to sulfur compounds over 5 orders of magnitude, does not participate in quenching reactions with co-eluting hydrocarbons, and has a selectivity for sulfur over carbon of greater than 10⁸ (Gaines et al, 1990, Shearer and Meyer, 1999). The sulfur chemiluminescence detector is the most selective instrument available for sulfur analysis (Shearer and Meyer, 1999).

The two chemical reactions utilized by the sulfur chemiluminescence detector are:

Primary combustion:  \( S \text{ (with oxidation state of 4 or less)} + O_2 \rightarrow SO \)

Secondary combustion:  \( SO + O_3 \rightarrow SO_2 \) \( + hv \)

Two commercial sulfur chemiluminescence detectors have been developed for the detection of volatile sulfur compounds. They are marketed by Seivers Instruments and Antek Instruments. Both of these instruments are based upon the chemiluminescence derived from the formation of SO₂ from SO and ozone. Both instruments are generally configured as detectors for a gas chromatograph.

In a commercial sulfur chemiluminescence detector, after volatile compounds containing sulfur elute from the separation column in the gas chromatograph, the compounds are drawn under partial vacuum into a primary burner, where they are combusted in a hydrogen rich flame at 800° or 1000°C to produce SO, sulfur monoxide.

The combustion in the primary burner occurs within two concentric ceramic burner tubes. The ceramic material catalyzes the reaction and greatly reduces the detectable limits for sulfur. All molecular species of sulfur with an oxidation state of 4 or less are then combusted to SO. The SO species is drawn under vacuum into a second
combustion chamber, where it reacts with ozone to form SO$_2$, which then chemiluminesces to evolve a photon of light in the 260 - 480 nanometer region. The maximum chemiluminescence for SO$_2$ occurs at approximately 350 nm.

A photomultiplier tube placed in the second combustion chamber measures the chemiluminescence. The photomultiplier tube for the Seivers sulfur chemiluminescence Detector, model 355, is equipped with a UV pass filter that allows greater than 1% transmittance between 240 to 410 nanometers, with 85 % transmittance at 320 nm.

There are a few chemiluminescent emitters that appear to have the potential to interfere with detection of sulfur compounds by SO$_2$ chemiluminescence. However, when one considers the principles of operation of the Seivers 355 sulfur detector, their effects are largely negated. Organic selenium, would be combusted to more oxidized or reduced, non-chemiluminescing forms by the primary burner. Any S$_2$ present should be combusted to SO in the primary burner. Nitrogen containing compounds could potentially be combusted to NO, nitrous oxide, in the primary burner, and react with O$_3$ to form NO$_2$, a chemiluminescing species. The photomultiplier is equipped with a band pass filter that would prevent detection of this wavelength of light.
Table 2.3 Analytically useful chemiluminescent Emitters

<table>
<thead>
<tr>
<th>Chemiluminescent Emitter</th>
<th>Wavelength Region, nanometers</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>CN</td>
<td>383 - 388</td>
<td>Sutton et al., Anal. Chem. 51, 1399 (1979)</td>
</tr>
</tbody>
</table>

2.10. Difficulties associated with the analysis of volatile sulfur compounds

2.10.1. Static headspace sampling

Analysis of volatile sulphydryl compounds identified in Cheddar cheese has traditionally relied upon either static or dynamic sampling of the headspace above Cheddar cheese. The two volatile sulphydryl compounds identified in Cheddar cheese, H$_2$S and methane thiol, have relatively low boiling points, - 60°C and 6°C, respectively
Analytical procedures are complicated by reactions of thiols with materials used to construct headspace-sampling devices, such as borosilicate and quartz glass. These chemical reactions result in the irreversible loss of sulfur containing compounds to glass containers (Schute and Farwell, 1978, Farwell, 1999, personal communications).

The identification of volatile sulfur compounds in food systems is complicated by their chemical reactivity and low concentration. Thiols, for example, are susceptible to oxidation reactions with molecular oxygen and hydrogen peroxide (Barret, 1978). Metallic impurities can rapidly catalyze the formation of disulfides from thiols (Oae, 1991). These metallic impurities in the form of oxides may be present on the surface of lab glassware, G.C. injection ports, or metal surfaces (Farwell and Gluck, 1980, Farwell, personal communications, 1999). Furthermore, quartz and borosilicate glasses that have been rigorously treated with various combinations of acids and bases in order to remove metal oxide impurities from their surfaces still show pronounced reactivity towards thiols and hydrogen sulfide (Farwell and Gluck, 1980). Appendix figure C.1 demonstrates potential mechanisms for the formation of di and polysulfides in Cheddar cheese exposed to air.

Techniques for the analysis of volatile sulfur compounds by headspace sampling have several limitations. For example, H₂S and CH₃SH may adsorb irreversibly onto glass, “deactivated” glass, and stainless steel (Schutte, 1978). Under the most ideal circumstances, the detection limits of CH₃S and H₂S with a purge and trap system with deactivated and silanized glass and metal surfaces are in the low parts per billion range (Schutte, 1978). Several potent thiol compounds have been identified in a number of different foods that have aroma detection thresholds in the low ppb to ppt range (Bolens
and van Gemert, 1993). It is possible that reactive sulfur compounds could make significant contributions to the flavor or aroma of a food product, but not be detected by purge and trap methods.

It has been demonstrated that oxidation of thiols may occur on glass or metal surfaces in a purge and trap type gas chromatograph, resulting in formation of disulfides (Schutte, 1978). Other types of chemical reactions involving sulfur compounds and glass surfaces may occur. An inverse relationship was seen with increased sample holding time between concentrations of H$_2$S and CH$_3$SH -Vs- both COS and CS$_2$, suggesting that C$_2$S and COS are artifacts of the G.C. analytical procedure and are formed from H$_2$S and CH$_3$SH by unknown mechanisms involving quartz and borosilicate glassware (Schutte, 1978). The above observation is significant, since COS and CS$_2$ have been observed in the headspace analysis of Cheddar cheese (Lindsay and Rippe, 1986, Urbach, 1993). These compounds then may actually be artifacts rather than compounds that naturally in Cheddar cheese.

Furthermore, all of the sample preparation techniques traditionally used in the analysis of volatile flavor compounds in Cheddar cheese would expose sulfur compounds to oxidizing conditions. The techniques include solvent extraction, simultaneous distillation extraction, and static or dynamic headspace sampling.

The utility of headspace sampling and gas chromatography for the study of volatile sulfur compounds in Cheddar cheese is limited, even under the most ideal conditions. Even if no oxidative loss of volatiles were to occur, low concentrations of volatile thiols in the headspace could allow these compounds to elude detection.
Recently, it has been demonstrated that the sample matrix may affect the analysis of volatile SH- compounds. Nedjema (1997) demonstrated that addition of either 20% ethanol, caramel coloring or transition metals resulted in the suppression of volatile thiols in the headspace. Presumably, ethanol increases the hydrophobicity of the medium, caramel coloring reversibly binds thiols through the formation of thioacetals and transition metals form a chelation complex with the volatile thiols (Nedjema, 1997).

2.10.2. Artifact formation

A number of sulfur containing flavor compounds in food systems have been shown to be sensitive to heat. For example, the principal character impact compounds of freshly cut onions and garlic, thiosulfinates, have been shown to undergo heat-induced rearrangement to form linear and cyclic polysulfides during analysis by conventional G.C methods. This is most likely due to the high temperatures (200 – 300 °C) encountered in the injection port of a capillary G.C. or G.C. /M.S. (Block, 1992, 1993). Therefore, a large number of sulfur containing artifacts have been reported as actual components of fresh onion and garlic flavor in publications dealing with the analysis of onion and garlic extracts (Block, 1993).

2.10.3 Methods for isolation and detection of thiols

2.10.3.1. Mercury trapping

The difficulties associated with the analysis of organic sulfur compounds in food systems suggest that any approach to extraction of these compounds from a food matrix requires a procedure that would protect the sulfur compounds from oxidation and heat. Recently, a method was developed for the extraction of organic thiols from wine, using p-hydroxymercuribenzoic acid as a chemical trap for thiols. (Tominaga et al, 1998).
Mercury has a strong affinity for sulfur. The word ‘mercaptan’ is derived from ‘mercury capturer’ because of the ability of mercury to capture thiols. Mercury reacts with sulfur containing compounds by forming inorganic or organometallic clusters (Carlton and White, 1990). A mercury atom may form a cluster with 2 to 6 organo-sulfur ligands. Mercury in the oxidation state of (II) is capable of forming complexes with thiourea, dithiocarbamates, thiocarboxylic acids, thiocarboxylates, mercaptides, thiones, and thioethers (Carlton and White, 1990).

Para-hydroxymercuribenzoic acid is an organic mercury compound with an oxidation state of (II). This compound has a specific affinity for thiol anions. The thiolate ion displaces the hydroxyl group from the mercury atom via an SN$_2$-like mechanism. This reagent is therefore unable to react with other biologically occurring sulfur compounds such as disulfides or thioesters (Jocelyn, 1972, Zygment and Stazewski, 1976).

2.10.3.2. Phosphine reagents

Thiols oxidize rapidly when present in small concentrations or when they exist in the form of a thiolate anion (Oae, 1991). In order to work with thiols in a synthetic or analytical environment, it is necessary to use special precautions to protect these compounds from oxidation (Oae, 1991)

Phosphines are a group of organophosphorous compounds that contain three organic ligands and phosphorous, and have the general formula P(R)$_3$. (Cobb et al, 1995). In the presence of water, phosphines will cleanly and quantitatively reduce a disulfide bond to two free thiols (Overman and O’Connor, 1975, Burns et al, 1991). Phosphines are generally utilized as reagents in organic chemistry, their use in protein chemistry is
often limited by the strong odors and poor water solubility associated with these compounds (Burns et al, 1991). A nonvolatile, odorless, water-soluble phosphine, Tris-(carboxyethyl phosphine), TCEP, has been used to cleave disulfide linkages in proteins (Burns et al, 1991). A similar odorless, sparingly water soluble reagent, Tris-(Cyanoethyl phosphine), has been used to cleave disulfide linkages in hydrophobic regions of protein molecules (Burns et al, 1991).

2.11. Retention indices

The position of a peak in a GC chromatogram is influenced by many factors, including the diameter of the gc column, length of the gc column, temperature, carrier gas flow rate, thickness of the stationary phase film, etc (Ettre and Hinshaw, 1994). However, in a given stationary phase, the relative retention of two consecutive peaks is constant at a given column temperature and is independent of the column type and dimensions (Ettre and Hinshaw, 1994).

As no two sets of conditions for an analysis with different columns of the same phase are exactly the same, a convention has been developed to account for differing retention times. This convention was originally proposed by Kovats in 1958, and is based on relative retention times of a series of homologous compounds, the linear hydrocarbons (Ettre and Hinshaw, 1994). With this system, the chromatographic behavior of a given compound may be expressed in a uniform scale, like that of a common temperature scale such as Kelvin, Centigrade or Fahrenheit (Ettre and Hinshaw, 1994). For a more in depth discussion of the mathematical derivation of the Kovats Index System, the reader is referred to the text Basic Relationships of Gas Chromatography by Ettre and Hinshaw.
Relationships necessary to determine the Kovats’ Index of a given compound are reported below. All retention times and related terms are reported in seconds.

1) \[ t_R = t_M + t'_{R} \]

where \( t_R \) is the total retention time in seconds of the analyte

\( t_M \) is the holdup time in seconds for methane, or retention time for methane

\( t'_{R} \) is the adjusted retention time.

Equation 1) is rearranged to determine the adjusted retention time for a given compound

2) \[ t_R - t_M = t'_{R} \]

\( t'_{R} \) is a derived term, and is used to calculate the actual Kovat’s retention index number, \( I \).

\[
100 \times \frac{\log t'_{R_i} - \log t'_{R_z}}{\log t'_{R(z+1)} - \log t'_{R_z}} + Z = I
\]

\( t'_{R_i} \) is the adjusted retention time for the compound of interest

The two \( t' \) terms with a \( z \) subscript are the hydrocarbons that bracket the compound of interest, i. \( z \) is the last hydrocarbon eluting prior to the analyte I, and \( (z+1) \) is the first linear hydrocarbon eluting after I, the analyte of interest.
Z is the hydrocarbon number for the last hydrocarbon that eluted prior to the compound of interest, i. As an example, if the hydrocarbon that eluted prior to i was hexane, the hydrocarbon carbon number would be the integer 6.

2.12. Electronic nose instrumentation

An electronic nose is defined as a system that comprises an array of electrochemical sensors with partial specificity and an appropriate pattern recognition system capable of recognizing simple or complex odors, and it is used primarily for sensory analysis applications. Typical applications of the electronic nose include screening of raw materials for odor taints and defects and the evaluation of consistency of finished products in the food beverage and cosmetic industries.

Current generation electronic noses have the capability to evaluate large numbers of samples; be trained to recognized differences between samples and operate with a high degree of accuracy and precision. Furthermore, it is possible for an electronic nose to interact in real time with automated production processes by linking the electronic nose and production process software programs.

Electronic nose sensors are generally configured in an array of 8 to 32 individual sensors. As the electronic nose technology is still in its relative infancy, there is no standard type of sensor. Sensors may be based upon conducting organic polymers, metal oxides, piezoelectric crystals, quartz crystal surface acoustic wave phenomena, or Mass spectral detection.

Typically, an electronic nose generates a large amount of raw data that includes several simultaneously occurring variables. A computer running a multi-variable statistical analysis software package is required to interpret and analyze the data. This
software is also capable “learning” sensor response profiles of acceptable samples as opposed to unacceptable samples. Methods of data analysis include graphical analysis, Multivariable analysis and neural network analysis.

2.13. Multivariable analysis

Hierarchical Cluster Analysis, HCA, and Principal Component Analysis, PCA, are two statistical methods that can be utilized to evaluate large quantities of multivariable data (Infometrix, 1998). They are useful for exploratory analysis, where it is necessary to check the quality of data, determine its information content, and pinpoint key measurements. It is also useful for establishing whether data may be used for regression analysis or classification model building (Infometrix, 1998).

These types of analyses allow for the graphical display of patterns of association in the independent variables of the multivariable data sets; the statistical algorithms reduce the large and complex data to visual graphics that show correlations among independent variables (Infometrix, 1998).

The primary purpose of HCA is to present data in a manner that emphasizes natural groupings. HCA calculates and compares the distances between pairs of samples, those which are relatively close together in terms of distance are similar, dissimilar samples are separated by relatively larger distances (Infometrix, 1998). The HCA algorithm processes the data by first measuring the multivariable distance between samples. Then inter-sample distances are then standardized, to determine the similarity of data sets or samples (Infometrix, 1998). Finally, after the distances between all pairs of samples have been calculated, the two most similar samples are linked into a cluster.
Separate clusters are then linked together based upon distance of separation, until all samples and clusters have been linked (Infometrix, 1998).

Pirouette software uses a graph known as a dendrogram to show the similarity of samples or variables. Similarity is measured along the top of the dendrogram, with 1.0 corresponding to an exact duplicate, and 0.0 showing maximum dissimilarity. The horizontal lines on the far left of the diagram, leaves, symbolize individual samples. Clusters of individual samples are joined together by horizontal lines known as branches; for a shorter branch, a greater similarity exists between samples or clusters.

Principal Component analysis, PCA, is a multivariable statistical approach that is useful for graphical representation of inter-sample and inter-variable relationships. An important attribute of PCA is that it combines in a linear fashion original independent variables that account for maximum variation.
CHAPTER 3

SENSORY AND ELECTRONIC NOSE EVALUATION OF COMMERCIAL CHEDDAR CHEESE

Application of an electronic nose to correlate with descriptive sensory analysis of aged Cheddar cheese


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3.1. Abstract

The ability of an electronic nose, using a mass detector, to correlate with descriptive sensory analysis of aged Cheddar cheese was determined. A sensory panel (n=12) received 75 hr of training on 17 flavor terms for Cheddar cheese. Eleven aged Cheddar cheeses (≥6 mo age) were acquired as ten to twenty pound blocks. Cheeses were evaluated in duplicate by an electronic nose and by the sensory panel. Data were analyzed by multivariate techniques including cluster analysis and principal component analysis. Differences were noted between the two techniques, but cheeses were placed into similar clusters by cluster analysis for both the sensory and the electronic nose data. While not providing as complete an evaluation of flavor as descriptive sensory analysis, the electronic nose does appear to provide a chemical basis for the differentiation of aged Cheddar cheese aroma.

Key Words: cheese, electronic nose, sensory analysis
3.2. Introduction

Cheese flavor and flavor quality is a critical parameter for marketing. Ultimately, flavor is a sensory perception. Flavor in the dairy industry is evaluated on the industrial level by graders who use a limited vocabulary of common flavor and texture defects to generate a quality grade. Grading is conducted by an individual and is somewhat subjective in nature although a useful tool for rapidly assessing overall quality. Alternatively, flavor can be more analytically described by descriptive sensory analysis. Descriptive sensory analysis is an analytical sensory technique where vocabulary to describe all flavors in a product is generated and then the product(s) are evaluated by a group of trained individuals. The descriptive sensory panel acts as an instrument.

Descriptive sensory analysis, while analytical in nature, can be time and resource-consuming. A minimum of seven panelists is recommended and the panel requires several hours of initial training and then maintenance training thereafter (Meilgaard et al., 1999; Drake and Civille, 2002). Instrumental analysis of flavor is equally time-consuming and often ambiguous in terms of the relevance to sensory analysis of flavor. The electronic nose, an instrument with an array of sensors for volatile compound detection, offers a potential alternative for discrimination of multiple samples. The instrument typically does not provide specific information of the qualitative and quantitative differences between samples, but when calibrated to sensory results or to specific instrumental data, the machine can be used to successfully monitor and differentiate products. A number of different sensors have been utilized in electronic noses, the most common being organic polymers, metal oxides and quartz crystal microbalance. Recently, the mass spectrometer has been used as a sensor, in which the
abundance of each mass analyzed provides a basis for some understanding of the chemical nature upon which the electronic nose differentiates aromas, potentially augmenting the information that can be obtained (Marsili, 1999; Harper, 2000).

Electronic noses have been applied to multiple applications including spoilage determination of beef (Blixt and Borch, 1999), monitoring of sausage fermentation (Eklov et al., 1998), microbial quality of grains (Jonsson et al., 1997; Borjesson et al., 1996), differentiation of strawberry and pear varieties (Hirshfelder et al., 1998; Wiesendfeld, 1997), differentiation of grapefruit juice (Bazemore et al., 1997), and milk microbial quality (Marsili, 1999). The electronic nose has also been applied to cheese (Wijesundera and Walsh, 1998; Jou and Harper, 1998). Previous studies with cheese utilized cheese graders rather than descriptive sensory analysis and utilized Swiss cheese or compared single varieties of several cheeses. The objective of this study was to determine the ability of an electronic nose with a mass spectrometer to correlate with descriptive sensory analysis of aged Cheddar cheese.

