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# Volatile sulfur compounds in Cheddar cheese determined by headspace solid-phase microextraction and gas chromatograph-pulsed flame photometric detection

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**Abstract**

The aim of this study was to develop a methodology for the analysis of volatile sulfur compounds (VSCs) in Cheddar cheese. Solid-phase microextraction (SPME) was employed to extract VSCs from the cheese matrix using a CAR-PDMS fiber. This extraction method was combined with gas chromatography–pulsed flame photometric detection (GC–PFPD) to achieve high sensitivity for sulfur compounds. The impact of extraction parameters, including time, temperature and sample size, was evaluated to determine the best conditions to analyze sulfur compounds in Cheddar cheese. Hydrogen sulfide, methanethiol, and dimethyl sulfide were found to constitute the majority of the overall sulfur profile while dimethyl disulfide and dimethyl trisulfide were present in lesser amounts. Artifact formation of volatile sulfur compounds was found to be minimal. Two commercial cheese samples were analyzed and differences in sulfur content were observed. Overall, SPME–GC–PFPD was found to be a highly sensitive technique for the analysis of sulfur compounds in Cheddar cheese.


*Keywords:* Volatile sulfur compounds; Cheese; Food analysis; Pulsed flame photometric detection

## 1. Introduction

Volatile sulfur compounds (VSCs), most with low odor thresholds, are considered important flavor contributors to cheeses [1–6]. Methanethiol, hydrogen sulfide, and dimethyl sulfide have all been found to be significant to the flavor of Cheddar cheese [7–11]. The ripening process of Cheddar cheese involves, in part, the decomposition of sulfur-containing amino acids, cysteine and methionine. An increase in the concentration of methanethiol as Cheddar cheese ages has been reported [12] and that it is postulated that other sulfur-containing compounds may also follow a similar trend with aging. Methanethiol not only contributes to Cheddar cheese flavor but also is a precursor for several other sulfur compounds. Once formed, methanethiol can be readily oxidized to create dimethyl sulfide, dimethyl disulfide, dimethyl trisulfide and other sulfur compounds [13,14].

While there is definite evidence that various sulfur compounds are present in Cheddar cheese, their significances to Cheddar cheese flavor are still poorly understood. This contributes to the fact that the volatile sulfur profile of Cheddar cheese highly relies on the specific method used for extraction and/or concentration techniques. Many conventional techniques including solvent extraction, static headspace, and purge-and-trap are not quite suitable for the analysis of VSCs in cheese. Each of these techniques has associated problems when it comes to extracting highly volatile sulfur compounds from cheese matrices including, respectively, the loss of analytes during the concentration stage, particularly compounds with high volatility; insufficient sensitivity for trace components; and great potential of thermal artifact formation [15–17].

Solid-phase microextraction (SPME) can effectively extract and concentrate aroma compounds and also provides

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high sensitivity with minimum artifact formation. With the use of SPME fibers, there is no requirement for organic solvents, and sample preparation can be completed in minimal time. In addition, SPME equipment can be automated. While there are a growing number of available fiber coatings, the Carboxen-polydimethylsiloxane (CAR-PDMS) fiber has repeatedly demonstrated its exceptional ability to extract sulfur compounds including methanethiol and dimethyl sulfide from food [18–25]. The process of concentration with the CAR-PDMS fiber is *adsorption* of small molecules into micro-pores by the Carboxen phase in addition to *absorption* by the PDMS coating [22], lending to its greater capacity for extracting highly volatile, low molecular weight molecules, which includes most VSCs.