3.3. Methodology

3.3.1 Electronic nose

Instrumental differentiation of cheese aroma was conducted with an Agilent Technology Chem Sensor 4400 operating as an Electronic Nose (Kenner, LA). It was comprised of a headspace auto-sampler unit (HP 7649), a mass selective detector (MSD 9753), and a gas chromatograph (GC) (HP 6890). All operating parameters were set directly either from the Chemstation’s instrument control screen or on the GC control panel. Mass scans (MS) were from mass 35 to 200 in negative chemical ionization mode.
(NCI), with methane as the ionizing gas. Each mass unit served as a sensor, with the output being the abundance of the given mass. [Operation in electronic ionization mode (EI) mode did not differentiate as well.]

Shredded cheese (2.5g) was placed in 20 ml headspace vials and capped with a Teflon faced silicon rubber cap. Triplicate vials were randomly placed in the automatic sampler for the head space system. Each vial was equilibrated at 60°C at for 30 minutes. This temperature provided better reproducibility for replicate samples than 40°C, without altering the ratios of the mass abundance for the different mass units. The head space volatiles were then transferred to the GC. The transfer loop was maintained at 90°C to prevent condensation. The loop was filled for 0.15 minutes and total injection time was 1.3 minutes. The GC was equipped with a capillary column (5% Phenyl Methyl Siloxane) with 30.0 m x 250 µm. x 0.25 µm film thickness. Helium was used as the carrier gas at a pressure of 40 psi. One microliter of head space was introduced in a pulsed splitless mode, with a pulse pressure of 75 psi, and the injection temperature was 250°C. The column temperature was 220°C for 6 minutes. No separation of individual components occurred. A purge time of 1.5 minutes was used between samples. Masses that were associated with differentiation of the head space volatiles of the different cheeses were determined by using the PCA loading plot (Beebe et al., 1998) followed by analysis of variance with means separation (PROC GLM) to determine significant differences of these masses between cheeses. Tentative identification of differentiating compounds was determined on the basis of expected masses formed during methane negative chemical ionization mass spectroscopy (Harrison, 1992) using a
list of reference Cheddar cheese compounds compiled by the flavor lab at the Ohio State University.

3.3.2. Descriptive sensory analysis

Twelve individuals, seven male and five female, 21 to 44 years of age were selected from university staff and students based on availability, interest, and a demonstrated liking for cheese. The panel received seventy-five hours of training using the Spectrum™ method on a previously identified language for the sensory evaluation of Cheddar cheese flavor (Drake *et al*., 2001; Suriyaphan *et al*., 2001). One noted addition was that the descriptor sulfur was further subdivided into sulfur/egg-like and sulfur/match-like (*Table 3.1*). Panelists received food treats and monthly gift certificates at local restaurants for their participation. During training, panelists were presented with and evaluated references (food or chemical) and cheese examples for the identified descriptive flavor terms. Panelists also evaluated and discussed cheeses to minimize within-group variability. Cheeses were cut into 1-inch cubes and placed into 2-oz soufflé cups with lids for sensory evaluation. Cheeses were tempered at room temperature for 30 min prior to evaluation and were evaluated individually under white lights using paper ballots. Following evaluation of each cheese, panelists rinsed their mouths with spring water. Expectoration was encouraged but was optional. Each panelist evaluated four cheeses per session. Each cheese was evaluated in duplicate on different days in a randomized balanced block design.
3.3.3. Cheeses

Eleven aged pasteurized milk Cheddar cheeses were selected from six different companies in four geographical regions (Table 3.2). Cheeses varied in age but were aged a minimum of 6 months and were characterized retail as “sharp or aged” prior to sensory or instrumental evaluation. Cheeses were obtained based on selection and availability and were purchased or donated as ten to twenty pound blocks. Cheeses from the same company were produced at the same plant location but were marketed as different types of aged Cheddar. All cheeses were labeled as Cheddar cheeses. Blocks were sliced in half, vacuum sealed and one block of each cheese shipped overnight on blue ice (gel packs) to Mississippi State University. Cheeses were stored at 4°C in the dark until analysis at each site. Instrumental analyses were conducted at the Ohio State University; sensory analyses were conducted at Mississippi State University. Instrumental and sensory analyses of individual cheeses were conducted within 2 weeks of each other to minimize variation due to age differences of cheeses.

3.3.4. Statistical analysis

Analysis of variance with means separation (PROC GLM) and correlation analysis (PROC CORR, Pearson’s product moment correlation) were conducted on sensory data. A one-way model with an unstructured treatment arrangement was used for sensory data. Principal component analysis (PROC PRINCOMP) and cluster analysis (PROC CLUS) were conducted on treatment means for sensory and instrumental data individually. Examination of scree plots was conducted to determine which principal components to retain for further examination (Lawless and Heymann, 1999). For cluster analysis, the average linkage method was used. The cubic clustering criteria in
conjunction with pseudo F and t² was used to evaluate the number of clusters present. Significance was established at p≤0.05. Data were analyzed using SAS Version 8.0 (Cary, NC).

3.5. Results

Both electronic ionization (EI) (data not shown) and methane negative chemical ionizations (NCI) modes differentiated the aroma of the eleven cheeses (Figs 1, 2). Operation in the NCI mode gave considerably better differentiation, which was considered to be due to less fragmentation occurring in the mass detector. Based on the loading plot for the PCA analysis, the differentiation of the cheeses was associated with 26 of the 166 mass units, with the remaining masses being in one very tight cluster (data not shown). These differentiating masses and the compounds associated with them on the basis of most probable mass units formed during chemical ionization are presented in Table 3.3. All of these compounds are known to be present in Cheddar cheese (Maarse et al, 1991).

Chemical ionization mass spectroscopy was originally developed to confirm molecular weights (M) of organic molecules and the most probable masses formed during NCI from a compound of molecular weight M are [M+1] [M+C₂H₅], [(M+1)- H₂O], [(M+1)-CO₂], together with varying amounts of the base compound (M) (Harrison, 1992). In many cases, more than one compound has the same mass unit (Harrison, 1992). M+1 is the most abundant species in most cases, with [(M+1)-H₂O] being common for alcohols, [(M+1)+C₂H₅] being common for esters and [M+1]- C₂H₅] being associated with acids (Harrison, 1992). In terms of tentative identification, the most
credence was given to compounds associated with three of the expected masses. The masses found to be significantly different in amount between cheeses were 59, 61, 73, 73, 77, 89 and 133 (Table 3.3). Compounds associated with these masses included butane thiol, dimethyl sulfide and 4 mercapto-4 methyl-pentan-2-one. The latter compound has been used as a reference compound for sensory analysis of catty aroma and flavor in cheese (Drake et al., 2001).

3.5.1. Sensory analysis

Individual treatment means for the twenty descriptors are shown in Table 3.4. Principal components analysis revealed three components that described 79% of the variance among the cheeses (Figs. 3, 4). Principal component one, which explained 39% of the variance was comprised of the aged flavors overall sulfur, free fatty acid, catty, and age intensity inversely associated with milkfat/lactone flavor. Cheeses that exhibit high intensities of sulfur, catty and aged flavors are not likely to have high intensities of the mild flavor milkfat, which explains why these terms are inversely related. Principal component two (26 % of the variance) was associated with fruity, match-like flavor, sweetness and umami with egg-like flavor and cowy negatively associated. Cheeses that exhibited match-like flavor also tended to exhibit fruity flavor and sweet taste explaining the association of these terms. Match-like flavor and egg-like flavor were inversely related in cheeses (Tables 3.4, 3.5) also explaining their inverse relationship by principal component analysis. Principal component three (14 % of the variance) was positively associated with cooked, whey, diacetyl, and brothy flavors and salty and sour tastes. Nutty flavor was inversely associated with principal component
three. Aged Cheddar cheeses generally are not characterized by the young undeveloped flavors cooked, whey, and diacetyl, hence their loading on principal component three.

Young undeveloped flavors, cooked, whey, diacetyl, milkfat played a consistent but minor role in the overall flavor impact of these aged cheeses. Less noticeable intensities of these flavors would be expected in aged cheeses since stronger aged/developed flavors such as sulfur and brothy develop as Cheddar cheeses age (Drake et al., 2001). Age intensity was high among the cheeses, not surprisingly since the cheeses were very aged. Age intensity was shown to be a meta-term with correlation to multiple descriptors (Drake et al., 2001). Of aged/developed flavors, cowy flavor was only noted at low levels (>1) in two cheeses. Cowy is not a typical flavor in Cheddar cheeses and was noted only in raw milk or international Cheddar cheeses in two previous studies (Drake et al., 2001; Suriyaphan et al., 2001). Free fatty acid (ffa) and fruity flavors were also not detected at high intensities and are not typically detected at high intensities in Cheddar cheese.

Overall sulfur flavor was a dominant flavor in the cheeses. Sulfur flavor is considered a characteristic flavor of Cheddar cheeses (Fox, 1993). The cheese flavor lexicon identified by Drake et al. (2001) also reported sulfur as a key flavor in Cheddar cheese. The term catty was also noted as a separate sulfurous flavor in Cheddar cheese and the chemical compound 4-mercapto-4-methyl pentan-2-one is one mercaptan compound used as a chemical reference to train sensory panelists on this descriptor (Drake et al., 2001) (Polak et al., 1988). The potential to further separate and characterize sulfur flavor into egg-like or match-like was noted (Drake et al., 2001). In the current study, we more closely examined sulfur flavor in aged Cheddar cheeses by
further categorizing sulfur flavor as egg-like or match-like. Catty was considered a separate flavor and overall sulfur was also quantified by panelists.

The aged Cheddar cheeses varied widely in overall sulfur intensity and in egg-like, match-like, and catty flavor intensity. Both overall sulfur and catty flavor were correlated with age intensity (Table 3.5, Fig. 1) as reported previously (Drake et al., 2001). The four sulfur related terms (overall sulfur, egg-like, match-like, catty) appeared to be non-redundant in respect to each other (Figs.1, 2). Intensities varied independently of each other (Table 3.4) and were not correlated with the exception of match-like and catty (Table 3.5).

3.5.2. Cluster analysis and principal component analysis

Both sensory and electronic nose data yielded three clusters with most cheeses being in the first main cluster (Table 3.5). The cheeses are listed in the order of their position within the cluster (from left to right). Cheeses in clusters 1-3 are identical for enose and sensory data when sensory data is analyzed using just the four sulfur-related terms (overall sulfur, egg-like, match-like, catty). Sensory and enose clusters are similar with one exception when all sensory data is included. Cheeses NE3-NE4 and NE5-NE7 each came from the same company. Enose cluster data identified these cheeses as closely related, with grouping in order within cluster 1. Sensory data when all sensory descriptors were included also identified these cheeses as similar with all these cheeses grouped within cluster 1 however NE3 and NE4 were more differentiated within cluster 1. When just sensory sulfur flavors were considered, these cheeses were again grouped within cluster 1, but more differentiated.
Principal component plots (Figs. 1-4) confirmed cluster analyses indicating some similarities in differentiation between the two techniques but also differences. Examination of the sensory principal components provides potential explanation of some of these differences. Cheese M2 was distinct from the other cheeses by both sensory and instrumental techniques. This cheese was most distinct from other cheeses by sensory analysis on principal component two and was characterized by intense egg-like flavor and bitter taste. Cheeses NE1, NE7, and NE5 were positively associated by sensory analysis with principal component two and exhibited high intensities of match-like and fruity flavors (Figs 3, 4). These three cheeses were also closely associated with each other by enose analysis on principal component one (Figs. 1, 2). Cheeses M1, NW2 and NE3 were closely related by sensory analysis on principal component one where they were characterized by moderate levels of overall sulfur and age intensity. These cheeses were also clustered together on principal components one and two by enose analysis. Cheeses NE2, NW1, NE2 were grouped by sensory analysis on principal component one where they exhibited low to moderate sulfur and age intensity flavors (Fig. 3). Cheese NE2 was differentiated from cheeses NW1 and NE4 by the presence of nutty flavors (Fig. 4). These cheeses were not clustered by enose analysis at all (Figs 1, 2).

3.6. Discussion

The electronic nose used in this investigation differed from other studies primarily in respect to the use of a mass spectrometer to evaluate the mass fragments formed during ionization. This provides a basis for gaining some understanding of the chemical basis for aroma differentiation not possible by the electronic noses using the more common
sensors, such as organic polymers, metal oxides and the quartz microbalance (Marsili, 1999; Harper, 2000). To the best of our knowledge this specific sensor (MS/NCI) has not been used previously to make an electronic nose. Negative chemical ionization provides more information about the probable nature of the compounds that contributed to the differentiation of the aroma of the cheeses. There is a lack of mass libraries to aid in the exact identification of compounds from NCI (Harrison, 1992), thus the exact identification of the compounds differentiating the aroma of the cheese will require future research.

Determination of the specific compounds associated with the electronic nose used in this investigation is complicated by the fact that different classes of compounds give different fragmentation patterns and that more than one compound is frequently associated with a specific mass. For example, although M+1 is the most common fragment, [(M+1)-water] is a common fragment for alcohols, (M+C2H5) for esters and [(M+1)-( C2H5)] for acids (Harrison, 1992). Most of the statistically significant mass units, had more than one compound that could have contributed that mass. Mass 58 could arise from propionaldehyde, acetone and/or valeric acid; 73 from valeraldehyde, butanone, 1-butanol thiol and/or isovaleric acid; and 89 could result from fragmentation of butyric acid, methyl propionate, and/or pentanol. We propose that the presence of 3 separate expected masses for a given compound does provide tentative identification that the compound is involved in the aroma differentiation of the cheeses. This would include the compounds ethane thiol, dimethyl sulfide, butyric acid, and 4-mercapto-4-methyl pentan-2-one (Table 3.3).
The eleven aged (>6 months) Cheddar cheeses that were designated as sharp in retail were differentiated by both sensory analysis and by the electronic nose, with similarities and differences between the two analyses. The lack of exact match between the two methods is not unexpected, since there is a good probability that the two methodologies do not measure exactly the same volatile components. Harper and Kleinhenz (1999) demonstrated that electronic nose and sensory thresholds were similar for volatile compounds. However, the electronic nose measures volatile headspace components while mastication in the mouth during sensory analysis will release further volatiles (Delahunty et al., 1996; Druaux and Voilley, 1997). In addition, basic tastes are not evaluated by electronic nose and the cheeses did differ in tastant intensities (Table 3.5). Cheeses that were extremely distinct were differentiated by both sensory and electronic nose (e.g. cheese M2) indicating that the electronic nose would work well for screening cheeses for consistency and quality control. Minor flavor nuances that can be important from a human sensory perspective may require further research between the electronic nose and a trained descriptive analysis panel to appropriately interpret results.

Sulfur compounds are well known to be important in Cheddar cheese flavor (Fox, 1993; Dias and Weimer, 1999). Sulfur/eggy has also been previously described in sensory analysis of Cheddar cheese (Muir et al., 1996; Hulin-Beraud et al., 2000). However, the subdivision of sulfur flavor into total sulfur, sulfur/egg, sulfur/match, and catty has not been previously reported. Sulfur compounds are associated with age and flavor development in Cheddar cheese. All of the cheeses in the current study exhibited some type or types of sulfur flavors as would be expected in aged Cheddar cheese. The individual sulfur flavor compounds, egg-like, match-like, and catty, varied independently
in the eleven cheeses. Clearly, some aspects of sensory and electronic nose
differentiation appear to be similar since 4-mercapto-4-methyl pentan-2-one was a key-
differentiating compound by electronic nose and catty flavor was also a key-
differentiating flavor by descriptive sensory analysis. Further similarities may become
apparent as sensory descriptive language becomes more strongly associated with causant
chemical flavor volatiles and as additional research is conducted to explore and determine
differentiating components by enose analysis rather than application of the instrument as
a “black box”. Additional work in the area of sulfur flavor development and Cheddar
cheese flavor is warranted. Studies are in progress to determine sulfur compounds in
Cheddar cheese in more detail and to determine which of these contribute to the different
sulfur sensory flavor descriptors. Based on this study, the electronic nose using MS
sensors may be used to screen and differentiate aged Cheddar cheeses from a variety of
sources.

3.7. Acknowledgments

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and Forestry Experiment Station (MAFES), Mississippi State University, the North
Carolina State University Experiment Station, and by the J. T. Parker Chair in Dairy
Foods, The Ohio State University. Appreciation is expressed to Dr. P. M. T. Hansen for
his assistance with the statistical analyses of the Electronic Nose data.
3.8. References


**Young/undeveloped flavors**

<table>
<thead>
<tr>
<th>Term</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cooked</td>
<td>Aromatics associated with cooked milk</td>
</tr>
<tr>
<td>Whey</td>
<td>Aromatics associated with Cheddar cheese whey</td>
</tr>
<tr>
<td>Diacetyl</td>
<td>Aroma associated with diacetyl</td>
</tr>
<tr>
<td>Milkfat/Lactone</td>
<td>Aromatics associated with milkfat</td>
</tr>
</tbody>
</table>

**Aged/developed flavors**

<table>
<thead>
<tr>
<th>Term</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fruity</td>
<td>Aromatics associated with different fruits</td>
</tr>
<tr>
<td>Sulfur/eggy</td>
<td>Sulfur aroma associated with hard-boiled eggs</td>
</tr>
<tr>
<td>Sulfur/match</td>
<td>Sulfur aroma associated with a freshly struck match</td>
</tr>
<tr>
<td>Free fatty acid</td>
<td>Aromatics associated with short chain free fatty acids</td>
</tr>
<tr>
<td>Brothy</td>
<td>Aromatics associated with boiled meat or vegetable soup</td>
</tr>
<tr>
<td>Nutty</td>
<td>Nut-like aromatic associated with different nuts</td>
</tr>
<tr>
<td>Catty</td>
<td>Aroma associated with tomcat urine</td>
</tr>
<tr>
<td>Cowy/phenolic</td>
<td>Aromas associated with barns and stock trailers</td>
</tr>
<tr>
<td>Aged</td>
<td>Flavors associated with aged Cheddar cheeses</td>
</tr>
<tr>
<td>Bitter</td>
<td>Basic taste sensation elicited by caffeine, quinine</td>
</tr>
<tr>
<td>Salty</td>
<td>Basic taste sensation associated with salts</td>
</tr>
<tr>
<td>Sour</td>
<td>Basic taste sensation associated with acids</td>
</tr>
<tr>
<td>Umami</td>
<td>Chemical feeling factor associated with certain peptides and nucleotides</td>
</tr>
</tbody>
</table>

**Table 3.1. Sensory evaluation terms for cheese flavor**

Drake et al., 2001
<table>
<thead>
<tr>
<th>Cheese</th>
<th>Company</th>
<th>Geographical location</th>
<th>Age</th>
</tr>
</thead>
<tbody>
<tr>
<td>NW1</td>
<td>1</td>
<td>Northwest</td>
<td>&gt;9 months</td>
</tr>
<tr>
<td>NW2</td>
<td>1</td>
<td>Northwest</td>
<td>&gt;15 months</td>
</tr>
<tr>
<td>M1</td>
<td>2</td>
<td>Midwest</td>
<td>&gt;6 months</td>
</tr>
<tr>
<td>M2</td>
<td>3</td>
<td>Midwest</td>
<td>&gt;6 months</td>
</tr>
<tr>
<td>NE1</td>
<td>4</td>
<td>Northeast</td>
<td>&gt;6 months</td>
</tr>
<tr>
<td>NE2</td>
<td>4</td>
<td>Northeast</td>
<td>&gt;14 months</td>
</tr>
<tr>
<td>NE3</td>
<td>5</td>
<td>Northeast</td>
<td>&gt;11 months</td>
</tr>
<tr>
<td>NE4</td>
<td>5</td>
<td>Northeast</td>
<td>&gt;9 months</td>
</tr>
<tr>
<td>NE5</td>
<td>6</td>
<td>Northeast</td>
<td>&gt;6 months</td>
</tr>
<tr>
<td>NE6</td>
<td>6</td>
<td>Northeast</td>
<td>&gt;6 months</td>
</tr>
<tr>
<td>NE7</td>
<td>6</td>
<td>Northeast</td>
<td>&gt;6 months</td>
</tr>
</tbody>
</table>