Pulsed flame photometric detection (PFPD) is a very sensitive sulfur-specific method of detection that uses a pulsed flame for the generation of flame chemiluminescence. Unlike traditional flame photometric detection (FPD), which uses a continuous flame, the PFPD utilizes low gas rates so that the flame is ignited, propagated and self-terminated 2–4 times per second. Specific elements have their own emission profile: hydrocarbons will complete emission early while sulfur emissions begin at a relatively later time after combustion. Therefore, a timed "gate delay" can selectively allow for only emissions due to sulfur to be integrated, producing a clean chromatogram. This timed "gate delay" greatly improves the sensitivity; the PFPD can detect sulfur-containing compounds at a much lower detection limit than nearly all other methods of detection [26]. The combination of SPME with GC–PFPD greatly enhances the ability to successfully extract and detect VSCs in cheese at low concentrations. The objective of this study was to develop a methodology using SPME–GC–PFPD to analyze the overall sulfur profile of Cheddar cheese.

## 2. Experimental

### 2.1. Chemicals

Pure standards were obtained for proper identification of chromatographic peaks: dimethyl sulfide was purchased from TCI America (Portland, OR, USA); dimethyl disulfide, dimethyl trisulfide, dimethyl sulfoxide, and dimethyl sulfone were all obtained from Aldrich (Milwaukee, WI, USA). Carbon disulfide was obtained from EMD Chemicals Inc. (Gibbstown, NJ, USA) and methional (3-methylthiopropionaldehyde) was purchased from Sigma (St Louis, MO, USA). Ethyl methyl sulfide (TCI-EP, Tokyo, Japan) was used as an internal standard for the analysis of VSCs in cheeses with various aging levels (Section 2.4.3).

Gaseous methanethiol was purchased in a cylinder from Aldrich (St Louis, MO, USA) and a solution was prepared by bubbling into methanol. Hydrogen sulfide was prepared by dissolving $Na_2S{\cdot}9H_2O$ (Sigma, St Louis, MO, USA) in acidic water (pH 3). Carbonyl sulfide (COS) was prepared according to the method described in Metrohm Information [27] with some modifications: concentrated sulfuric acid was added dropwise from a dropping funnel to potassium thiocyanate (both from Mallinckrodt/J.T. Baker Inc, Phillipsburg, KY, USA) in a stoppered Erlenmeyer flask; the generated COS (g) was passed through a small diameter glass transfer-line immersed in a cold water bath (for exothermic nature of reaction) and the COS (g) was trapped by bubbling into a separate flask containing distilled water.

### 2.2. Equipment

#### 2.2.1. GC–PFPD analysis

A Varian CP-3800 gas chromatograph (Varian, Walnut Creek, CA, USA) equipped with a 1077/1079 split/splitless injector port and a pulsed flame photometric detector was used for this study. The volatile compounds extracted by the SPME fiber were thermally desorbed in the 300 °C injector port for 10 min. The injector was in splitless mode for the first four minutes, after which the split valve was opened. Separation of the analytes was performed using a DB-FFAP fused silica capillary column (30 m × 0.32 mm, 1.0 μm film; Agilent, Palo Alto, CA, USA), with nitrogen as the carrier gas (constant flow at 2.0 mL/min). The oven temperature program was as follows: 35 °C held for 5 min, heated to 150 °C at a rate of 10 °C/min, held for 1 min, then heated to 220 °C at a rate of 20 °C/min with a final hold time of 5 min. The PFPD was held at 300 °C and 450 V with the following flow rates: Air1 at 17 mL/min, $H_2$ at 14 mL/min, and Air2 at 10 mL/min. The detector response signals were integrated using computer software (Star Workstation 6.2, Varian). Statistical analyses were performed with STATGRAPHICS*Plus* software (version 5.0, Manugistics Inc., Rockville, MD, USA).

#### 2.2.2. SPME extraction

A Stableflex 85 μm CAR-PDMS fiber (Supelco, Bellefonte, PA, USA) was used in this study. Prior to use, the fiber was conditioned at 300 °C for 90 min. The fiber was then placed into the SPME adapter of a CombiPAL autosampler (CTC Analytics AG, Zwingen, Switzerland) fitted with a vial heater/agitator. For all analyses, agitation during equilibration (10 min prior to fiber exposure) was set to 500 rpm while agitation during extraction was held at 250 rpm.