**Table 3.2. Cheeses used in the study**
<table>
<thead>
<tr>
<th>Compounds</th>
<th>Associated Mass</th>
</tr>
</thead>
<tbody>
<tr>
<td>45</td>
<td>Acetaldehyde&lt;sup&gt;b&lt;/sup&gt;, Ethane thiol&lt;sup&gt;c&lt;/sup&gt;, dimethyl sulfide&lt;sup&gt;c&lt;/sup&gt;, butyric acid&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>57</td>
<td>butanol&lt;sup&gt;c&lt;/sup&gt;, propionic acid&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>59*</td>
<td>Acetone&lt;sup&gt;b&lt;/sup&gt;, propionaldehyde&lt;sup&gt;b&lt;/sup&gt;, valeric acid&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>60</td>
<td>Acetic acid&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>61*</td>
<td>Acetic acid&lt;sup&gt;b&lt;/sup&gt;, propanol&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>62</td>
<td>Dimethyl sulfide&lt;sup&gt;a&lt;/sup&gt;, Ethane thiol&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>63</td>
<td>Dimethyl sulfide&lt;sup&gt;b&lt;/sup&gt;, ethane thiol&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>71</td>
<td>Pentanol&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>72</td>
<td>Butanone&lt;sup&gt;a&lt;/sup&gt;, valeraldehyde&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>73*</td>
<td>Butanone&lt;sup&gt;b&lt;/sup&gt;, valeraldehyde&lt;sup&gt;b&lt;/sup&gt;, 1-butanethiol&lt;sup&gt;d&lt;/sup&gt;, isovaleric acid&lt;sup&gt;e&lt;/sup&gt;</td>
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<td>74</td>
<td>Butanol&lt;sup&gt;a&lt;/sup&gt;, propionic acid&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>75*</td>
<td>Butanol&lt;sup&gt;b&lt;/sup&gt;, propionic acid&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>77**</td>
<td>Dimethyl sulfide&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>79*</td>
<td>Unknown</td>
</tr>
<tr>
<td>87</td>
<td>diacteylb</td>
</tr>
<tr>
<td>88</td>
<td>Butyric acid&lt;sup&gt;a&lt;/sup&gt;, methyl propionate&lt;sup&gt;b&lt;/sup&gt;, pentanol&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>89*</td>
<td>Butyric acid&lt;sup&gt;b&lt;/sup&gt;, methyl propionate&lt;sup&gt;b&lt;/sup&gt;, pentanol&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>97</td>
<td>2-heptanone&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>100</td>
<td>Hexanone&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>103</td>
<td>Hexanolb, Valeric acid&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>115</td>
<td>4-mercapto-4-methyl- pentan-2-one&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>117</td>
<td>Ethyl butanoate&lt;sup&gt;a&lt;/sup&gt;, Isovaleric acic&lt;sup&gt;b&lt;/sup&gt;, capric acid&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>118</td>
<td>ethyl butanoate&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>131</td>
<td>Caproic acid&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>132</td>
<td>4-mercapto-4-methyl- pentan-2-one&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>133**</td>
<td>4-mercapto-4-methyl- pentan-2-one&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Table 3.3. Compounds associated with the differentiation of aged Cheddar cheese based on matching expected masses {M+1 or [(M+1)- 18] or [(M+29) or M}

*Indicates differences in amounts of this mass between cheeses (p<0.05)
** Indicates differences in amounts of this mass between cheeses (p<0.01)

<sup>a</sup> M  
<sup>b</sup> M+1  
<sup>c</sup> (M+1)-18 [-H2O]  
<sup>d</sup> (M + 29) [+C2H5]
### Table 3.4. Sensory analysis individual treatment means.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>NW1</th>
<th>NE6</th>
<th>M2</th>
<th>NE1</th>
<th>NE7</th>
<th>NE3</th>
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</table>

15-point universal intensity scale (Drake et al., 2001)

¹ abbreviations are cheese treatment designations, NW1, NW 2 – cheeses from Northwest geographical region, M1, M2 - cheeses from Midwest, NE1-NE7 – cheeses from Northeast

² LSD – least significant difference, p≤0.05
Table 3.5. Correlation of sulfur sensory descriptors

<table>
<thead>
<tr>
<th>Descriptor</th>
<th>Egg-like</th>
<th>Match-like</th>
<th>Catty</th>
<th>Age</th>
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***P<0.001

**P<0.01
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<tr>
<th>Cluster number¹</th>
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<th>Sensory (sulfur)</th>
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<td>NW1</td>
<td>NW1</td>
</tr>
<tr>
<td>1</td>
<td>NE4</td>
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<tr>
<td>3</td>
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<td>NE1</td>
<td>M2</td>
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</tbody>
</table>

Table 3.6. Cluster analysis of cheeses

² abbreviations are cheese treatment designations, NW1, NW 2 – cheeses from Northwest geographical region, M1, M2 - cheeses from Midwest, NE1-NE7 – cheeses from Northeast
Fig. 1. Principal component plot from electronic nose analysis of cheeses.
Fig. 2. Principal component plot from electronic nose analysis of cheeses.
Fig. 3. Principal component plot from sensory analysis of cheeses.
Fig. 4. Principal component plot from sensory analysis of cheeses.
CHAPTER 4

METHOD DEVELOPMENT FOR DETERMINATION OF THIOL COMPONENTS IN AGED CHEDDAR CHEESE

4.1. Evaluation of the air sensitive flavor notes of Cheddar cheese

It has been reported that it is necessary for Cheddar cheese to maintain a low oxidation/reduction potential in order to develop a characteristic “Cheddar like” flavor (Kristofferson and Gould, 1959; 1960, Kristofferson et al, 1964; and Kristofferson et al, 1966. Furthermore, Kristofferson et al (1964) reported that the characteristic “Cheddar like” flavor is degraded by exposure of the cheese to air during cutting and wrapping of ripened blocks into consumer packages.

In order to test the observations of Kristofferson et al, (1964) concerning the importance of oxidation/reduction potential in relation to specific sulfur related flavor notes of Cheddar cheese, the following experiment was conducted: A 1lb piece of aged, extra sharp Cheddar cheese was evaluated by two trained dairy products judges for intensity of the following flavor attributes: Cheddar-like flavor, Egg-like flavor, Cat-like, and Sulfide-like flavor. The cheese was then cut in half, with one half being sealed in a 3 mil Nylon/Polyethylene barrier pouch, a packaging material with low gas transmission rates, and the other half was sealed in a Cryovac E-bag pouch. The E-bag pouch is used
for packaging fresh fruits and vegetables, and has a very high gas oxygen transmission rate.

The cheese packaged in the E-bag was then placed in a stainless steel cylinder equipped with a pressure gage and pressurized to 150 psi. The cylinder and nylon/polyethylene packaged Cheddar was then placed in a refrigerator and held at 5°C for 2 weeks. At the end of this time period, the two Cheddar cheeses were then evaluated for the above flavor attributes. For the cheese packaged in the E-pouch and stored under 150 psi, The Cheddar-like aroma was diminished in comparison to the nylon/polyethylene packaged Cheddar, while the egg-like aroma, cat-like aroma, and sulfide-like aroma disappeared in the Cheddar cheese stored in the E-pouch. Savory and salty flavor notes were unchanged. The flavor of cheese stored in the E-bag was still recognizable as that of Cheddar; however, it was more reminiscent of that of a macaroni and cheese powder than an aged sharp Cheddar cheese.

These observations strengthened a hypothesis that there are air sensitive flavor compounds, perhaps thiols, which contribute to the flavor Cheddar cheese. A proposed mechanism for the loss of thiols in Cheddar cheese upon exposure to air is shown in Appendix C, Figure 14.

**4.2. Development of method for detection of very low levels of thiols in Cheddar cheese**

An attempt to directly apply the method of Tominaga et al (1998) to extraction of thiols from Cheddar cheese was not successful. Tominaga et al (1998) extracted thiols from wine by first extracting wine with dichloromethane, and then extracted thiols from
the dichloromethane with a solution of aqueous sodium hydroxide and p-hydroxymercuribenzoic acid.

While wine can be thought of as a food with one continuous phase or fraction, Cheddar cheese can be thought of as a food system with 3 fractions, that of fat, water, and protein. Of the three fractions of Cheddar cheese, the fat phase is perhaps most readily separated from the other 2 phases. The fat phase is also the most likely phase to contain organic thiol compounds, due to their hydrophobic nature.

The water soluble phase of Cheddar cheese contains approximately 5% sodium chloride (Weimer and Dias, 1998), other inorganic ionic salts, as well as lactic acid, free amino acids, and water soluble peptides. These ionic compounds generally have stronger water binding properties than weakly polar organic compounds such as thiols. All of these dissolved ions will increase partitioning of organic flavor compounds from the water phase into the fat phase. Therefore, it is reasonable to assume that the fat phase of Cheddar cheese will contain the highest concentration of thiol compounds.

An attempt to recover thiols from the fat fraction of Cheddar cheese by using the method of Tominaga et al (1998) was unsuccessful. Addition of aqueous sodium hydroxide and p-hydroxymercuribenzoic acid solution to Cheddar cheese oil resulted in the formation of a stable emulsion. Attempts to dilute the Cheddar cheese oil with hexane and then extract with aqueous sodium hydroxide were also unsuccessful, due to the formation of a stable emulsion. Although it was possible to recover the aqueous phase from the Cheddar cheese and oil mixture by centrifugation, this approach was abandoned due to the potential explosion hazard.
4.2.1. Recovery of oil from cheddar cheese

200 gm of Cheddar cheese per bottle was weighed into Nalgene 250 ml wide mouth polypropylene centrifuge bottles and then centrifuged for 40 minutes at 38°C. The Cheddar cheese was centrifuged in a DuPont Sorval centrifuge equipped with a GSA rotor at 10,000 rpm. Approximately 3.5 pounds of cheese was centrifuged per extraction, with a recovery of approximately 75% of the fat, with a total yield of approximately 400 grams of oil.

In order to quantitatively recover the oil fraction from Cheddar cheese, it was necessary to warm the cheese to a point where the fat fraction melts and then centrifuge it at high speeds. Experimentation with centrifugation of a sharp Cheddar cheese showed that optimum recovery, approximately 65-75% of the cheddar oil, occurred at a temperature of 38°C and 10,000 rpm. Centrifugation above this temperature and speed resulted in deformation of the centrifuge bottles.

Recovery of the oil fraction varied among cheeses. No attempt was made to investigate the variability in recovery further, however, it is likely that a number of factors may be responsible for this observation, including the degree of proteolysis of the cheese, pH of cheese, salt content of cheese and amount of water in the cheese.

4.2.2. Solvent preparation

The solvent used to dilute the cheese fat, Optima™ grade Hexane (Fisher Scientific, Pittsburg, PA) has a purity specification for total Sulfur not to exceed 0.005%. All commercially available research grades of hexane originate from petroleum feedstocks. These feedstocks contain organic sulfur in a number of forms, including thiols, disulfide, and thiophene (Zgmunt and Staszewski, 1976). Although research grade
hexane is purified by a number of chemical means, including distillation, it is not possible to obtain research grade hexane that is certified to be free of thiol, disulfide or other polysulfide contaminants. It is possible during the extraction of thiols from Cheddar cheese oil diluted with hexane that hexane could introduce sulfur artifacts. Therefore, as a precaution, the Optima grade hexane was refluxed for 24 hours over fresh sodium metal in order to remove thiol, disulfide and polysulfide contaminants. It was not necessary to address the issue of other organic sulfur contaminants. Only thiols or compounds that can be chemically reduced by the phosphine reducing reagent to thiols, such as disulfides or polysulfides, would be extracted from the hexane by the p-hydroxymercuribenzoic acid.

4.3. Extraction of thiols from Cheddar cheese

During the above lengthy procedure of obtaining the fat from Cheddar cheese, it was unavoidable that air sensitive compounds present in the cheese were exposed to atmospheric oxygen, since oxygen is capable of rapid diffusion into fats, oils, and organic solvents. Thiols present were potentially oxidized, forming disulfides or polysulfides.

The intensity of the “sulfide” flavor note diminished considerably or disappeared in the water and fat during the fractionation process, which included sample preparation and centrifugation. The above observations suggest that in order to recover the thiol compounds from the fat fraction of Cheddar cheese, it would be necessary to utilize a recovery strategy that will maintain the thiols in a chemically reduced state.

4.3.1. Phosphine reducing reagent

Tri-alkyl phosphines are known to reduce organic disulfides smoothly and quantitatively in water (Burns et al, 1991). However, many of these alkyl phosphine reducing reagents possess a very strong, disagreeable odor, somewhat limiting their
usefulness as reducing reagents (Burns et al, 1991). A water soluble, odorless phosphine reagent, tris–(2-carboxyethyl) phosphine, TCEP (Molecular Probes, Eugene, OR), can be synthesized from tris-(2-cyanoethyl) phosphine (Alfa Aesar, Ward Hill, MA), which is also an odorless phosphine (Burns et al, 1991).

TCEP cleanly and quantitatively reduces protein disulfides, oxidized lipoic acid, and oxidized dithiothreitol to thiols. TCEP is useful for reducing disulfide linkages in the hydrophilic regions of proteins, and tris-(2-cyanoethyl) phosphine may be used to reduce disulfide linkages buried within hydrophobic regions of proteins (Molecular probes, 2002). The reaction of TCEP with disulfides is so fast at pH 4.5 that it is difficult to measure (Burns et al, 1991). TCEP is also an excellent reducing reagent for disulfides under basic conditions, and has been found to be a better reducing reagent than dithiothreitol at a variety of pH values (Han and Han, 1994). Aqueous TCEP oxidizes rapidly in an open container at high pH, 9.0 –10.0, in the presence of air (Burns et al, 1991), however, when aqueous TCEP is stored at a low pH in a capped vial, it is stable (Han and Han, 1994).

Experiments in this laboratory have demonstrated that tris-(2-carboxyethyl) phosphine and tris-(2-cyanoethyl) phosphine are suitable for use as a reducing reagents in an analytical strategy to extract organic thiols from a fatty sample matrix. tris-(2-carboxyethyl) phosphine was used in a concentration of 0.05 Moles. It should be noted that tris-(2-cyanoethyl) phosphine is only sparingly soluble in H₂O, and is not suitable for use as a reducing reagent unless it is dissolved in a solution containing at least 10% acetonitrile. The mechanism for the reduction of a disulfide by a phosphine is demonstrated in Appendix C, Figure 15.
4.3.2. Sonication and nitrogen purge of samples

In order to protect thiols in the Cheddar cheese solution from oxidation, some sample handling procedures were devised. The solution of Cheddar fat and hexane was degassed by sonication in a Fisher Scientific Sonication bath for 15 minutes. For an additional 5 minutes, the solution was sonicated and 50 ml/minute of 99.999% pure nitrogen was used to purge the headspace.

The aqueous solution containing phosphate buffer, tris-(2-carboxyethyl) phosphine, and p-hydroxymercuribenzoic acid was treated in a similar manner.

Note: As a precaution, lights in the laboratory were dimmed whenever p-hydroxymercuribenzoic acid was handled as a neat powder or in solution, as a precaution against causing degradation of this light sensitive reagent.

4.3.3. Internal standard

An internal standard was added to the hexane/cheese oil solution prior to addition of the phosphine and mercury salt solution, to determine the concentrations of sulfur compounds extracted from the Cheddar cheese oils. 20 ml of pentane was measured by weighing out 12.52 grams of pentane into a silanized 27 ml crimp top Carlo Erba headspace vial (Restek, Bellefonte, PA). The headspace vial was previously silanized by treatment with Aquasil™ (Pierce Chemical, Rockford, IL) by the procedure of Farwell and Gluck (1980). 10 ul of 3-methoxythiophenol, 99% (Lancaster Synthesis, Pelham, NJ) was then added to the pentane solution with a 10 ul Hamilton model 1701 Teflon tipped plunger syringe (Reno, NV). The solution was then capped with a Restek crimp top PTFE/Silicon seal (Bellefonte, PA), and stored at −20°C.
A Teflon coated magnetic stir bar, 51 mm x 13 mm, (Fisher Scientific, Pittsburg, PA) was added to the combine cheese oil and extraction solutions. Finally, a 10 ul aliquot of the internal standard solution was added to achieve a concentration of 3.2 ppb as S in the cheddar cheese oil. The headspace of the combined solution was then purged with nitrogen for 2 minutes at 200 ml/minute, and the solution was stirred at a speed of 5, using a Fisher Small Vessel Stirrer (Fisher Scientific, Pittsburg, PA). The solution is stirred for a total of 6 hours.

\[
\frac{(10 \times 10^{-3})(32 \text{gms sulfur} / 140 \text{gms/mole of ISTD})(1.13 \text{ gms/ml ISTD}) \cdot (10^{-3})}{(20 \text{ ml})(400 \text{ ml})} = 3.2 \times 10^{-9} \text{[S]} \text{ or 3.2 ppb Sulfur}
\]

### 4.3.4. Effect of buffering agents on extraction

In order to trap a thiol with p-hydroxymercuribenzoic acid, it is necessary to maintain the thiol in a reduced state and maintain the pH of the medium so that the thiolate anion (RS⁻) will be formed. Organic thiols have pKa’s of approximately 10.6, therefore, it is necessary to utilize a buffer that will maintain the extraction medium in a pH above 8.6. In addition, p-hydroxymercuribenzoic acid is a very weak acid, due to inductive donation of electrons to the benzoic acid portion of the molecule by the mercury atom. In order to maintain p-hydroxymercuribenzoic acid in the form of a water-soluble salt, the pH of the extraction solution should be maintained above at least 9.0.
Appendix C Figure 16 demonstrates the capture of a polyfunctional thiol by p-hydroxymercuribenzoic acid, under conditions of basic pH.

Sodium hydroxide was suitable for use as a buffer in water/ethanol solutions, however, with acetonitrile/water solutions, it was observed to cause excessive fat hydrolysis.

0.1 Molar Dibasic sodium phosphate buffered with NaOH was found to be suitable for maintaining the extraction solution at a pH of 10 with minimal fat hydrolysis.

In order to minimize formation of esters during the workup of thiol extracts for Mass Spectral analysis, acetonitrile/water was used as the extraction solvent, and dibasic Sodium Phosphate was used as the buffer. 0.15 gm dibasic sodium phosphate, 0.105 gm NaOH, and 0.025 gm p-hydroxymercuribenzoic acid was dissolved in 75 mls H2O in a 125 ml Erlenmeyer flask with a 24/40 ground glass neck. Then a silicon septum was used to seal the flask, and it was sonicated and purged with nitrogen for 5 minutes. 25 ml acetonitrile was then added, and the solution was sonicated and purged with nitrogen for 5 minutes. Finally, 0.25 gm of TCEP was added, and the solution was sonicated and purged with nitrogen.

4.3.5. Effect of solvents on extraction

An emulsion is formed when an aqueous solution containing tri-sodium phosphate, TCEP, and p-hydroxymercuribenzoic sodium salt is added to a mixture of hexane and cheddar cheese oil and stirred by use of a magnetic stir bar. This emulsion becomes unstable when a water-soluble organic solvent such as methanol, ethanol or acetonitrile is added. Therefore, in order to extract thiol compounds from the oil fraction
of Cheddar cheese, it is necessary to add a water-soluble solvent to prevent the formation of a stable emulsion.

The number of commonly available solvents that are both miscible in H$_2$O and immiscible in hexane is limited to methanol, acetonitrile, and dimethyl formamide. Ethanol is miscible in both water and hexane. Three solvents were studied for use in the extraction of thiols from Cheddar cheese oil, methanol, ethanol and acetonitrile.

Methanol and ethanol are both alcohols, and classified as hydroxylic solvents, i.e. polar solvents that contain an OH group and may act as proton donors under basic conditions. Acetonitrile is a polar, aprotic solvent. All 3 solvents were determined to be suitable in preventing the formation of an emulsion. Ethanol was the best, followed by methanol and then acetonitrile.

Ethanol, when used as a solvent, caused less hydrolysis of the cheese fat than did acetonitrile. However, use of ethanol complicated attempts to determine the Kovat’s indices of the sulfur compounds that were extracted. A large number of peaks were observed in the Flame Ionization chromatograms of extracts that were obtained by using ethanol and water as extraction solvents. These peaks were so large that they made estimation of Kovats’ indices impractical. These peaks may be caused by the formation of fatty acid esters when the extraction solution pH is lowered to 6.5 and the extract is eluted through an ion exchange column. Although the pH of the solution is still at least two log units above the pKa for saturated fatty acids, the pH within the ion exchange resin was 4.3 pH units. At this pH, formation of esters by the reaction of free fatty acids with methanol was possible.
Although acetonitrile was observed to cause more fat hydrolysis than ethanol, it was judged to be suitable for use as an extraction solvent. FID chromatograms were relatively clean of interfering peaks, so this solvent was suitable for extraction of thiols and determination of their Kovat’s retention indexes.

Acetonitrile, when used in the above procedure with NaOH as a pH buffering agent to extract volatile thiols from Cheddar cheese oil, at concentrations of 25% and 50 % in an aqueous solution, was observed to cause excessive fat hydrolysis. The pH of the acetonitrile/water solution, when buffered with NaOH, is approximately 13.0.

Results were not determined quantitatively, however, it was difficult to elute the extraction solution through the ion exchange resin, due the extremely low flow rate caused by the build up of free fatty acids above and within the ion exchange resin bed. Acetonitrile caused less fat hydrolysis when the pH of the extraction medium, (approximately 9.5) was controlled by using di-sodium phosphate buffer.

4.3.6. Post extraction sample handling

After the above solution completed stirring, it was allowed to settle for 1 hour. Then the mercury salt containing water layer was pipetted off of the bottom of the flask. Next, the solution was diluted with 2 volumes of de-ionized water. Then, the solution was poured into a 250 ml Nalgene narrow neck centrifuge bottle and centrifuged in a DuPont Sorval centrifuge equipped with a GSA rotor at 8,000 rpm and 25°C.

The bottom layer was then carefully removed from the centrifuge bottle by pipetting, so as to not disturb the top layer. If oil droplets were visible at the top of the extract or a hazy material visible within the resulting extract, the solution was again centrifuged and handled by the above methods to remove this material.
4.4. Effect of ion exchange resin form on extraction

The method of Tominaga et al (1998) for the extraction of volatile thiols from wine calls for the use of the chloride form of the Dowex 1X2 Strongly basic anion exchange resin, in the chloride form. The pH of the extraction solution containing the p-hydroxymercuribenzoic acid is to be carefully lowered to a pH of 7.0, and then eluted through the ion exchange resin. Presumably, p-hydroxymercuribenzoic acid is in the form of its sodium salt, and it undergoes an ion exchange with the Dowex 1X2 resin, and is immobilized on the resin, and the net product of the reaction is NaCl.

However, titration of 0.025 gms p-hydroxymercuribenzoic acid, sodium salt, with 0.05 N HCl, suggested that the majority of the p-hydroxymercuribenzoic acid was in the acid form, not the sodium salt form. The beginning point of the titration was at approximately 9.5, and the endpoint of the titration was at approximately 6.5, with all of the sodium form of the p-hydroxymercuric acid mercury salt being converted to the acid form. Therefore, if the mercury salt was captured by the chloride form of the ion exchange resin, it was by filtration rather than ion exchange.

The mercury trapping salt, p-hydroxymercuribenzoic acid, is a weaker acid than benzoic acid, due to the covalent bonding of mercury to the aromatic ring. At a pH of 7.0 as called for in the procedure of Tominaga et al, (1998), p-hydroxymercuribenzoic acid exists mainly in the form of the H₂O insoluble protonated acid, rather than as the water soluble sodium salt.

In order for the mercury salt to bind to the ion exchange resin, the weak acid, p-hydroxymercuribenzoic acid, must ionize and give up its proton and bind to the ion exchange resin, liberating a chloride ion. The “spectator ions”, a proton and a chloride.
ion, constitute a strong acid, hydrogen chloride. Simple acid-base chemistry suggests that the protonated form of the mercury salt is not capable of quantitative binding to the ion exchange resin, for as a general rule, the salt of a strong base does not react with a weak acid to form a strong acid.

Some attempts were made to manipulate the acid/base chemistry of the ion exchange resin and the p-hydroxymercuribenzoic acid salt in order to affect the quantitative capture of the mercury salt on the ion exchange resin. A solution containing the protonated form of the mercury salt, p-hydroxymercuribenzoic acid, was quantitatively captured by the strongly basic form of the ion exchange resin.

However, when Cheddar cheese was extracted with ethanol/water based solution, and the pH lowered to 6.0, and solution was run through the Dowex 1x2 ion exchange resin, OH form, and the eluate pH was monitored with a combination pH electrode (Acumet, Denver, CO) and Acumet pH meter (Denver Instruments, Denver, CO). The slightly acidic solution overwhelmed the ion exchange capacity of the resin, after about 25 - 30% percent of the material had passed through the column.

4.4.1 Preparation of ion exchange resin column

First, 10 ml of de-ionized water is added to a 25 ml burette (Fisher Scientific) with 1/10 ml graduations, equipped with a Teflon stopcock. Then, a plug of pesticide grade glass wool is inserted into the column with a 100 cm long glass rod. The glass rod is then used to tap the glass wool against the bottom of the burette to remove pockets of air trapped in the glass wool. Next, the ion exchange resin is fully hydrated with 18 mΩ deionized water, and poured into the burette, to a volume of 12 ml of resin.
The ion exchange resin is then rinsed with 150 ml of 3 N HCl, and then 150 ml of de-ionized, 18 mΩ D.I. H₂O.

4.5 Release and recovery of volatile thiols from ion exchange resin

The pH of the mercury salt solution is reduced to 6.5 by adding, drop wise, a 0.5 N solution of HCl. Then the mercury salt solution is slowly added to the ion exchange resin, and allowed to percolate through the resin at the rate of 1 ml/min. Then the resin is then washed with 75 ml of pH 6.0 Sodium Acetate, 0.1 M, and NaCl, 0.1 M.

The volatiles are released from the ion exchange resin by percolating for 30 minutes using a cysteine solution (1.5 gm cysteine HCl/60ml D.I. H2O) previously extracted 3 times with pesticide grade diethyl ether (Fisher Scientific), 1.5 mls/extraction. Appendix C Figure 17 demonstrates the liberation of a thiol bound to p-hydroxymercuribenzoic acid. A control was run, by taking hexane refluxed over sodium metal, combining it with TCEP, p-HMBA, water, ethanol and sodium hydroxide. The water-soluble fraction was separated and applied to the ion exchange column. Subsequently, it was eluted with the cysteine solution. The eluate was extracted with ether: pentane (1:1) and then concentrated by evaporation. The concentrate was then run on the GC with the sulfur detector. No sulfur peaks were detected.

The eluate containing the volatile thiols released from the column by cysteine is collected in a 100 ml round bottomed flask that is placed in an ice-water bath. The eluate is then extracted with 7.5, 3, and 3 mls of a solution of 1:1 pesticide grade diethyl ether (Fisher Scientific) and pesticide grade pentane (Fisher Scientific). The organic phases are combined and dried over anhydrous sodium sulfate 99.99% (Alfa Aesar). The volatiles
are then transferred to a silanized concentration tube, placed in a modified gas bubbling tube, and concentrated under a flow of nitrogen.

4.6. Analysis of volatile thiols by gas chromatography with SC detection and mass spectrometry detection

Method 1: Gas chromatography analysis of the thiols was performed using a Hewlett Packard 5890 Series II plus GC, equipped with electronic pressure control. Data integration was performed using Hewlett Packard 3365 Series II Chemstation software, on a Hewlett Packard Vectra VL2 486/50 personal computer. The column used for chromatographic separations was a Restek RT-5, 5% diphenyl/95% dimethyl polysiloxane (Restek, Bellefonte, PA) of the following dimensions: 30 meter length, 0.32 mm internal diameter, with a film thickness of 1.0 microns. The RT-5 column was fitted with a 5 meter x 0.32 mm I.D Siltek guard column. The guard column was connected to the analytical column with a Restek Siltek Press-tight connector (Restek, Bellefonte, PA). The injection port was equipped with a Merlin High Pressure Microseal (Restek, Bellefonte, PA) and a Siltek Double Gooseneck inlet liner (Restek, Bellefonte, PA).

A Seivers model 355 Sulfur Chemiluminescence Detector (SCD), equipped with a quartz burner tube, was utilized for detection of sulfur compounds. Oxygen was used as a combustion gas, and the oxygen flow rate into the sulfur detector was maintained at 8.5 ml/minute. The flow of hydrogen into the sulfur detector was maintained at 100 ml/minute. The pressure in the ozone chamber of the sulfur detector was maintained at a pressure of 190 - 250 Torr.

Initially, samples were run under the following conditions:

Injection mode: splitless, 8 ul sample per injection

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Inlet pressure: 80 psi

Temperature program:
Inlet temperature: 230°C
Initial Oven temperature: 27°C
Initial oven hold time: 2.00 minutes

Temperature ramp: increase 5°C/minute, to 250°C. Hold at final temperature for 10 minutes

Pressure Program:
Initial Pressure: 80 psi
Initial time: 0.50 minutes

Decrease pressure 99.0 psi/min to final pressure of 11.5 psi, hold 2.0 minutes
Increase pressure at 0.11 psi/min to final pressure of 16.5 psi. Hold 9.75 minutes

A problem was encountered with the SCD, in that it began to experience a buildup of Pyrolized carbon on the burner tubes. This Pyrolized carbon, also known as “coke” causes a loss in detector sensitivity of several orders of magnitude. In order to overcome this problem, the method was changed so that less solvent was injected into the GC, and the sample was also split between the FID and SCD to measure Kovat’s retention indexes. The new method developed to overcome the coking problem is outlined below.

Method 2: This method is identical to the above method, with the exception of the following changes:
Injection mode: splitless, 4 ul thiol sample + 1 ul Kovat’s Index hydrocarbons per injection

Inlet pressure: 50 psi

Temperature program:

Inlet temperature: 230°C

Initial Oven temperature: 6°C

Initial oven hold time: 15.00 minutes

The outlet of the RT-5 GC column was fitted with a Restek Siltek treated Universal Angled “Y” connector. Two ends of the “Y” connector were fitted with a 40 cm long 0.25 mm ID section of Restek Siltek tubing (Restek, Bellefonte, PA), and a 60 cm long section of Restek 0.10 mm ID Intermediate Polarity tubing (Restek, Bellefonte, PA). The 0.10 section of tubing was connected to the inlet for the sulfur detector, and the 0.25 mm ID tubing was connected to the Flame Ionization Detector of the gas chromatograph.

Kovats’ Index hydrocarbons were detected by use of the HP Flame Ionization Detector (FID).

4.7. Approaches to control SCD coking

The Seivers sulfur Chemiluminescence Detector uses a hydrogen rich flame at 800°C to convert Sulfur compounds in an oxidation state of 4 or less to sulfur monoxide. Hydrocarbons eluting from the GC column and passing into the sulfur detector, if present in sufficient quantity, will overwhelm the limited oxidative capacity of the burner. Instead of being converted to carbon monoxide and passing into the ozonolysis chamber, the hydrocarbons undergo pyrolysis reactions and deposit carbon on the surface of the
quartz or ceramic burner tubes. This will reduce the efficiency by which the burner tubes catalyze the conversion of sulfur compounds to sulfur monoxide, and decrease the sensitivity of the detector.

The Seiviers model 355 SCD is designed for use as a detector for a GC operating in a split mode of sample injection. In this mode of operation, the vast majority of solvent is diverted from the flow path of the sulfur detector. A pulsed pressure injection of 8 ul, as was used in the injection of samples in this research project, will introduce 80x more sample than typical injection conditions of 1 ul of solvent injected with a 10:1 split ratio.

Decreasing the amount of sample injected in the splitless mode from 8 ul to 4 ul and finally 1 ul still resulted in the formation of coke deposits on the burner tubes.

The Seivers SCD can utilize high purity air or oxygen for supporting combustion in the primary burner, and for ozone generation. When air is used as a combustion gas, the recommended flow is 40 ml/minute. The recommended flow for Oxygen is 8 ml/minute. Use of Oxygen has some advantages. It is easier to maintain a proper vacuum in the detector, since the total volume of hydrogen fuel and combustion gas flowing into the Sulfur Chemiluminescence Detector is reduced by approximately 23 %. Second, it allows the use of the decoking valve. The decoking valve is designed to increase the flow of oxygen into the detector for a short time period, in order to burn off coke deposits from the burner tubes. The use of Oxygen also would allow for the use of more oxidizing conditions in the burner.

Oxygen flow may be increased to 10 ml/min without appreciable loss of sensitivity. Increasing oxygen flow to 12 ml/min results in the loss of some detector
sensitivity of approximately 20–30%. Increase of oxygen flow above 12 ml/min resulted in an increase in the detector baseline signal, from 0.3–0.6 to 1.2–1.7. This is attributed to oxidation occurring on the tip of the burner probe. Increasing the relative amount of oxygen increases the temperature of the combustion zone within the burner. Disassembly of the burner after running at 14 ml/min of O₂ for approximately 2 hours showed significant damage to the inner burner probe. The tip of the probe was deformed and appeared to have melted and had a smooth glassy appearance on the tip. Increasing the amount of oxygen in the burner increases the temperature of the combustion zone.

Decreasing the rate of flow of hydrogen produced a similar effect. Increasing the net amount of oxygen will increase the temperature of the combustion flame and result in damage to the inner probe.

Several attempts were made to overcome the coking problem by increasing the amount of oxygen used in the burner, however, none of these attempts were successful, even when sample volume was reduced to 1 ul.

Other attempts at elimination of coking included: regeneration of ceramic probes after each analysis, installation of a quartz outer probe, and decreasing the temperature of the oven at injection to 4°C. None of these methods were successful in elimination of coking, however, they slowed the onset of the problem significantly.

4.8. Multidimensional gas chromatography

Multidimensional Gas Chromatography is a term used to describe the application of pressure balancing to gas chromatography, which, in turn, allows the chromatographer several new options. Portions of a GC analysis may be diverted from the flow path of the gc to a separate analytical column, a detector, or a cryogenic trap. Additionally,
Multidimensional Gas Chromatography may be used to vent excessive amounts of solvent, i.e. a solvent front, from the flow path of the GC.

Attempts were made to use a multidimensional GC approach to overcoming the coking problem caused by the hydrocarbon solvent. These approaches were successful. The multidimensional GC was able to successfully split of the hydrocarbon solvent from the system and prevent coking. However, a significant band broadening and peak tailing effect was observed with thiols, so this approach was abandoned.

4.9. Analysis of volatile thiols by gas chromatography with EI and NCI MS detection

A Hewlett Packard 6890 GC equipped with a Hewlett Packard 5973 Mass Selective Detector, capable of operating in Electron Impact or Chemical Ionization modes of detection, is used for analysis of samples for identification of sulfur compounds. The conditions used in the analytical procedures are as follows:

Injection mode: splitless, 4 ul sample per injection
Inlet pressure: 40 psi
Inlet temperature: 230°C
Initial Oven temperature: 27°C
Initial oven hold time: 2.00 minutes
Mass spectrometer Solvent Delay: 5.0 minutes
Temperature ramp: increase 5°C/minute, to 250°C. Hold at final temperature for 10 minutes
Pressure Program:
Initial Pressure: 40 psi
Initial time: 0.50 minutes

Decrease pressure to 9.76 psi, maintain constant flow conditions for the remainder of the analysis.

Mass Spectrometer Voltage: 70 electron Volts potential, with Hewlett Packard

The conditions for sample analysis in Negative Chemical ionization mode are identical to those used for in Electron impact mode, except that 40 ml/minute of 99.999% pure methane was supplied to the Hewlett Packard 5973 Mass Selective Detector as an ionization buffering gas.

4.10. Conversion of raw sulfur chemiluminescence data to concentration of a given sulfur compound

The sulfur chemiluminescence detector is linear in its response to sulfur compounds over 5+ orders of magnitude of concentration (Shearer and Meyer, 1999, Gaines et al, 1990). The Sulfur Chemiluminescence detector deviates from this linear response only at very high concentrations of sulfur (Gaines et al, 1990), which is well above the concentration of compounds measured in this study.

In order to determine the concentration of individual compounds appearing in the chromatograms in terms of sulfur, the raw data, given in terms of “HP Counts” obtained from the total peak area underneath each peak, was compared to the total peak area underneath the internal standard peak. The range of concentration of compounds could be determined from high parts per trillion to at least 100’s of parts per billion. The lower limit of concentration was reached when the signal to noise ratio was below 3:1, at an approximate concentration of high parts per trillion of sulfur. Also, the concentration of compounds tentatively identified by Kovat’s Indices was generally within +/- 1 order of
magnitude of the concentration of the internal standard. Some later eluting compounds had concentrations of sulfur in the hundreds of parts per billion; however, this is still within the linear range of the sulfur detector.

Data was expressed in terms of ppb in the oil phase of the Cheddar cheese. The peak area for the internal standard compound was assigned the numerical value in parts per billion in which it was added to the cheddar cheese fat prior to extraction, either 3.89 or 9.72 ppb. Concentration of sulfur in a given thiol compound could then be determined by comparing the peak area for that compound to that of the internal standard.