### 2.3. Initial sample preparation

Cheddar cheeses with varying ages ("medium", "sharp", and "extra sharp" of two different commercial brands) were purchased from a supermarket and were refrigerated at 3 °C. Samples were used within one month from purchase date. For each cheese sample, a layer of 2 cm was removed from the surface in order to eliminate any possible fluctuations in volatile composition and contamination from packaging. The cheese was then cut into small cubes, measuring approximately 0.5 cm on each side. All vials used in this study were flushed with argon prior to the addition of sample. Following

the addition of samples, the vials were immediately sealed with Teflon-lined silicone caps (8 mm hole). All samples were analyzed in triplicate.

### 2.4. Preparation for parameter evaluation and extraction methods

#### 2.4.1. Time and temperature parameters

For the analysis of the time and temperature parameters, 5 g of "sharp" cheese cubes were individually weighed into 20 mL headspace vials with the addition of 0.5 g water to improve agitation. For the time trials, the equilibration/extraction temperature was held constant at 50 °C while the following lengths of time for fiber exposure to the headspace were evaluated: 5, 15, 30, 45, 60, 90, and 120 min. For the temperature trials, the fiber exposure time was held constant at 30 min while the following equilibration/extraction temperature range was evaluated: 30–70 °C in 5 °C increments.

#### 2.4.2. Sample size parameter

The indicated sample sizes (0.5, 1, 2, 3, 4, and 5 g of "sharp" cheese cubes) were prepared with the addition of vegetable (100% soybean) oil, rather than water as above, at 10% of the sample weight (for example, 0.05 g oil was added to the 0.5 g cheese sample while 0.4 g oil was added to the 4 g cheese sample). Using a Teflon resin-coated spatula, samples were ground directly in the vial using caution to keep all samples in the bottom of the vial. For the sample size trials, the vials were held at a constant 50 °C for equilibration/extraction and the fiber was exposed to the headspace over the varying sample sizes for 30 min.

#### 2.4.3. Cheese at various aging levels

Sulfur contents of two commercial brands of cheeses at different levels of aging ("medium" = 60 days, "sharp" = 9 months, "extra sharp" = 15 months) were analyzed. For each aged sample, 2 g cheese cubes with the addition of 0.2 g vegetable oil containing the internal standard, ethyl methyl sulfide, was ground as described previously in the evaluation for sample size. These samples were analyzed using an equilibration/extraction temperature of 50 °C and an extraction time of 30 min.

## 3. Results and discussion

### 3.1. Evaluation of SPME extraction parameters: time, temperature, sample size

Parameters for SPME extraction were evaluated to determine the best conditions to use for the extraction of VSCs from the cheese sample matrix. Extraction time, temperature, and sample size were all taken into consideration for overall extraction efficiency. The values for peak area (arbitrary units) were square-rooted because the PFPD response is from the emission of two excited sulfur atoms ($S_2^*$) corresponding to a second-order, or quadratic, response.

#### 3.1.1. Evaluation of time parameter

For the time trials, a fixed temperature was chosen (50 °C) and the time of extraction was varied. The results, as shown in Fig. 1, demonstrated that as time increased so did the amount of volatiles extracted by the SPME fiber, as seen from the PFPD response, but the relationship was not the same for all sulfur compounds. While most sulfur compounds, such as methanethiol, hydrogen sulfide and dimethyl sulfide, seemed to follow a logarithmic-type trend, the data for dimethyl disulfide and dimethyl trisulfide was better fit with a power function. In all cases, adsorption rates progressively decreased with no immediate evidence of reaching equilibrium within the tested time range (up to 2 h).