Since the SCD only measures the concentration of sulfur, it was necessary to convert the concentration of sulfur to total concentration of the compound in question. This was done by using the following equation:

\[
\frac{(\text{Estimated molecular weight of compound, gms/mole})}{(\text{Concentration of sulfur}) \times \text{(Weight of sulfur atom, 32 gms/mole)}} = \text{total concentration of compound}
\]

### 4.11. Development and use of specialized glassware

In order to reduce the loss of thiol compounds during the various steps of the analytical procedure, a number of approaches were taken. First of all, the GC inlet liner, press tight connectors, y-shaped splitting tubes, and guard column were all treated with Siltek, a proprietary polymeric silanized coating developed by Restek Corporation (Bellefonte, PA). This silanized coating material has been demonstrated to be the most
chemically inert surface available for the coating of GC inlet liners, guard columns, and connectors, and was recommended by the manufacturer of the Sulfur Chemiluminescence detector (Randy Shearer, personal communications).

An evaporative concentration tube is used to concentrate samples for GC analysis. In order to decrease oxidation of thiols at the surface of the concentration tubes, they were treated with a procedure to remove metallic contaminants from the surface of the glass by soaking them in solutions of Nitric Acid, Hydrochloric acid, and ammonium hydroxide. The flint glass concentration tubes were then treated with a silanizing reagent, Aquasil (Pierce Chemical, South Belloit, Il), found to be the best commercially available material for passivation of glass toward oxidation of thiols (Farwell and Gluck, 1980).

Finally, in order to retard the oxidation of thiols during the evaporative concentration step, an evaporation chamber that would enclose the sample in a nitrogen blanket was constructed from a Pyrex Tall-form bottle. A Pyrex Tall-form bottle was modified by cutting off approximately 2 inches from the bottom of the central tube. Also, the outlet of the bottle was fitted with a 1 cm section of silicon tubing, and a Fisher borosilicate disposable pipette was inserted into the tubing to serve as a back flow restrictor.

4.12. Confirmation of selectivity of SCD

A number of peer-reviewed articles have reported that the sulfur chemiluminescence detector has a selectivity of sulfur -vs.- hydrocarbons of approximately $10^9$ to 1. However, it was not known if this response was similar for
oxygenated organic volatile compounds, such as free fatty acids, which may be generated as an artifact of extraction of the volatile sulfur compounds from the fat fraction.

In order to test the selectivity of the SCD for oxygenated organic compounds, 1 ul of a solution of 5000 ppm of acetic acid was injected a total of 5 times into the GC, using a 0.32 mm ID x 30 meter HP FFAP column. No discernable deflection was noted in the Chromatogram for the Sulfur detector upon elution of acetic acid. Furthermore, in injections where the effluent from the GC column was split between the Sulfur Chemiluminescence detector and the Flame Ionization Detector, it was noted that the solvent peak would cause a maximum response for the flame ionization detector; the simultaneous signal on the Sulfur Chemiluminescence detector registered a slight increase in the baseline. However, this increase in the baseline was less than 1% of the total scale for the Sulfur Chemiluminescence detector. It was concluded that the SCD was highly selective for sulfur compounds -vs.-highly oxygenated hydrocarbons.

4.13. **Confirmation of sensitivity of extraction**

A moderately polar thiol-containing internal standard, 3-methoxythiophenol, was selected due to its ability to form sharply eluting peaks on both a relatively non-polar 5%Diphenyl 95% dimethylpolysiloxane GC column, as well as a polar FFAP column. Other compounds were evaluated for use as the internal standard. 1-octanethiol gave sharply eluting peaks on a 5%Diphenyl 95% dimethylpolysiloxane GC column, however, it exhibited significant peak tailing on the FFAP column. B-Mercaptoethanol formed sharply eluting peaks on the polar FFAP column, but exhibited significant peak tailing on the 5%Diphenyl 95% dimethylpolysiloxane GC column.
The internal standard, when added in concentrations of 3.89 ppb as total concentration of sulfur to the Cheddar cheese oil in the Group 1 Cheeses discussed in chapter 6, and added as 9.72 ppb of sulfur to the cheeses in Group 2 discussed in chapter 6, was readily recovered from a fat matrix using the procedures outlined in Chapter 3. The concentration of internal standard was increased in the Cheeses in group 2, since the effluent from the GC column was split between the Sulfur Chemiluminescence Detector and the Flame Ionization Detector, whereas the effluent from the cheeses in group 1 passed directly into the Sulfur Chemiluminescence Detector.
CHAPTER 5

IDENTIFICATION AND CONCENTRATION OF POLYFUNCTIONAL THIOLS
IN FRESH CURD AND AGED CHEDDAR CHEESES

A novel method for the extraction of thiols from a fatty matrix was developed using the chemical reagents Tris-Carboxyethyl Phosphine (TCEP) and p-hydroxymercuribenzoic acid to extract thiols from the fat fraction of Cheddar cheese. TCEP was used to maintain thiols in a chemically reduced state, and p-hydroxymercuribenzoic acid was utilized as a selective trapping agent for organic molecules with a thiol functional group.

This represents the first successful reported use of these reagents in combination to facilitate the extraction of thiol compounds from a food matrix and provides a general approach for recovery of thiol containing flavor compounds responsible for air sensitive and possibly transient flavor notes in a number of food systems. This approach also allowed thiols present in concentrations of low parts per billion to high parts per trillion to be extracted from the fat fraction of Cheddar cheese, or another fatty food matrix. In all, over 40 separately eluting thiols were extracted from 11 aged Cheddar cheeses and
fresh curd and detected using capillary gas chromatography with Sulfur Chemiluminescence Detection.

A number of these compounds are believed to be polyfunctional thiols, compounds with a thiol and an additional organic functional group, such as an aldehyde, ketone, or alcohol. Additionally, 4-mercapto-4-methylpentan-2-one was also tentatively identified in Cheddar cheese by matching retention times and Kovats Retention Indexes from samples of Aged Cheddar with that of a pure sample of this compound. While 15 thiol compounds were tentatively identified by matching Kovat’s Retention Indexes with those from polyfunctional thiols published in scientific research journals, the majority of the thiol compounds remain unidentified.

5.1. Identification of thiols

5.1.1. GC/MS

GC/MS is an investigative tool commonly used in various branches of analytical chemistry to both identify unknown volatile and semi-volatile compounds, and confirms the suspected identity of a given compound. Attempts were made to positively identify some of the thiol compounds extracted from Cheddar cheese by using GC/MS in the both the Electron Impact (EI) mode and Negative Chemical Ionization (NCI) mode.

Three basic problems were encountered in the attempts to identify polyfunctional thiol compounds using GC/MS. These include: low sensitivity of GC/MS in E.I. mode, no library standards for most of the compounds in question, and high concentrations of other compounds present in the sample matrix.

A GC/MS in electron impact (E.I.) mode is capable of detecting nanogram concentrations of compounds, while a properly tuned SCD should be able to detect the
presence of sulfur in low pictogram quantities. It appears as if a number of the polyfunctional thiols were not present in sufficient concentration to be detected by EI GC/MS.

Since no library standards were available to us for use in methods development, it was not practical to utilize other more sensitive GC/MS techniques, such as Selected Ion monitoring, or Negative Chemical Ionization.

Additionally, high concentrations of other compounds were also present in the samples that were analyzed. A 5% match was found for 4-mercapto-4-methyl pentan-2-one in one sample. This matched the retention time of a pure standard. However, co-eluting compounds appear to have interfered with the attempts to positively identify this and other polyfunctional thiol compounds by GC-MS.

Over 250 individual peaks were detected in an analysis of samples by using the scanning EI mode of detection. Large amounts of fatty acids were present, which gave more than an 80% match with the GC/MS library. It will be necessary to devise a better method of sample clean up in order to detect volatile thiol compounds extracted from Cheddar cheese using GC/MS and Electron Impact ionization or Negative Chemical Ionization.

5.1.2. Kovat’s retention indexes

Kovats Indexes were used to tentatively identify a number of thiols in this study. Although polyfunctional thiols have been found in a number of heated and fermented food systems, information concerning published Kovat’s Retention Indexes of these compounds is incomplete. Recently, Vermeulen et al (2001, 2002) synthesized a number of polyfunctional thiols that contained alcohol, aldehyde and ketone functional groups.

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Vermeulen et al (2001, 2002) published the Kovats Retention Indexes for these compounds, using a 100% PDMS stationary phase capillary GC column. Unfortunately, a 5% Diphenyl 95% PDMS stationary phase column was used in this study. Therefore, it was necessary to devise a method to convert the Retention Index values obtained by Vermeulen et al (2001, 2002) to those that would be obtained by the same compounds on a 5% Diphenyl 95% PDMS column.

There is no standard format that has been published for conversion of Kovat’s Indexes among different GC column stationary phases. However, Flavornet (Acree and Arn, 1997) is a database available on the World Wide Web that lists the retention indexes by Kovat’s hydrocarbons and ethyl esters on four different GC stationary phases. A number of compounds have not been measured on all GC phases for their respective Retention Indexes (Acree and Arn, 1997). However, the retention indexes are listed on all 4 phases, suggesting that Acree and Arn (1997) have developed a convention for conversion of retention indexes from one phase to another. By comparing the retention indexes for a number of compounds on 100% PDMS -vs.- 95% Polydimethyl 5%Diphenyl siloxane, it is possible to suggest a conversion value encompassing a narrow range that can be used to convert retention times from one of the above phases to another.

A conversion factor was then adopted in order to convert the retention indexes of compounds measured on 100% PDMS to 95% Polydimethyl 5%Diphenyl siloxane. In order to convert aldehydes, ketones and alcohols, a conversion factor of (1.025 x Retention Index on PDMS) was used.

The retention index data in Table 5.1 was obtained from the work of Vermeulen et al (2001, 2002) with the exception of 1 compound (Vermeulen et al 2001, 2002).
retention index for 4-methyl-4-mercaptopentan-2-one was obtained from a pure sample of this compound. All of the retention indexes published in these two papers was obtained using a 100% polydimethyl siloxane (PDMS) stationary phase GC column. In order to convert the retention indexes from the above references to a retention index for these compounds on a 95% polydimethyl siloxane 5% polydiphenyl siloxane GC column, the Retention indexes obtained from the 100% PDMS phase for polyfunctional thiol aldehydes and alcohols were multiplied by 1.025 and then tabulated. Retention indexes for polyfunctional thiol ketones were obtained by multiplying the number for the 100% PDMS phase by 1.036. Careful comparison of retention indexes for actual compounds occurring in Cheddar cheese demonstrates that the 1.036 conversion factor gives more tentative matches than the 1.025. Comparing the Kovat’s index number for 4-mercapto-4-methylpentan-2-one on 100% polydimethyl siloxane and 5% Diphenyl 95% polydimethyl siloxane GC columns arrived at this number. Matches were made by applying a margin of error to the calculated Kovats Index of approximately +/- 10 Kovats Index units for compounds with a thiol and ketone functional group or thiol and alcohol functional group. For compounds with a thiol and aldehyde functional group, a margin of error of +/- 15 Kovats Index units was used. It was not possible to identify compounds with a Kovat’s index number below 700, due to variability in elution time for the hexane (C-6) G.C. peak.
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<th>Compound name</th>
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<th>Estimated K.I. 95% PDMS</th>
<th>% difference between 100% PDMS and 95% PDMS 5%DP phase</th>
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<td>1016 / 1020</td>
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Table 5.1 Conversion of Retention Indexes
5.2. Qualitative separation

Selected chromatograms obtained from the SCD detection of thiol compounds in Cheddar cheese are shown in Appendix A. These chromatograms include Figure 5, Cheddar cheese NE1; Figure 6, Cheddar cheese NE5; Figure 7, Cheddar cheese NE7; Figure 8, Cheddar cheese NW1; and Figure 9, Cheddar cheese NE6.

5.2.1. Identified compounds

A number of polyfunctional thiol compounds have been tentatively identified for the first time in Cheddar cheese (see Table 6.2). Only one of these compounds, 4-methyl-4-mercaptopentan-2-one is available commercially, and has been previously reported in cheese (Badings, 1967). Furthermore, the majority of these compounds cannot be found in a Mass Spectral data base. Only recently have these compounds been synthesized and analyzed by GC/MS, with GC/MS fragmentation patterns being reported (Tominaga et al, 1998; Vermeulen et al, 2001, 2002). The compounds synthesized by Vermuelen et al (2001, 2002), with the exception of 4-mercapto-4-methyypentan-2-one and 4-methyl-4-mercaptopentan-2-ol, are so new to the literature that they were not synthesized individually, but rather in a batch approach, and information on their aroma properties has been determined only by sniffing at a GC port equipped for olfactometry (Vermuelen et al, 2001, 2002). Therefore, any identification of these compounds in a food product by GC/Kovat’s or GC/MS is quite novel.

Compounds containing a thiol functional group were extracted in concentrations as low as high parts per trillion levels. Furthermore, an unexpected large number of thiols were extracted from the fat fraction of the 12 cheeses and fresh cheddar curd that were analyzed according to the methods mentioned in chapter 3. Over 40 separately eluting
compounds were detected. Using the Kovat’s Retention Index (R.I.) system tentatively identified several of these compounds.

No thiol was found in every cheese. However, 4-mercapto-4-methylpentan-2-one was found in a majority of the cheeses, as was 4-mercapto-3-methylpentan-2-one and 3-mercapto-3-methylbutanal. A number of polyfunctional thiols were tentatively identified only 1 or 2 times, including 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.

Some cheeses possessed, in terms of occurrence, a relatively large number of polyfunctional thiols. Samples NE1 and NW1 possessed a total of 8 polyfunctional thiols each, and samples NE2 and NE5 possessed a total of 7 polyfunctional thiols each. The samples NE2 and NE3 possessed relatively few polyfunctional thiols, with only 2 occurring in NE4 and 3 occurring in NE3. The remainder of the 6 cheese samples possessed an intermediate number of polyfunctional thiols ranging from 3 to 5 in each cheese.

A total of 15 polyfunctional thiol compounds have tentatively been identified in the Cheddar cheeses that were analyzed. These compounds include 5 compounds with both a thiol and ketone functional group, 5 compounds with a thiol and alcohol functional group, and 5 compounds with a thiol and aldehyde functional group.

5.2.1.1. Compounds with ketone and thiol functional groups

4-mercapto-2-pentanone was found in a total of 4 of the Cheddar cheeses originating in the Midwest and Northeast. 4-methyl-4-mercapto-pentan-2-one occurred in a total of 8 of the 12 cheese analyzed, and appears to be one of the 2 most abundant polyfunctional thiols in terms of occurrence. This compound was found in the cheeses
from the Northeast, Midwest, and Northwest geographic regions. 4-mercapto-3-methylpentan-2-one was the other of the 2 most abundant poly-functional thiols, in terms of occurrence and was detected in a total of 10 of 12 cheeses. This compound was found in cheeses from all 3 geographical regions.

5-mercaptohexan-3-one occurred in fewer cheeses, being detected in 3 of the 12 cheeses analyzed. This compound occurred in cheeses from the Northeast and Midwest geographical regions. 5-mercapto-4-methylhexan-2-one occurred in only 2 of the cheeses, and was the least abundant compound tentatively identified containing a thiol and ketone functional group. This compound occurred in 1 cheese from the Northwest, and 1 cheese from the Northeast.

5.2.1.2. Compounds with alcohol and thiol functional groups

As a class, compounds with an alcohol and thiol functional group occurred fewer times than the compounds containing ketone and aldehyde functional groups. 4-mercapto-3-methylpentan-2-ol, 4-mercapto-4-methyl-pentan-2-one, and 5-mercaptohexan-3-ol were the most abundant alcohols, with each occurring in 3 of the 10 cheeses. Two of the cheeses that contained 4-mercapto-4-methylpentan-2-ol also contained the analogous ketone. Furthermore, this compound occurred only in cheeses originating in the Northeast. 4-mercapto-3-methylpentan-2-ol occurred in cheeses containing the ketone analog. This compound occurred in cheeses originating in all 3 geographical regions. 5-mercaptohexan-3-ol occurred in 1 cheese that also contained the ketone analog, and occurred in all 3 geographic regions. 4-mercaptopentan-2-ol was the least abundant, occurring in only one cheese from the Midwest.
5.2.1.3. Compounds with an aldehyde and thiol functional group

A total of 5 compounds containing an aldehyde and thiol functional group were observed in the cheeses. 3-mercaptobutanal was tentatively identified in 3 cheeses, and occurred only in the Northeastern geographical region. 3-mercapto-3-methylbutanal was tentatively identified in 7 of the cheeses, and was found in all 3 geographical regions. 3-mercaptoheptenal was tentatively identified in 4 cheeses, one from the Northwest, and one from the Northeast. 3-mercapto-2-butylpropanal was tentatively identified in 4 cheeses, occurring in all geographical regions. Finally, 3-mercaptooctanal was tentatively identified in 1 cheese from the Northeastern U.S.
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<th>NE2</th>
<th>NE3</th>
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<th>NE5</th>
<th>NE6</th>
<th>NE7</th>
<th>NE8</th>
<th>M2</th>
<th>M3</th>
<th>NW1</th>
<th>Fresh Curd</th>
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Table 5.2 Polyfunctional Thiols Tentatively Identified in Cheddar, Mean concentrations in Fat Fraction Continued
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<th>K.I.</th>
<th>Compound Description</th>
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<th>1124/1135</th>
<th>1136</th>
<th>1161</th>
<th>1268</th>
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<td>1107</td>
<td>5-methyl-4-mercaptohexan-2-one</td>
<td>+</td>
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<tr>
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<td>+</td>
<td>+</td>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>1268</td>
<td>3-mercaptooctanal</td>
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<td></td>
<td>+</td>
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</table>

**Table 5.2 Polyfunctional Thiols Tentatively Identified in Cheddar, Mean concentrations in Fat Fraction**

K.I. - Kovat’s retention index number
5.2.2. Unidentified thiols

5.2.2.1. Low molecular weight thiols

While no lower molecular weight thiols such as ethane thiol, 1 or 2-propane thiol or 1 or 2-butane thiols were tentatively identified in Cheddar cheese, it is likely that lower molecular weight thiols are present, as there were several thiol peaks with relatively low boiling points that were observed. However, it was not possible to tentatively identify these compounds by retention times, as they co-eluted with a large solvent peak composed of diethyl ether and pentane. This large co-eluting solvent peak appears to cause variability in the retention times of the lower molecular weight thiols. Also, great variability was seen in the retention times for the Kovat’s index hydrocarbon C-6, hexane.

The variability in the elution of hexane made it impossible to assign a Kovat’s index number to thiols eluting between the C-6 and C-7 hydrocarbons. Thiol compounds that may elute prior to the C-7 hydrocarbon, the first Kovat’s hydrocarbon with a reproducible retention time and a boiling point of 98°C, may include methane thiol, ethane thiol, and isomers of propane thiol and butane thiol. As many as 7 or as few as 3 different lower molecular weight thiol compounds with a Kovat’s retention index number for 700 or less were observed in a single sample of Cheddar cheese.

5.2.2. Unidentified thiols

5.2.2.2. Unidentified medium and higher molecular weight thiols

A number of unidentified intermediate and higher molecular weight thiols were observed in chromatograms from the Sulfur Chemiluminescence detector. Although the work of Vermeulen et al (2001 and 2002) was extremely useful in allowing for the
tentative identification of a number of polyfunctional thiols, the majority of the thiols could not be tentatively identified. However, since a number of these compounds could be assigned a Kovat’s retention index value on a 5% Diphenyl 95% polydimethyl siloxane column, it is also possible to estimate the molecular weight of the unknowns. The molecular weight of these unidentified compounds was estimated by using the following formula:

\[
\text{Estimated M.W.} = \left( \frac{\text{R. T. of unknown} - \text{R.T. of lower bracketing hydrocarbon}}{\text{R. T. of higher bracketing hydrocarbon} - \text{R. T. of lower bracketing hydrocarbon}} \times 14 \text{ gm/mole} \right) + \text{M.W. of lower bracketing hydrocarbon}
\]

This was accomplished by first identifying the Kovats’ index hydrocarbons bracketing the thiol compound in question. Then, the period of time in seconds, elapsed between the lower molecular weight bracketing hydrocarbon, and the higher molecular weight hydrocarbon was determined from the chromatogram report, as well as the period of time in seconds between the lower molecular weight bracketing hydrocarbon and the thiol. Then the period of time between the lower molecular weight bracketing hydrocarbon and the thiol was divided by the period of time between the two bracketing hydrocarbons. Finally this number was multiplied by 14 grams/mole, and added to the molecular weight of the lower molecular weight bracketing hydrocarbon.
By using this method to predict the molecular weight of 4-mercapto-4-methylpentan-2-one, a molecular weight of 135 grams per mole was predicted, based upon a Retention Index number of 948. This is only 3 grams per mole above the actual molecular weight of 4-mercapto-4-methylpentan-2-one, 132 grams per mole.

When this formula was applied to the internal standard, 3-methoxy benzenethiol, it showed a significant deviation between the predicted molecular weight and the actual molecular weight for this compound. The actual molecular weight of this compound is 140 grams per mole, and the predicted molecular weight was 177 grams per mole. However, it is known that small aromatic compounds such as benzene generally possess a higher boiling point that that of the corresponding linear alkanet. As an example, the boiling point of hexane is 68.7°C (Aldrich, 2002a), and the boiling point of benzene is 80.7°C (Aldrich, 2002b).

The difference in the actual molecular weight of 3-methoxy benzenethiol and the predicted molecular weight may be partially accounted for by the difference in boiling points of the alkane and the aromatic compound. Additionally, the stationary phase of the GC column contains 5% polydi phenyl siloxane. The inclusion of an aromatic functional group in the stationary phase may also in part account for the discrepancy between the estimated and actual molecular weight of the internal standard compound. Due to this discrepancy, it is suggested that this method is accurate for only predicting the molecular weights of compounds with an alkane backbone.

The estimated molecular weights of the unidentified compounds are listed in Table 5.3. It is not possible at this time to assign a tentative identification to these compounds. They did not match the molecular weights for ethane thiol, butane thiol, or
octane thiol. Compounds with calculated molecular weights of 748, 1180, 1295, 1379 and 1824 were present in at least 2 of the cheeses listed in Table 5.3. The compound with an estimated molecular weight of 114 grams/mole was the only thiol that was observed in the Fresh Cheddar Curd sample.

As stated earlier, the identities of the thiols in Table 5.3 are not known. Furthermore, it is not known if any of these thiols contain an additional chemical functional group such as an aldehyde ketone or alcohol. It may be useful, however, to compare the retention times of polyfunctional thiols with those of the thiols listed below.

The retention index of the lowest molecular weight tentatively identified polyfunctional thiol, 3-mercaptobutanal, was 822. Two unidentified compounds fall slightly below the K.I. number for this compound. The retention indexes for thiol compounds with 5 carbons and an additional oxygen containing functional group fall between 847 and 914. No unidentified thiols fall near or within this range. The retention indexes for thiol compounds with 6 carbons and an additional oxygen containing functional group fall between 948 and 1063. No unidentified thiols fall near or within this range. For thiol compounds with 7 carbons and an additional functional group, estimated Retention Indexes fall between 1107 and 1161. No unidentified thiols fall within this range, however, a thiol appears close to the upper limit of the observed range with a retention index of 1180. The remainder of the thiols elute under conditions that suggest that they would have more that 8 carbons if they are polyfunctional thiols.

The retention indexes for the cheeses that are shown in Table 5.3 were obtained by splitting the GC column effluent between the chemiluminescence detector and an FID.
detector. Additionally, linear hydrocarbons from C-6 through C-19 were added to the sample prior to injection onto the GC column.

<table>
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<th>K.I.</th>
<th>Estimated Molecular Weight</th>
<th>NE2</th>
<th>M3</th>
<th>NE6</th>
<th>NE8</th>
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<td>x</td>
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</tr>
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</table>

Table 5.3. Retention Index and Estimated Molecular Weights of Unknown Thiols
5.3. Quantitative estimation of concentrations of polyfunctional thiols in Cheddar cheese

As mentioned in the previous sections, a number of thiols have been tentatively identified in Cheddar cheese for the first time. The mean values of the analyses of the polyfunctional thiols found in the cheeses are presented in Table 5.4.

5.3.1. Concentrations of volatile polyfunctional thiols tentatively identified in Cheddar cheese

Of the 15 polyfunctional thiols found in this study, 4-mercapto-4-methylpentan-2-one generally occurred in or near the highest concentration for a polyfunctional thiol in the 8 cheeses where it was identified (6 – 33 ppb). Although 4-mercapto-3-methylpentan-2-one occurred in more cheeses than the above compound, it generally occurred in lower concentrations (1.7 – 29 ppb). The remainder of the polyfunctional thiols tentatively identified occurred in fewer instances and/or in lower concentrations than these two compounds.

The Cheddar cheese with the designation NE1 was also relatively rich in compounds that were tentatively identified as polyfunctional thiols. This cheese possessed 3-mercapto-3-methylbutanal in a relatively low concentration, 2.9 ppb. 4-mercapto-pentan-2-one was detected in low ppb concentrations, at an average value of 2.6 ppb. This cheese was relatively abundant in concentrations of 2 ketones possessing methyl branches. 4-mercapto-4-methyl pentan-2-one was found in concentration of 32.9 ppb. 4-mercapto-3-methyl-pentan-2-one was detected at 15.1 parts per billion, and 28.8 ppb. It is suggested that 2 isomers for this compound were present. 5-mercapto-hexan-3-one was detected at concentrations of 16.0 ppb. 4-mercapto-3-methylpentan-2-ol was
found in the highest concentration of all samples evaluated in this cheese, in 73.7 ppb. 3-mercaptoheptanal was tentatively identified to have an average concentration of 10.6 ppb, and 3-mercapto-2-butylpropanal possessed an average concentration of 18.3 ppb.

The sample with the designation NE2 is relatively rich in polyfunctional thiols compounds. 4-methyl-4-mercaptopentan-2-one is present in highest concentration in this sample, and occurs at an average concentration of 37.0 ppb. 2 compounds tentatively identified and possessing aldehyde functional groups, 3-mercaptobutanal, and 3-mercapto-3-methylbutanal, occurs at 23.4 and 14.0 ppb, respectively. 4-mercapto-4-methylpentan-2-ol was measure at an average concentration of 10.4 ppb. 4-mercapto-3-methylpentan-2-one, and 5-methyl-4-mercaptohexan-2-one, 2 ketones with branched methyl groups, possessed average concentrations of 5.7/8.3 and 17.5 ppb, respectively. 5-methyl-4-mercaptohexan-2-ol was possessed an average concentration of 14.4 ppb.

The cheese with the sample designation NE3 possessed only 3 tentatively identified polyfunctional thiols, and all of these compounds were at relatively low concentrations, compared to other cheeses. The concentrations were relatively low for all of these cheeses. 4-methyl-4-mercaptopentan-2-one was present in average concentrations of 11.0 ppb, 4-mercapto-3-methylpentan-2-one was present at an average concentration of 11.0 ppb, and 3-mercaptooctanal was present at approximately 6.5 ppb.

The cheese with the sample designation NE4 was also relatively deficient in polyfunctional thiols that could be tentatively identified. An isomer of 4-mercapto-3-methylpentan-2-one was suggested to have an average concentration of 16.9 ppb in this sample.
Cheese NE5 was relatively abundant in polyfunctional thiols with a ketone functional group. 4-methyl-4-mercaptopen-2-one was present in this cheese in the second highest concentration of 30.4 ppb. Furthermore, 5-mercapto-hexan-3-one was present at 10.2 ppb, and 4-mercapto-3-methylnpentan-2-one was present at a concentration of 5.3 ppb. 3-mercapto-3-methylbutanal was present in a relatively low concentration at 2.4 ppb. The other tentatively identified polyfunctional aldehyde 3-mercapto-2-butylpropanal was present at 10.4 ppb. The only alcohol tentatively identified in this sample, 5-mercaptohexan-3-ol, was present at a concentration of 0.9 ppb.

The Cheddar cheese with the sample designation NE6 possessed a relatively low concentration of 4-methyl-4-mercaptopen-2-one, at 10.7 ppb. Four other polyfunctional thiol compounds were tentatively identified in this cheese and possessed average concentrations above 10 ppb. These compounds include 4-mercapto-4-methylpentan-2-ol, at 15.9 ppb, 3-mercaptohexan-3-ol at 17.2 ppb, 3-mercapto-3-methylbutanal at 29.7 ppb and 3-mercapto-2-butylpropanal at 13.0 ppb.

The cheese with the sample designation NE7 possessed a relatively high concentration of 4-methyl-4-mercaptopen-2-one, at an average value of 31.0 ppb. The compound tentatively identified as 3-mercapto-2-butyl propanal was present in an average concentration of 11.4 ppb, and two relatively minor components, 3-mercapto-3-methylbutanal and 4-mercapto3-methylpentan-2-one, were present in concentrations of 3.2 and 2.7 ppb, respectively.

Cheddar cheese sample NE8 possessed relatively few polyfunctional thiols compounds. Only 3 were identified in this cheese, including 3-mercaptohexan-3-ol at 1.5 ppb, 4-mercaptopen-2-one at 1.7 ppb, and 4-mercapto-4-methylpentanol at 2.3 ppb.
The Cheddar cheese with the designation **M2** was relatively rich in the number of polyfunctional thiol compounds detected. 3-mercapto-3-methylpropanal was detected in low ppb concentrations, at 4.7 ppb. 4-methyl-4-mercaptopentan-2-one, a compound with a “catty” or “ribes” odor, was detected in an average concentration of 28.5 ppb. 4-mercapto-pentan-2-ol was detected in a relatively low concentration, 3.3 parts per billion. The rest of the compounds tentatively identified were also in low parts per billion concentration, with 4-mercapto-3-methylpentan-2-one at 1.7 ppb, 5-mercaptohexan-3-ol at 3.9 ppb, 3-mercaptopheptanal at 4.1 ppb, and 3-butyl propanal at approximately 3.5 ppb.

The cheese with the sample designation **M3** possessed relatively high concentrations of 3 tentatively identified polyfunctional thiol compounds. 4-mercapto-3-methylpentan-2-one was present at concentrations of 55.3 ppb. 5-mercaptohexan-3-one was present at an average concentration of 34.4 ppb, and 5-methyl -4 mercaptohexan-2-ol was present at an average concentration of 81.4 ppb. 4-mercaptopentan-2-one was present at a lower concentration of 9.9 ppb.

For the Cheese with the sample designation **NW1**, 4-methyl-4-mercaptopentan-2-one occurred in a relatively low concentration for this compound, 6.6 ppb. All other compounds, including 3-mercapto-3-methylbutanal, 4-mercapto-3-methylpentan-2-one, 5-mercaptohexan-3-ol, 4-mercapto-3-methylpentan-2-ol, 5-methyl-4-mercaptohexan-2-one, 3-mercaptopheptenal, and 3-mercaptop-2-butylpropanal were present in mean concentrations ranging from 1.6 to 4.2 ppb.
Of all of the polyfunctional thiols tentatively identified in Cheddar cheese, none were identified in the curd. One thiol with a Retention Index of 800 was identified in the curd.
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<th>NE5</th>
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Table 5.4 Mean Concentration in Cheddar Fat in Parts Per Billion of Polyfunctional Thiols  Continued
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<td>3-mercaptooctanal</td>
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Table 5.4 Mean Concentration in Cheddar Fat in Parts Per Billion of Polyfunctional Thiols
5.3.2. Estimated concentrations of thiols unidentified in Cheddar cheese

The majority of the thiols occurring in Cheddar cheese could not be tentatively identified. The chemistry associated with these compounds appears to be quite novel, as there is no mention of higher molecular weight polyfunctional thiols compounds in the scientific literature, with the exception of the medium to higher molecular weight polyfunctional aldehydes recently synthesized and reported by Vermuelen et al, (2002).

Also, it was not possible to partially characterize the unknown medium and higher molecular weight thiols in the majority of the Cheddar cheeses analyzed in this study, although a number of unidentified thiol containing compounds were observed in 5 Cheddar cheeses and Fresh Cheddar curd that were analyzed.

A total of 15 medium to higher molecular weight thiols were observed in 5 Cheddar cheeses and 1 Cheddar curd sample. Although it was not possible to tentatively identify these compounds, it was possible to estimate the molecular weight and concentrations of these compounds based on the techniques described in the above sections.

The greatest number of unidentified thiols was found in Cheese M3. It appears if at least 3 of these thiols may be present in sufficient concentration to have an effect upon the flavor. The thiol with a Kovat’s retention index number of 1180 was present in an approximate concentration of 47 parts per billion, and next two eluting unknown thiols were present in approximately 657 and 1353 parts per billion respectively. 5 more thiol compounds were found in this cheese at lower parts per billion concentrations where they could potentially have an impact upon flavor. The compound with K.I. of 1824 and a molecular weight of approximately 268 grams per mole occurs in a relatively high...
concentration, approximately 160 parts per billion, but relatively few flavor compounds are reported that have this high of a molecular weight, and Kovat’s Index. Therefore, it is difficult to speculate if this thiol can impact the flavor of Cheddar cheese.

Sample NE2 possessed a total of 5 unidentified thiols, spread over a range of molecular weights, from approximately 97 to 225 grams per mole. All of these compounds have low enough molecular weights where they could potentially contribute to the aroma of Cheddar cheese, if they are present at or above any of their respective odor thresholds.

Sample NE6 possessed only 2 unidentified medium or higher molecular weight thiols, one with an estimated molecular weight of 97 and the other with an estimated molecular weight of 167 grams per mole. These thiols were also present in relatively low parts per billion concentrations.

In the Cheddar cheese NE8, 4 unidentified thiols were observed. One of these thiols was present in a relatively low concentration, approximately 4 ppb, and possessed a relatively low molecular weight, approximately 97 grams per mole. Another potentially volatile thiol with a molecular weight of 195 grams per mole was present in a concentration of approximately 125 parts per billion. Two additional higher boiling thiols were present in somewhat higher concentrations, 32 and 2145 parts per billion. However, their estimated molecular weights, 242 and 268 grams per mole, and relatively high Retention Indexes, 1712 and 1824, suggest they may not be sufficiently volatile to impact Cheddar cheese flavor.

Cheddar cheese NE4 possessed a total of 3 unidentified thiols. The Lowest K.I. number unidentified thiol in this cheese possessed a molecular weight of approximately
195 grams per mole, and was present in an approximate concentration of 46 ppb. Two higher molecular weight thiols with estimated molecular weights of 239 and 268 grams, respectively, were present in concentrations of 28 and 1969 ppb.

The only thiol found in Cheddar cheese curd was present in a concentration of approximately 162 ppb. This compound had an estimated molecular weight of 114 grams per mole.
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Table 5.5 Retention Index and Estimated Concentrations in Parts Per Billion of Unknown Thiols
5.4. Variability in analysis of polyfunctional thiols by GC/SCD

Analysis of solutions of a solvent such as pentane, ether, or a 2:1 mixture of pentane and ether containing part per billion levels of octane thiol, beta mercaptoethanol, 3-methoxythiophenol and 4-mercapto-4-methylpentan-2-one were reproducible, with a standard deviation of less than 10%.

However, the analysis of thiols extracted from Cheddar cheese was not as reproducible. A number of factors may be responsible for this. Free fatty acids and orange and yellow pigments, presumably from the annatto food coloring used in orange colored Cheddar cheeses, were extracted from the Cheddar cheese fat. It was difficult to completely separate this material from the p-hydroxymercuribenzoic acid salt used to capture the polyfunctional thiols.

The carry over of the lipid materials appeared to result in the rapid buildup of Pyrolized material in the injection port of a GC. The build-up of Pyrolized material in a GC injection port is known to introduce a significant source of error (Rood, 2002). In order to minimize contamination, the injection port liner was removed and cleaned after triplicate analysis of a sample. However, this did not fully alleviate the problem, as evidenced by the variability in data.

In addition to the difficulty associated with removing the lipid material from the samples, a great deal of difficulty was encountered with the sulfur detector. For some unknown reason, the sample tubes developed severe coking problems after about ½ of the Cheddar cheeses had been analyzed. This drastically affected the reproducibility of the samples that were analyzed after the severe coking problem was observed. Two cheeses,
NE6 and NE8, were not measured in replicate analysis due to a severe loss in detector sensitivity that was attributed to coking.

Among tentatively identified polyfunctional thiols, coefficients of variation were as high as 105% or as low as 1.8%. Sample variability appears to be independent of sample class, compounds tentatively identified as polyfunctional thiol aldehydes, ketones and alcohols all were seen to exhibit variability in concentration measured within a given cheese sample over a wide range. Furthermore, wide variability was seen among the same compound measured in different cheeses.

5.4.1. Polyfunctional thiol/aldehyde variability

Among the 5 polyfunctional thiols with an aldehyde functional group, the Coefficients of variation occurred over a wide range of values. For 3-mercaprobutanal, the lowest coefficient of variation for any polyfunctional thiol with an additional aldehyde group, 1.8%, was observed in the NE2 sample. This compound was not observed in multiple occurrences in any other cheeses.

3-mercapto-3-methylbutanal occurred in a total of 7 cheeses, and the coefficients of variation for this compound were as low as 8.8 %, and the highest was 78.2 %. Sample NE6 also possessed this compound, but as multiple samples were not observed for this cheese, no data is available for the coefficient of variation in this sample. Three of the coefficients of variation for this compound were above 25%, and occurred in cheeses NE7, M2, and NW1, at 48.6, 78.2 and 45.3% respectively.

3-mercaptoheptenal was tentatively identified in a total of 3 cheeses, and the coefficients of variation were relatively low for this compound. Only one was above
25%, occurring in sample M2 at 30.7%. This compound possessed relatively low variation in samples NE1 and NW1, at 11.2% and 18.2%, respectively.

3-mercapto-2-butylpropanal was identified in a total of 8 cheeses. In samples NE6 and NE8, no data is available concerning multiple analyses. 4 of the remaining samples possess a coefficient of variation lower than 25%, including NE1, NE5, M2 and NW1. Only one sample, NE7, possessed a coefficient of variation above 25%, at 30.7%.