The volatile composition of Cheddar cheese is complex therefore numerous volatile compounds can be in competition for the adsorption sites of the fiber. Although the PFPD reports only signals from sulfur-containing compounds, the SPME fiber is not similarly selective and will extract a wide range of volatile compounds. When using a CAR-PDMS fiber to extract analytes from a complex matrix, the porous structure of the fiber can easily become saturated when using prolonged extraction times [28]. Once this occurs, compounds with a higher affinity for the fiber will essentially displace those compounds with lower affinity. The occurrence of this phenomenon is well known [21,29] and is often referred to as competitive adsorption. This can be minimized when shorter extraction times are used [21,28], although sensitivity is usually decreased since concentrations of analytes will not have approached their maximum levels within the fiber.

In this study, the detector response for most VSCs of interest was found to be sufficient with extraction times as low as 5 min due to the enhanced sensitivity of the PFPD toward detecting sulfur compounds. One-way analysis of variation (ANOVA) and Tukey's multiple-range tests demonstrated that, for many VSCs, significant improvement for extraction efficiency ($p \leq 0.05$) was observed when the fiber exposure time was increased from 5 to 30 min. The extraction efficiency improved less significantly with further increases in exposure time. For practical reasons, the shortest successful extraction time was desired, therefore, 30 min was chosen as a good compromise between overall sensitivity and runtime efficiency for the instrument.

#### 3.1.2. Evaluation of temperature parameter

A relationship between temperature and extraction efficiency was not easy to establish for all sulfur compounds. Fig. 2 illustrates the outcome from changing the temperature of extraction. Higher temperatures typically increase the volatility of volatile compounds, thus increasing their concentrations in the headspace. However, for some highly volatile compounds, further increases in temperature are