3-mercaptooctanal occurred in a total of 2 cheeses, and the coefficient of variation for this compound was 10.9% in sample NE3. No data is available for NE4, the other cheese where this compound was observed.

5.4.2. Polyfunctional thiol/ketone variability

4-mercaptopenan-2-one was tentatively identified in 5 cheeses, however, in 3 of the cheeses, NE3, NE8, and M3, it was only detected 1 time. In the cheeses where this compound was observed in more than 1 individual measurement, NE1 and NE5 the coefficients of variation were relatively low, 10.9 and 15.9%, respectively.

4-mercapto-4-methylpentan-2-one was one of the most frequently occurring polyfunctional thiols, and it occurred in a total of 8 of the cheeses. The lowest coefficient of variation for this compound was in the NE2 sample, at 0.9%. This compound occurred in 5 other cheeses, NE1, NE3, NE5, M2, and M3 at less than 25% coefficient of variation. In sample NE7, this compound had a very high coefficient of variation of 105.2%. In sample NE6, which was only analyzed 1 time, no data is available for the coefficient of variation.
4-mercapto-3-methylpentan-2-one was observed in a total of 8 cheeses. The lowest coefficient of variation occurred in cheese NE1, where the coefficients of variation for 2 suspected isomers of this compound were 7.5 and 2.5%. In sample NE2, this compound was observed only once, so no data is available for the coefficient of variation. In samples NE3, NE5, M2, M3 and NW1, the coefficients of variation were all less than 25%. Sample NE7 possessed a coefficient of variation of 46.4, the highest observed for this compound.

5-mercaptohexan-3-one occurred in a total of 3 cheeses, and was relatively reproducible in each one. In sample NE1, this compound occurred with a coefficient of variation of 25.3%. The coefficient of variation was lower in the 2 other cheeses, 6% in NE3 and 16.1% in M3.

5-methyl-4-mercaptohexan-2-one occurred in a total of 2 cheeses, and both coefficients of variation were above 25%. NE2 possessed a coefficient of variation of 28.8% for this compound, and NW1 37.2%.

5.4.3. Polyfunctional thiol/alcohol variability

4-mercaptopentan-2-ol was observed in only 1 cheese, M2, and the coefficient for variation in this sample was 4.3%.

4-methyl-4-mercaptopentan-2-ol occurred in a total of 3 cheeses. The only coefficient of variation measured for this compound was in sample NE2, at 9.5%. In samples NE6 and NE8, this compound was not observed more than 1 time in each.

4-mercapto-3-methylpentan-2-ol was observed in 2 samples, NE1 and NE8. The coefficient of variation was 1.6% in sample NE1. This compound was observed only 1 time in sample NE8.
5-mercaptohexan-3-ol occurred in a total of 3 cheeses. Sample NE5 had only 1 occurrence of this compound. In samples M2 and NW, this compound had coefficients of variation of 18.0 and 1.7%, respectively.

5-methyl-4-mercaptohexan-2-ol occurred in two cheeses. In sample NE2, it possessed a coefficient of variation of 44%. In sample M3, a coefficient of variation of 24.1% was observed for this compound.

5.4.4. Variability of cheeses

Sample NE1 appears to be the most reproducibly analyzed cheese. This compound contained a total of 9 polyfunctional thiols, all of which were analyzed with a coefficient of variation near or below 25%.

Cheeses NE3, NE5, ME3 and M2 were also very reproducible in their analysis. In the first 3 samples, only 1 compound was not reproducibly analyzed in each. Otherwise, each polyfunctional thiol compound occurring in each of these cheeses possessed a coefficient of variation of less that 25%. In sample M2, of the 7 polyfunctional thiols tentatively identified, only 2 compounds had a coefficient of variation higher than 25%.

Samples NW1 and NE2 were relatively reproducible as well. A total of 8 polyfunctional thiols were tentatively identified in the NW1 cheese. 5 of these compounds possessed a coefficient of variation of less that 25%. Two compounds possessed coefficients of variation above 25%, and 1 was not reproducible. A total of 8 polyfunctional thiols were tentatively identified in NE2. 5 of these compounds possessed a coefficient of variation of less that 25%. Two compounds had coefficients of variation above 25%, and 1 compound was identified in only 1 sample.
Samples NE4 and NE7 could be considered to show poor reproducibility. Only 1 polyfunctional thiol was tentatively identified in this cheese, possessing a coefficient of variation of 70.1%. This sample appeared to be severely effected by coking. A total of 4 polyfunctional thiols were tentatively identified in NE7, and all of these compounds possessed a coefficient of variation above 25%.

Cheeses NE6 and NE8 were the least reproducibly analyzed cheeses. Only 1 chromatographic run for each of these cheeses was reported, due to a severe loss of sensitivity and repeatability observed in subsequent runs of the same sample. It is suggested that the severe coking associate with the Seivers SCD at this time is the most likely cause of this problem.

It is likely that the severe coking problem that was observed with the Seivers Sulfur Chemiluminescence Detector is at least partially responsible for the lack of reproducibility observed in some of the cheese samples. Of all of the cheeses that showed good reproducibility, NE1, NE3, NE5, M2 and M3, all of them except for M3 were analyzed before the onset of the coking problem. Furthermore, only one cheese that showed poor reproducibility was analyzed before the onset of the coking problem.
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| NE4  | Retention Index [PPB], 1 [PPB], 2  | |
|------|-----------------------------------|------|-------|-------|
| 994  | 4-mercapto-3-methylpentan-2-one  | 25.2 | 8.5  | 16.9  | 11.8 | 70.1 |
|      | 3-mercaptooctanal                | 1.7  | NA   | 1.7   | NA   | NA   |

| NE5  | [PPB], 1 [PPB], 2 [PPB], 3  | |
|------|-----------------------------|------|-------|-------|
| 847  | 3-mercapto-2-methylbutanal | 2.6  | 2.3   | 2.2   | 2.4  | 0.2  | 8.8  |
| 913  | 4-mercapto-2-pentanone      | 1.4  | 1.3   | 1.7   | 1.5  | 0.2  | 15.9 |
| 948  | 4-methyl-4-mercapto-2-pentanone | 33.3 | 30.8 | 27.1  | 30.4 | 3.1  | 10.3 |
| 1002 | 4-mercapto-3-methylpentan-2-one | 6.3  | 4.2   | 5.2   | 5.3  | 1.0  | 19.6 |
| 1019 | 5-mercaptohexan-3-one       | 10.1 | 10.9  | 9.7   | 10.2 | 0.6  | 6.0  |
| 1049 | 5-mercapto-hexan-3-ol       | 0.9  |       | 0.9   | NA   | NA   | NA   |
| 1169 | 3-mercapto-2-butyldialanal  | 11.3 | 10.5  | 9.3   | 10.4 | 1.0  | 9.6  |

**Table 5.6 Sample Variability**

Continued
Table 5.6 Continued

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Table 5.6 Sample Variability

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Table 5.6 Sample Variability
The importance of sulfur compounds to the flavor of Cheddar cheese is well established. However, this relationship has been built primarily around the presence of hydrogen sulfide, methane thiol, methyl disulfide, dimethyl disulfide, and methional. This study places a new perspective on the role of sulfur compounds to the flavor of aged Cheddar cheese. First, the recognition of multiple sulfur related descriptors (total sulfur, egg-like, match like and catty) by this study. Secondly, is the discovery of a large number of previously unrecognized poly functional thiols with a broad range of flavor descriptors.

These include cooked, broth, cheese, meaty, onion, broom and catty. Catty appears to be of particular significance, since it was found to be statistically correlated to age. Drake (2002 – personal communication) has indicated that this flavor descriptor is only noted in aged Cheddar cheese. There seems to be little doubt that the polyfunctional thiols are formed during the ripening process, since none of them were found in fresh Cheddar cheese curd.

In this study, the use of the reducing agent would have converted any di-sulfides into their reduced thiol forms. Therefore the actual form of these poly function sulfur
compounds cannot be established. However, given the low oxidation/reduction potential reported for Cheddar cheese in the scientific literature, and the fact that methane thiol is found in Cheddar cheese, it is likely that these compounds exist in an oxidation/reduction dependent equilibrium between free thiols and disulfides. The balance of these compounds in the thiol form as opposed to a more oxidized form is most likely determined, then, by the oxidation/reduction potential of the individual cheese.

Cheddar cheese has long been recognized to have flavor notes that are sensitive to changes in the oxidation/reduction potential of the cheese. As oxidation/reduction potential in a given piece of Cheddar increases, characteristic Cheddar flavor is lost (Kristofferson et al, 1964). It has long been known that low concentrations of thiols will oxidize rapidly in the presence of air or oxygen (Oae and Doi, 1991). In a biological system, an increase in oxidation/reduction potential in a given reaction system increases the number of disulfide bonds formed between thiols (Oae and Doi, 1991). Therefore, an increase in oxidation/reduction potential will decrease the odor potency of any thiols present in a given flavor system.

Hydrogen sulfide is also sensitive to oxidation/reduction potential. In the presence of oxygen, H$_2$S will polymerize to form sulfur molecules such as S$_8$ or S$_4$ (Oae and Doi, 1991). If polyfunctional thiols are formed by chemical reactions occurring within Cheddar cheese, then an increase in oxidation/reduction potential will not only effect the odor properties of thiols already present in the cheese, it will also effect the formation of these compounds from the possible precursor molecules such as H$_2$S.

The relative importance of the poly functional sulfur compounds cannot be determined at this time, since the odor thresholds of these compounds within a Cheddar
cheese matrix have not been determined under conditions of controlled oxidation reduction potential, where the concentration of free thiols relative to disulfides could be estimated. Measurement of odor thresholds of these compounds within a Cheddar cheese matrix and the oxidation/reduction potential would allow for an accurate estimation of the odor activity values of the individual compounds. A simpler, alternative approach could include measuring the odor thresholds of these compounds in oil and water sample matrices, and measuring the oxidation reduction potential of the cheese and correcting the concentration of thiols.

The flavor threshold values for the polyfunctional thiols tentatively identified in this study are probably much lower than those for all sulfur containing compounds previously reported in Cheddar cheese, including hydrogen sulfide, and methane thiol, dimethyl sulfide, dimethyl disulfide, methional, and methionol. Dimethyl sulfide, dimethyl disulfide, methional and methionol possess relatively high odor thresholds compared to 4-mercapto-4-methylpentan-2-one, and their odor thresholds in water range from approximately 20 to 1 ppb (Rychlik et al, 1998a). The odor threshold of H$_2$S is 10 ppb in water (Rychlik et al, 1998b). The odor threshold of methane thiol is 0.3 to 0.03 ppb in water (Rychlik et al, 1998c). The odor threshold of 4-mercapto-4-methylpentan-2-one is 0.00012 ppb in water (Tominaga and Dubourdieu, 1997), 250 to 2500 times more potent than methane thiol, and 8300 to 83000 time more potent than Hydrogen Sulfide. Given the powerful odors that may be associated with polyfunctional thiols, more in-depth study of their odor properties in association with Cheddar cheese flavor is warranted.
Compounds with retention times consistent with methane thiol were noted in all Cheddar cheeses and curd analyzed in this study, but could not be quantified. The SCD detector was run in the most sensitive mode in order to detect polyfunctional thiols. Methane thiol was present in all Cheddar cheeses in concentrations that overwhelmed the SCD detector when it was run in the most sensitive mode.

Hydrogen sulfide, considered to be important in Cheddar cheese flavor, would not be measured by the method used in this study, as H$_2$S reacts quantitatively with phosphines. As mentioned in Chapter 4, phosphines were used in the extraction procedure. The egg-like flavor of Cheddar cheese has long been associated with the concentration of H$_2$S measured in the cheese (Barnicoat, 1933, Kristofferson and Nelson, 1955).

The electronic nose work also appears to help substantiate the role of sulfur compounds in the flavor of aged Cheddar cheese, since these results were shown to be related to the sensory data and the masses used to differentiate the cheese aroma can be associated with methane thiol, methyl disulfide and 4-mercapto 4-methyl pentan-2-one.

The origin of these compounds in aged Cheddar cheese is not known at this time. The literature suggests a number of different pathways, which include enzyme mediated release of the thiols from S-conjugates with cysteine and/or glutathione. Addition of H$_2$S, to $\alpha,\beta$-unsaturated aldehydes and ketones are another potential source of these compounds. Of these potential pathways to free polyfunctional thiols, those related to hydrogen sulfide addition to unsaturated aldehydes and ketenes would appear to be most likely to occur.

More detailed discussion of some aspects of the study follows.
### 6.1. Poly functional thiol/flavor descriptor relationships

Based on our sensory evaluation and the flavor descriptors associated with polyfunctional thiols, it appears that sulfur compounds can be related to 6 of the 15 descriptors that are related to the aroma of Cheddar cheese. These include cooked, broth, egg-like, match-like, catty and total sulfur. Broth, cooked and catty flavor descriptors were represented by 4 of the compounds that were present in more than 7 of the cheeses. These were: four-mercapto-3-methyl petan-2-one (cooked milk), 4-mercapto-4 methyl pentan-2-one (catty), 3-mercapto-2-butylpropanal (pungent, rhubarb), 3-mercapto-3-methyl butanal (cheese, broth) were present in 9, 8, 8 and 7 cheeses respectively. Neither match-like or egg-like was found to be flavor descriptors for any of the known polyfunctional thiols listed in Table 2.2. As noted earlier, egg like is most likely associated with sulfur compounds other than the polyfunctional thiols. The fact that catty and match-like were shown to be statistically correlated, suggest that these might be considered together.

#### 6.1.1. Catty flavor

Catty appears to be one of the most important of the sulfur related descriptors, as it was strongly correlated to the sensory perception of the age of the Cheddar cheese.

The sensory experiment was designed in part with the assumption that a single catty flavored compound, 4-mercapto-4-methylpentan-2-one may be present in Cheddar cheeses possessing a detectable “catty” flavor. This was based upon the work of Badings (1967) who detected this compound in Gouda cheese, and determined that it was
responsible for a catty off flavor. Furthermore, we intended to look for a statistically significant relationship between the concentration of this compound and the intensity of the catty flavor note.

Therefore, an attempt was made to correlate concentrations of 4-methyl-4-mercaptopen
tan-2-one, with the intensity of the catty flavor note observed by the sensory panel. No statistically significant relationship was observed between this compound and the catty flavor attribute in aged Cheddar cheeses.

A careful examination of recently published scientific literature related to polyfunctional thiol aroma or flavor compounds in foods, some which entered into the public domain only after the conclusion of the above sensory portion of this study (Vermuelen et al, 2001, 2002), suggests that there may be more than 10 polyfunctional thiol compounds with a “catty” or similar odor. First of all, Polak et al, (1988) demonstrated that several polyfunctional thiol compounds possess a “catty” odor quality. When 4-mercapto-4-methylpentan-2-one, a compound isolated from tomcat urine and responsible for its characteristic odor, was presented as a reference sample to human subjects, it was readily confused with other thiol compounds including 3-mercapto-3-methylpentan-2-one, \textit{trans}-8-mercapto-\textit{p}-menthan-3-one, and tert-amyl mercaptan (Polak et al, 1988). Furthermore, Vermuelen et al (2001) synthesized 5 polyfunctional thiol compounds with “catty” or similar odor properties. Careful examination of literature presented below suggests that there are several descriptive terms used to describe the odor qualities of polyfunctional thiols that appear to have significant overlap.
First of all, various researchers, in order to describe the odor quality of several polyfunctional thiol compounds, the descriptive terms “ribes,” “catty,” and “black currant” have been used by different researchers to describe the same compounds. “Ribes” is short for *Ribes nigrum*, the taxonomic genus and species name for the Black currant (Clapperton, 1976); therefore, “ribes” and “black currant” should be equivalent terms. Additionally, “Ribes” and “black currant” should be equivalent to “catty.” The term “ribes” is used when this odor is found to be desirable, such as in the Black Currant, whereas the term “catty” or “ribes” may be used to describe this odor when it appears as a taint or undesirable odor. (Clapperton, 1976). Therefore, all compounds with descriptors of “ribes,” “catty,” or black currant most likely possess similar odor properties.

Additionally, Vermuelen et al (2002) suggested that 4 polyfunctional thiol compounds possess a black currant (i.e. ribes or catty) aroma, including 4-mercaptopentan-2-one, 4-mercapto-4-methylpentan-2-one, 4-mercaptopentan-2-ol, and 4-mercapto-4-methylpentan-2-ol. Tominaga and Dubourdieu (1997) reported that 4-mercapto-4-methylpentan-2-one possesses an odor characteristic of the broom or Box tree. Additionally, 5-mercaptohexan-3-one is reported to possess a “box tree, fresh, emphyreumatic” odor (Collin et al, 2002). Tominaga and Dubourdieu (1997) used “The odor of Broom or Box Tree” as a descriptive term for the compound 4-mercapto-4-methylpentan-2-one, which has been described by several researchers as possessing a “ribes” aroma. 3-mercaptohexyl acetate, a thiol compound recently identified in Cabernet Sauvignon and Merlot wines of the Bordeaux region of France, possesses an odor that is described as “grapefruit, box tree, broom, passion fruit” (Bouchilloux et al, 1998).
The odor threshold of these polyfunctional thiols with a “ribes” or “catty” aroma may differ by several orders of magnitude. Bouchilloux et al (1998) reported an odor threshold in a 12% aqueous ethanol solution for 4-mercapto-4-methylpentan-2-one of 0.8 parts per trillion, whereas the odor threshold of the analogous alcohol, 4-mercapto-4-methylpentan-2-ol was reported as 55 parts per billion. Vermeulen et al (2001) determined odor thresholds for polyfunctional thiols using a technique known as BE-GC-LOADS to determine odor thresholds of these compounds with a GC odor sniffing port. The odor thresholds of the various polyfunctional thiols with potential “catty”-like odors differed over one order of magnitude from the highest odor threshold to the lowest odor threshold. Since the odor thresholds of these compounds appear to vary by individual compound and according to the type of media in which they are presented to the assessors, it is not possible at this time to correlate the concentration of any or all of these compounds to the “catty” flavor note in Cheddar cheese. Further work must be done in order to determine the odor properties of these compounds in a Cheddar cheese matrix, as well as the odor threshold of the individual compounds.