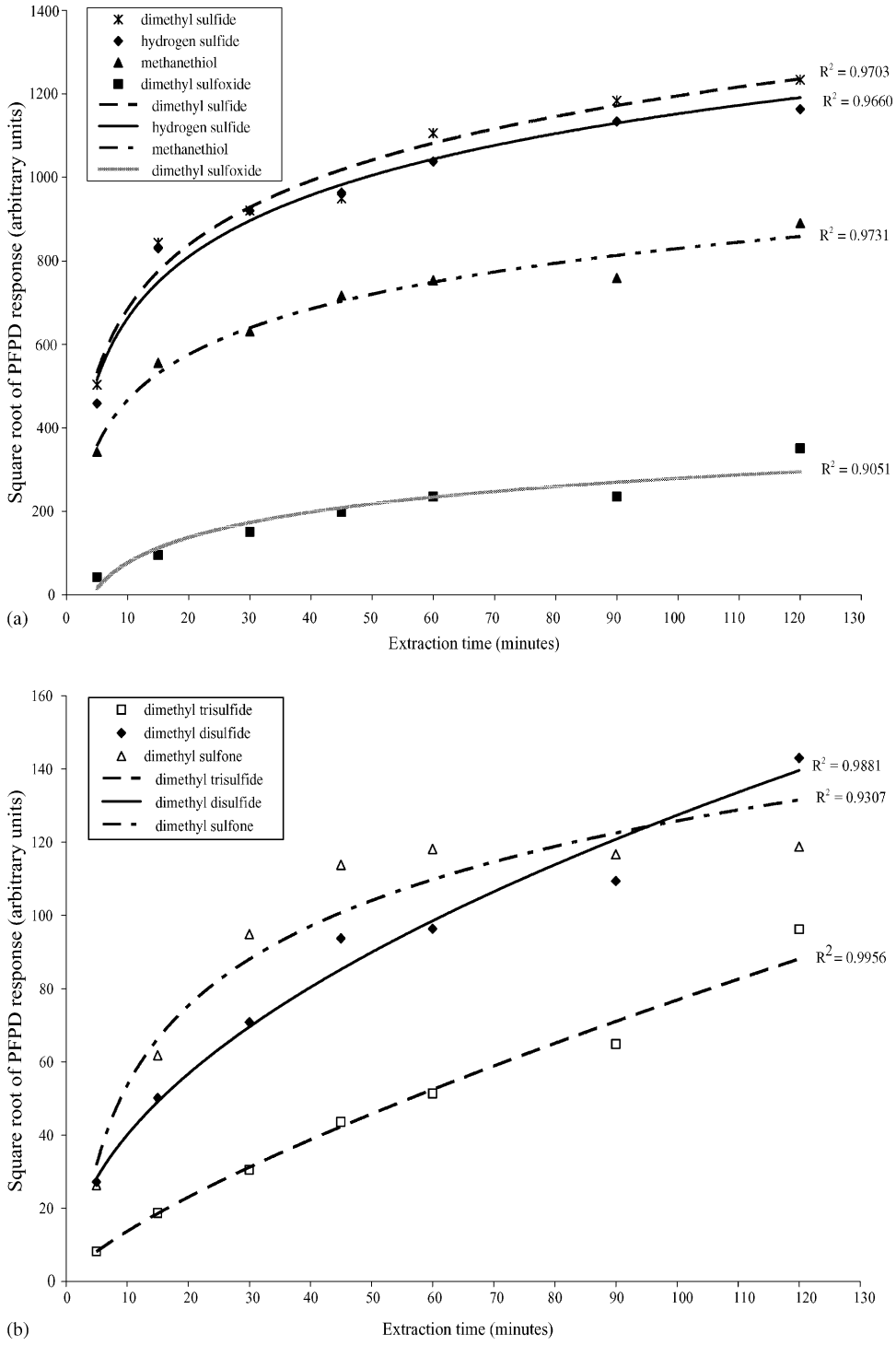


Fig. 1. Effect of time on SPME extraction of VSCs from Cheddar cheese with temperature held at 50 °C: (a) hydrogen sulfide, methanethiol, dimethyl sulfide, dimethyl sulfoxide; (b) dimethyl disulfide, dimethyl trisulfide, dimethyl sulfone.

expected to have minimal impact on their concentrations in the headspace. This hypothesis was confirmed for methanethiol, dimethyl disulfide and dimethyl trisulfide. As temperature increased from 30 to 70 °C, the responses for these compounds stayed almost the same. Since higher temperatures generally allow less volatile compounds, such as the larger molecular weight compounds, to be more easily released from the cheese matrix, this should theoretically increase the extraction efficiency of these compounds. The results in Fig. 2b showed that a definite increase in response occurred for dimethyl sulfone and methional when higher temperatures were used. The presence of methional in the overall sulfur profile chromatogram was only observed when the extraction temperature was raised above 50 °C with sat-

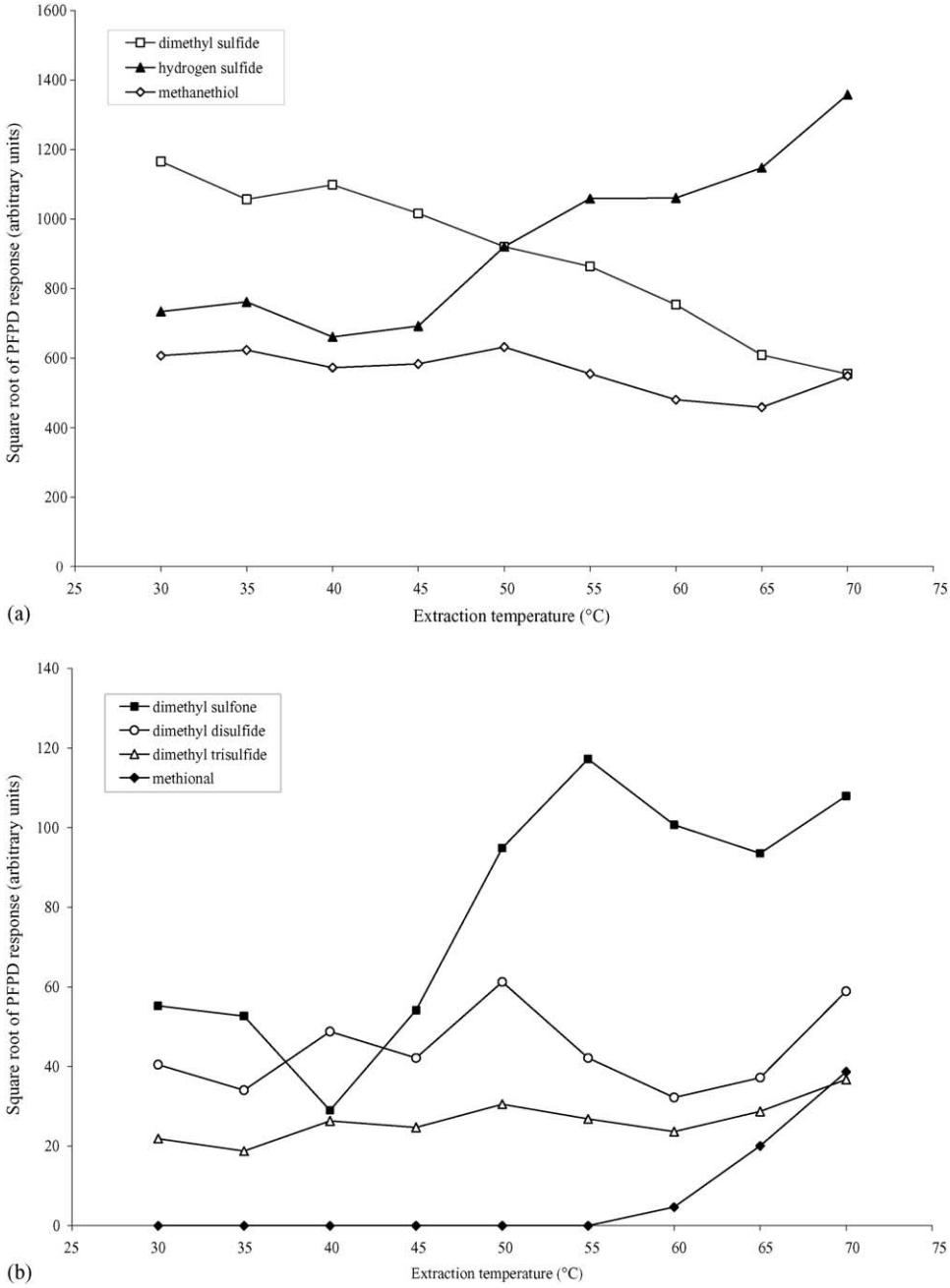


Fig. 2. Effect of temperature on SPME extraction of VSCs from Cheddar cheese with extraction time set at 30 min: (a) hydrogen sulfide, methanethiol, dimethyl sulfide; (b) dimethyl disulfide, dimethyl trisulfide, methional, dimethyl sulfone.

isfactory peak shape only seen with the highest temperature tested (70 °C).

For many of the higher molecular weight VSCs, an increase in PFPD response was observed with corresponding increases in temperature, however it was interesting to note that a decreased response occurred at 40 °C for dimethyl sulfone. When the cheese samples were heated at temperatures around 35–40°, the physical state of the cheese began to change causing fats in the cheese to exude from the matrix to form an oily layer that covered the surface of the sample. It is possible that, at this temperature, the oily layer had a negative effect on extraction efficiency for some sulfur compounds, namely dimethyl sulfone, since the presence of lipids can slow certain volatiles from volatizing into the headspace. Because the lipid content of Cheddar cheese is about one-third of its total mass [30], distinct interactions can occur between VSCs and the lipid phase, depending on the partition coefficient ($K_{\text{oil-headspace}}$) of the molecule [31,32]. When the temperature was further increased, the energy became high enough for these molecules to overcome any energy barriers that had kept them within the matrix [33]. Therefore, the successful extraction of dimethyl sulfone and methional from

the cheese matrix is highly temperature dependent as seen from the results.