Future work will attempt to address several of the issues presented in the above discussion (Drake 2003, personal communications). In order to determine the sensory properties and odor thresholds of these compounds, they will be added individually and in various combinations and concentrations to Cheddar curd. Furthermore, the oxidation/reduction potential of the curd will be adjusted to that of a mature Cheddar cheese (Drake, 2003, personal communications).
<table>
<thead>
<tr>
<th>Compound</th>
<th>Odor description</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-mercapto-4-methylpentan-2-one</td>
<td>box tree, cat urine¹, black currant, catty, Broom, vinaigrette, citrus fruit³, catty⁵, ribes, characteristic odor of Box tree, Broom²</td>
</tr>
<tr>
<td>4-mercapto-4-methylpentan-2-ol</td>
<td>Broom, black currant, solvent, fresh, sweet³</td>
</tr>
<tr>
<td>4-mercapto-pentan-2-one</td>
<td>Greenery, potato, black currant³</td>
</tr>
<tr>
<td>4-mercapto-pentan-2-ol</td>
<td>Broom, black currant, catty³</td>
</tr>
<tr>
<td>5-mercaptohexan-3-one</td>
<td>Box tree, fresh, empyreumatic³</td>
</tr>
<tr>
<td>4-methoxy-2-methyl-2-butanethiol</td>
<td>Black currant⁴</td>
</tr>
<tr>
<td>3-mercaptohexyl acetate</td>
<td>Reminiscent Of Box tree with grapefruit and passion fruit notes²</td>
</tr>
<tr>
<td>3-mercapto-3-methylpentan-2-one</td>
<td>Catty⁵</td>
</tr>
<tr>
<td>( trans )-8-mercapto-p-menthan-3-one</td>
<td>Catty⁵</td>
</tr>
<tr>
<td>tert-amyl mercaptan</td>
<td>Catty⁵</td>
</tr>
</tbody>
</table>

**Table 6.1 Polyfunctional Thiols with “Catty” aroma descriptors**

1) Darriet et al, 1995  
2) Tominaga et al, 1996  
3) Vermeulen et al, 2001  
4) Reiners, J. and Grosch, W. 1999  
5) Polak et al, 1998
6.1.2. Brothy flavor

Brothy is an odor quality of aged American cheddar cheeses that was also measured in this study. Little is known about the cause of broth flavor in Cheddar cheese; however, Lindsay and Dunn (1985) determined that methional, when added to Cheddar, caused a broth flavor defect.

Recently, a number of polyfunctional thiols with “brothy” odor qualities were identified by Vermuelen et al (2002). Two polyfunctional thiols with brothy odors, 3-mercaptopbutanal and 3-methyl-3-mercaptopbutanal, were tentatively identified in 3 and 7 cheeses respectively. However, all cheeses had a brothy note, with scores ranging from 1 to 2.2. Thus, it is not possible in this study to determine the significance of the brothy polyfunctional thiols in respect to the brothy note of a given cheese, since the possible contributions of non-thiol brothy odor compounds such as methional cannot be accounted for.

6.1.3. Cooked flavor

Vermeulen (2002, personal communications) noted that as the polyfunctional thiols that were synthesized in this study began to oxidize and form disulfides, the cocktail of compounds developed “cooked” flavor notes. One compound (4-mercato-3-methyl pentan-2-one) present in 9 cheeses is described as being “cooked milk”. The existence of other polyfunctional thiols in an oxidized state in the cheese and associated with cooked flavor cannot be determined from this study. In addition methyl sulfides have long been associated with cooked flavor of milk and may be major contributors to the “cooked” flavor note of aged Cheddar.
6.2 Possible origins of polyfunctional thiols

The origins of the polyfunctional thiols in Cheddar cheese are not known at the present time. A number of different possibilities exist, as noted in the following discussion.

Vermuelen et al (2001, 2002) synthesized polyfunctional thiols by utilizing α,β-unsaturated aldehydes and ketones (ene-als and ene-ones, respectively) that were previously formed via aldol condensations. These ene-ones and ene-als were then reacted with H₂S to form a number of polyfunctional thiols (Vermuelen et al, 2001, 2002). The origin of the polyfunctional thiols tentatively identified in this study is unknown, however, by drawing on scientific discoveries in this and related fields, it is possible to speculate concerning the mechanisms of their formation. Three mechanisms are discussed below that may result in formation of these polyfunctional thiol compounds. These mechanisms include the chemical reactions associated with the Aldol Condensation, formation of polyfunctional thiols from bound precursors, and reactions similar to those occurring in cooked meat.

6.2.1. Aldol condensation and related reactions

The aldol condensation reaction is the acid catalyzed or base catalyzed reaction between 2 carbonyl compounds, either aldehydes or ketones, and this type of chemical reaction is one of the most useful and most studied synthetic reactions for carbon-carbon bond formation (Carey and Sundberg, 1993). When this reaction occurs between two different carbonyl compounds, the term mixed aldol condensation is applied (Carey and Sundberg, 1993). The reaction product may undergo further transformations, especially
dehydration (Carey and Sundberg, 1993), to form an \( \alpha,\beta \) unsaturated aldehyde or ketone. Dehydration reactions are catalyzed by the presence of acid or base. The slightly acidic conditions in Cheddar cheese would favor the dehydration of aldol condensation products to an \( \alpha,\beta \) unsaturated aldehyde or ketone. Typically, a series of equilibriums exist between the free aldehydes and/or ketones, the aldol condensation product, and the \( \alpha,\beta \) unsaturated aldehyde or ketone (Carey and Sundberg, 1993).

There are a number of aldehyde and ketones present in Cheddar cheese that are capable of undergoing aldol or mixed aldol condensations. These compounds include acetaldehyde, acetone, butanone, 2-pentanone, 2-heptanone, 2-nonanone, 2-undecanone, 2-tridecanone, and 2-pentadecanone. All of these compounds occur in low parts per million in Cheddar cheese. The concentration ranges of these compounds as they occur in Cheddar are listed in Table 6.6. Interestingly, all of the polyfunctional thiols tentatively identified in Cheddar cheese could be formed by reactions of the compounds listed below with hydrogen sulfide.

Maillard-type reactions, specifically the Strecker Degredation between homocysteine and diacetyl was observed by Vermuellen et al (2002) to be a source of 2-mercaptopropanal. Although this amino acid has not been measured in Cheddar cheese, a reaction between free cysteine and diacetyl would produce the polyfunctional thiol 2-mercaptoethanal.
Carbonyl | Range of concentration, ppm
---|---
acetaldehyde | 0.1 - 7.5
acetone | 0.1 - 8.5
butanone | 0.1 - 67.1
2-pentanone | 0.1 - 2.9
2-hexanone | 0.3 - 0.9
2-heptanone | 0.58 - 6.6
2-nonanone | 0.2 - 1.7
2-undecanone | 0.2 - 1.5
2-tridecanone | 0.11 - 2
2-pentadecanone | 0.06 - 2.3

Table 6.2 Ranges of concentration of aldehydes and ketones in Cheddar cheese

6.2.2. Reactions of H$_2$S with aldol condensation products, $\alpha,\beta$ unsaturated aldehydes or ketones

The aldol condensation does not produce polyfunctional thiols. However, H$_2$S will readily react with the dehydration products ($\alpha,\beta$ unsaturated aldehydes or ketones) of an aldol condensation. The addition of nucleophiles to $\alpha,\beta$ unsaturated aldehydes or ketones is an extremely useful synthetic pathway and has been studied extensively within the scientific discipline of synthetic organic chemistry (Carey and Sundberg, 1993). Molecular orbital calculations demonstrate that in the $\alpha,\beta$ aldehyde or ketone, the molecular orbital on the carbon atom $\beta$ to the carbonyl functional group is the most electron deficient, and this will always be the site of attack by the nucleophile (Carey and Sundberg, 1993).

H$_2$S adds readily across the carbon - carbon double bond in a $\alpha,\beta$ unsaturated aldehyde, or ketone such as mesityl oxide, with the Sulfur atom in the $\beta$ position, and a
hydrogen atom adding in the $\alpha$ position to form 4-mercapto-4-methylpentan-2-one (Barret, 1978). The concentration of Hydrogen sulfide in the headspace of Cheddar cheese has been measured between 0.45 and 0.8 ppm (Lawrence, 1963).

**6.2.3 Evidence for the aldol condensation and addition of H$_2$S to $\alpha,\beta$ unsaturated aldehydes or ketones in Cheddar cheese**

Badings (1967) demonstrated that acetone producing non-starter lactic acid bacteria were responsible for the development of a catty off-flavor in Gouda. Badings (1967) suggested that some of the acetone produced by these bacteria reacted with H$_2$S to form the catty-smelling compound 4-mercapto-4-methylpentan-2-one.

Kristofferson and Gould, (1959), suggested that most carbonyl compounds occurring in Cheddar, rather than contributing directly to flavor, may serve as precursors for the formation of specific flavor components of Cheddar cheese. Furthermore, addition of a mixture of thioacetamide, a precursor to H$_2$S, and the methyl ketones acetone, butanone, pentan-2-one, heptan-2-one, nonan-2-one, undecan-2-one, and tridecan-2-one, to a 3-week-old cheese was observed to impart a “Cheddar-like” flavor to the young cheese. The cheese was also judged to be lacking in a “savory background flavor” over which the “Cheddar-like” flavor would be superimposed in an aged cheese (Walker, 1961).

These observations by Kristofferson and Gould (1959), Walker (1961), and Badings (1968) and the detection of a number of polyfunctional thiols in this study, suggest that polyfunctional thiols could be formed from the reactants that Walker (1961) added to 3-week-old Cheddar. The Aldol Condensation reaction, followed by the addition of H$_2$S to $\alpha,\beta$ unsaturated aldehydes or ketones may constitute the mechanism by which
these compounds are formed in Cheddar. Finally, a “retro synthetic analysis” (i.e. reverse synthesis or running the synthesis backwards) of the polyfunctional thiols tentatively identified in this dissertation, and previously synthesized by Vermuelen et al (2001, 2002) further suggests that they could be formed from reactions of H_2S with carbonyl compounds commonly found in Cheddar and listed in Table 6.2 on the previous page.

Appendix B contains several reaction schemes for the formation of polyfunctional thiols via the mechanisms described in the above sections. Figure 10 demonstrates the formation of 4-mercapto-4-methylpentan-2-one from acetone and hydrogen sulfide. Figure 12 demonstrates the formation of 3-mercapto-3-methylbutanal from acetone, acetaldehyde, and hydrogen sulfide. Figure 13 demonstrates the formation of 4-mercaptopentan-2-one from acetone, acetaldehyde, and hydrogen sulfide.

6.2.4 Formation of polyfunctional thiols with an alcohol functional group

Lactic acid bacteria may actively reduce aldehydes and ketones to the corresponding alcohols via an alcohol-reductase enzyme (Tidswell and Morris, 1993). A number of medium higher molecular weight alcohols derived from the corresponding methyl ketones have been identified in a number of cheeses (Tidswell and Morris, 1993). Heterofermentative lactobacilli are used in the chemical processing industry for the enzyme catalyzed formation of enantiomerically pure alcohols from various aldehyde and ketone substrates (Tidswell and Morris, 1993). It is suggested then that polyfunctional thiols with an alcohol functional group may be formed by the enzyme-catalyzed reduction of polyfunctional thiols possessing an aldehyde or ketone functional group (Appendix B., Figure 13)
6.2.5 Formation of polyfunctional thiols from bound precursors

Recently, several polyfunctional thiols compounds have been found in wine as well as fresh passion fruit juice (Bouchilloux et al, 1998). These polyfunctional thiols occur naturally in Passion fruit and certain cultivars of the wine making grape *Vitis vinifera*, in the form of S-cysteine precursors (Tominaga and Dubourdieu, 2000; Murat et al, 2001, Des Gachons, Tominaga and Dubourdieu, 2000). These precursors are released from the conjugate in the presence of the yeast *Saccharomyces cerevisiae* (Murat et al, 2001 des Gachons, Tominaga and Dubourdieu, 2000).

It is possible that the polyfunctional thiols may originate in forage or feed if the precursor molecules occur there. However, at this point it is merely speculation, as no evidence exists to suggest that these compounds occur in sources of feed. Furthermore, it is not known if it is possible for the S-cysteine precursors to pass through the digestive system of the cow into milk. However, a precursor to Dimethyl Sulfide, S-methyl-methionine sulphonium salt, is believed to pass from feed or forage into milk, and this precursor compound has been tentatively identified in milk by thin layer chromatography (Keenan and Lindsay, 1968).

6.2.6. Heated reaction-type flavors

The flavor of cooked or roasted foods is a very complex subject, and an in depth review of this topic is beyond the scope of this discussion. However, it is possible to speculate that chemical reactions responsible for generating thiol containing flavor compounds in cooked or roasted meat or grain may also give rise to similar flavor compounds in cheese. A number of polyfunctional thiols have been found in cooked meat.
and model systems of cooked meat. These compounds include 4-mercapto-4-methylpentan-2-one, 3-mercapto-2-butanone, 2-methyl-3-furanthiol, 3-mercapto-2-pentanone, 1-mercapto-2-propanone, 2-furfurylthiol, 2 methyl-3-furanthiol, 3-thiophenethiol, 2-methyl-3-thiophenethiol, and 2-thiophenemethanethiol (Hoffman and Schieberle, 1995; Zhang and Ho, 1991, Mottram and Nobrega, 2002).

There appear to be 3 basic types of reaction mechanisms that result in the formation of polyfunctional thiols in cooked or heated food systems. First of all, free H$_2$S may react with ene-ones or ene-als as already outlined earlier. Second, there appears to be a series of ring closure reactions resulting from the pyrolysis of a sugar molecule to form a 5-member furan ring. This series of reactions also results in the capture of sulfur atom from H$_2$S or cysteine. The Sulfur atom is incorporated into the molecule in a thiol functional group. Third, reactions between H$_2$S or cysteine and a sugar molecule result in formation of a polyfunctional thiol with adjacent ketone and thiol functional groups (Hoffman and Schieberle, 1995; Zhang and Ho, 1991, Mottram and Nobrega, 2002).
CHAPTER 7

SUMMARY

Attention was given to further elucidation of the role of sulfur compounds in the flavor of aged Cheddar cheese. Eleven aged, commercial Cheddar cheeses, over 6 months of age, were obtained from different regions in the country. These cheeses were:

- Evaluated by sensory descriptive analysis
- Evaluated an electronic nose using a mass spectrometer
- Comparison made between sensory and electronic nose analyses
- Analyzed for multifunctional thiol compounds

Sensory descriptive analyses of the 12 cheeses showed that all cheeses were different, especially in respect to the four descriptors that were related to sulfur notes (total sulfur, egg like, match-like and catty). Statistically, both total sulfur and catty were correlated to age. Catty and match-like were also correlated to one another. None of the other sensory descriptors were correlated to age.

The electronic nose differentiated the aroma of the eleven cheeses when the mass spectrometer was operated in the negative chemical ionization (NCI)
mode, but not in the electronic ionization (IE) mode. The statistically significant masses the aroma of these cheese were 59, 61, 73, 75, 77, 79, 89 and 133. Theoretical assignment of massed to chemical compounds known to be present in Cheddar cheese suggested that the sulfur related compounds could be dimethyl sulfide and 4-mercapo-4-methyl pentan-2-one.

Comparison of the results of the sensory and electronic nose results showed similarity. Both methods differentiated the cheese in the same three clusters. PCA results also gave differentiation, but suggested that different components might be involved in the differentiations.

In order to analyze for trace polyfunctional thiol compounds in Cheddar cheese, a new method was developed which was capable of analyzing these compounds in ppb and ppt concentrations in the presence of several hundred other compounds.

Cheddar cheese was centrifuged to extract the fat phase. This was then diluted with an equal volume of redistilled hexane. The combined oil and hexane, and a thiol containing internal standard was then extracted with an aqueous solution of ethanol, TCEP, p-HMBA, and NaOH. The extract was percolated through a cation exchange resin, previously regenerated with HCl. A solution of cysteine, previously washed 3 times with diethyl ether: pentane (1:1), was then percolated through the column. Free thiols were obtained from the eluate by extracting it 3 times with diethyl ether: pentane (1:1). The ether: pentane solution was then concentrated by evaporation to approximately 100 micro liters. Thiols were separated and quantified by capillary GC with SCD detection.
A total of 40 different poly functional thiols were found in the eleven aged Cheddar cheese, but none were found in fresh Cheddar cheese curd. However, the cheeses differed markedly in the specific compounds found in any given cheese. The numbers of thiols present in a given cheese varied from 2 to 8.

A total of 15 polyfunctional thiols with either an aldehydes, ketone, or alcohol functional group were tentatively identified by using Kovat’s retention indexes. These compounds were found to occur in concentrations ranging from 1 to 60 ppb. Four-mercapto-3-methyl petane-2-one (cooked milk), 4-mercapto-4-methyl pentan-2-one (catty), 3-mercapto-2-butylpropanal (cheese broth), 3-mercapto-3-methyl butanal (cheese, broth) were present in 9, 8, 8 and 7 cheeses respectively. [Add note on statistical correlation of concentrations to age, and sulfur notes]

Molecular weights could be estimated for another 15 unidentified thiol compounds. [Time permitting – a check to be made to see if any of compounds in Table 2.4 match these molecular weights]

GC/MS was not successful in the analysis of the polyfunctional thiols because: (a) lack of sensitivity for compounds in low ppb concentrations, (b) presence of >200 non-thiol compounds which include large concentrations of free fatty acids and (c) only one of the polyfunctional thiols was either commercially available or present the MS library.

The three analytical approaches all confirm the importance of sulfur compounds to the flavor of aged Cheddar cheese and all help support the
significance of the poly functional thiols in Cheddar cheese flavor, especially the 4-mercapto-4methyl pentan-2-one (catty) compound.

7.1. Concluding Remarks

The research presented in this dissertation represents several important advances that have been made in understanding the flavor chemistry of Cheddar cheese. More work is needed in order to verify the identity of these compounds, their role in the flavor of Sharp Cheddar cheese, and the mechanisms associated with their formation in a ripening cheese body.

Several follow up studies have already been planned. Drake (2003, personal communications) plans to determine the odor properties and odor thresholds of polyfunctional thiols in Cheddar by adding these compounds individually and in varying combinations and concentrations to Cheddar cheese curd, under conditions of controlled oxidation/reduction potential.

Harper (2002, personal communications) has suggested that a large study involving several research groups is needed in order to determine the mechanisms of formation associated with these compounds.

Clearly, this dissertation represents significant groundbreaking work in the field of Cheddar cheese flavor, and this preliminary work will continue to bear fruit in future studies.
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APPENDIX A

SELECTED CHROMATOGRAMS FOR VOLATILE SULFUR COMPOUNDS IN CHEDDAR CHEESE

Figure 5: Cheddar cheese NE1
Figure 6: Cheddar cheese NE5
Figure 7: Cheddar cheese NE7
Figure 8: Cheddar cheese NW1
Figure 9: Cheddar cheese NE6
APPENDIX B

PROPOSED MECHANISMS OF FORMATION OF VARIOUS POLYFUNCTIONAL THIOLS

Figure 10: Formation of 4-mercapto-4-methylpentan-2-one from acetone and hydrogen sulfide
Figure 11: Formation of 3-mercapto-3-methylbutanal from acetone, acetaldehyde, and hydrogen sulfide
Figure 12: Formation of 4-mercapto-4-methylpentan-2-one from acetone, acetaldehyde, and hydrogen sulfide
Figure 13: Formation of chiral alcohol by a reductase enzyme
APPENDIX C

MECHANISMS OF OXIDATION AND ISOLATION OF POLYFUNCTIONAL THIOLS FROM CHEDDAR CHEESE

Figure 14: Formation of di and polysulfides in Cheddar cheese exposed to air
Figure 15: Reduction of a disulfide by a phosphine to form two free thiols and a phosphine oxide
Figure 16: Capture of a thiol by p-hydroxymercuribenzoic acid
Figure 17: Liberation of a free thiol from p-hydroxymercuribenzoic acid