Uniquely, dimethyl sulfide was observed to have a continuous decrease in response with increasing temperature. Although the actual mechanism is still not clear, it has been reported that high temperature can have undesirable effects on the extraction efficiency of SPME fibers for some low molecular weight analytes with higher volatility [15,34,35]. This could be related to the exothermic nature of adsorption of analytes onto the fiber, where higher temperatures may actually decrease the adsorption [20,35]. It could also be that the distribution coefficient for dimethyl sulfide, between the fiber and headspace of the sample ($K_{\text{fiber-headspace}}$), may decrease with higher temperatures [34] so that analytes with higher $K_{\text{fiber-headspace}}$ values would essentially push dimethyl sulfide out of the fiber, a good example of competitive adsorption. Because higher temperatures generally cause an escalation in the headspace concentrations of many compounds, such as free fatty acids, the increased abundance of those compounds in the headspace could progressively exclude dimethyl sulfide from the fiber.

In general, increasing the extraction temperature allowed for most VSCs to be effectively extracted from the cheese matrix. However, when the extraction temperature was raised

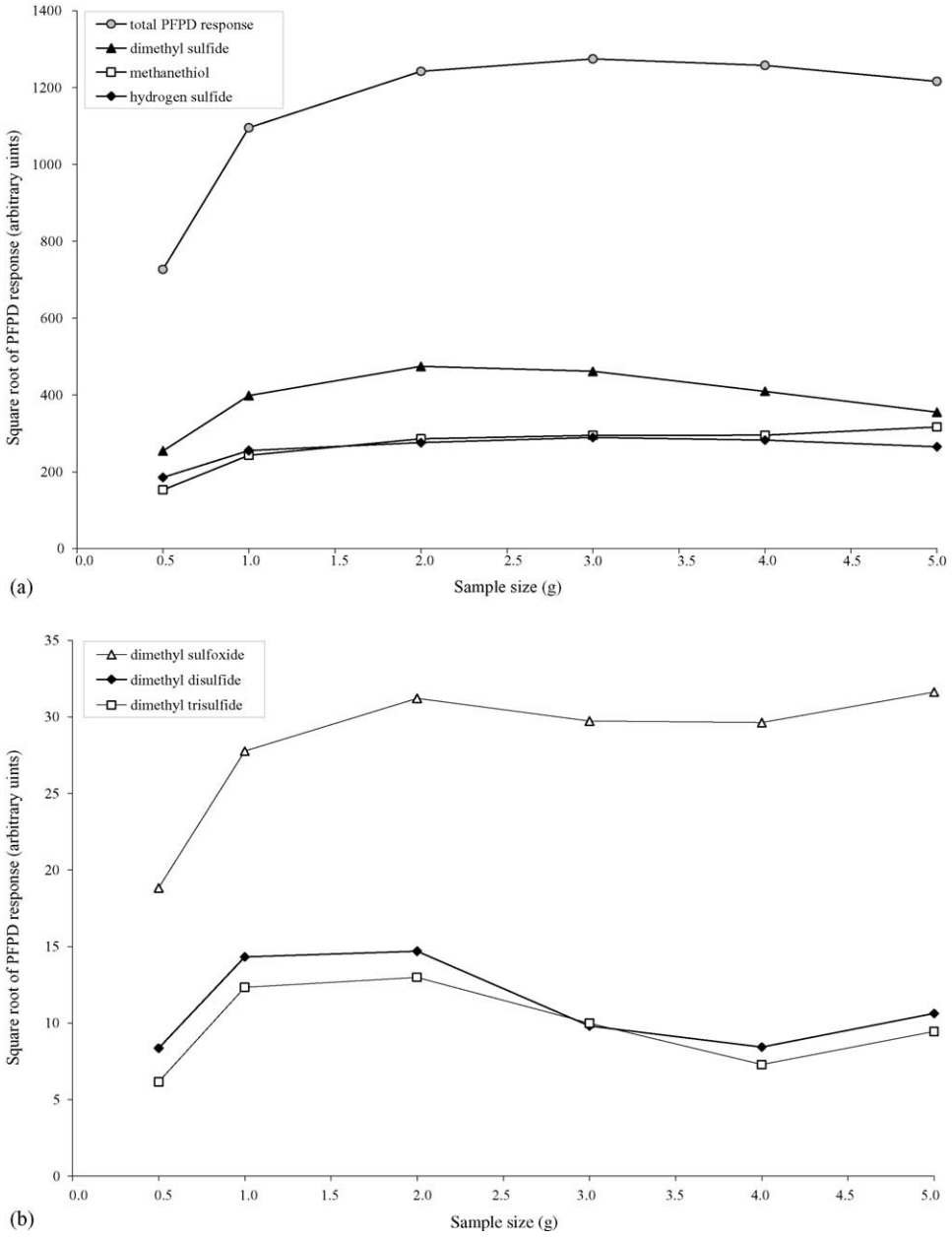


Fig. 3. Effect of sample size on SPME extraction of VSCs from Cheddar cheese, extraction performed at 50 °C for 30 min: (a) hydrogen sulfide, methanethiol, dimethyl sulfide, and total PFPD response (peak areas of all VSCs of interest combined, then square rooted); (b) dimethyl disulfide, dimethyl trisulfide, dimethyl sulfoxide.

above 55 °C, there was observed to be an increase in the number and/or size of unknown peaks, presumably the result of thermally generated compounds. Because of this, temperatures above 55 °C were deemed unacceptable whereas temperatures of 45 °C and below were not able to evenly melt the cheese samples, resulting in higher coefficients of variance (CV), where the average CV values ranged from 10 to 32% for all extractions below 45 °C. In the end, a satisfactory extraction temperature of 50 °C was selected, where most sulfur compounds of interest gave relatively high responses with low CV values (CV range = 2–9%, for extraction at 50 °C).

### 3.1.3. Evaluation of sample size parameter

Extraction of specific analytes from the headspace of a sample using SPME depends on numerous factors. In addition to time, temperature, and distribution coefficients (such as $K_{\text{sample-headspace}}$, $K_{\text{fiber-headspace}}$), the amount of sample and the headspace volume are also important [36]. Since more sample can contribute more volatiles to the headspace, the total amount of analytes that can be adsorbed by the fiber can be directly related to the sample size [36]. In this study, it was observed, as illustrated in Fig. 3, that sensitivity improved as the sample size increased from 0.5 to 2 g. Beyond that, further increase in sample size did not significantly increase overall sensitivity. For some compounds, such as dimethyl sulfide, dimethyl disulfide and dimethyl trisulfide, increasing the sample size beyond 2 g actually decreased sensitivity. Since agitation is not easy with a solid-type matrix, a uniform release of volatiles from larger sample sizes will be more difficult [32,33], especially from Cheddar cheese, which contains lipids and proteins that can slow the diffusion of some volatiles. Large variations were observed with 5 g sample size (CV range = 14–48%), which may be related to the fact that some molecules must diffuse a relatively long distance through the softened cheese mass, where many molecular interactions can take place, before reaching the headspace. Ultimately, because the PFPD responses for dimethyl disulfide and dimethyl trisulfide were generally low throughout the study, the 2 g sample size was chosen so that detection of these VSCs could be most successful with lesser variability overall.

## 3.2. Possible injector artifact formation

Literature has suggested that the extreme temperatures often used with the GC injector port can lead to artifact formation so this possibility was investigated for the VSCs involved in this study. Pure standards of methanethiol and dimethyl sulfide were injected at 300 °C where their oxidation products (dimethyl disulfide and dimethyl sulfoxide, respectively) were not generated to any degree of significance. This result agrees with a previous study that sulfur compounds undergo minimal thermal conversion in the injection port by SPME fiber [37]. While this evidence was initially promising, the inconsistent presence of a compound, which eluted after methanethiol but prior to dimethyl sulfide, was not anticipated. Retention times from pure standards suggested it to be carbon disulfide. It was found that the addition of water,

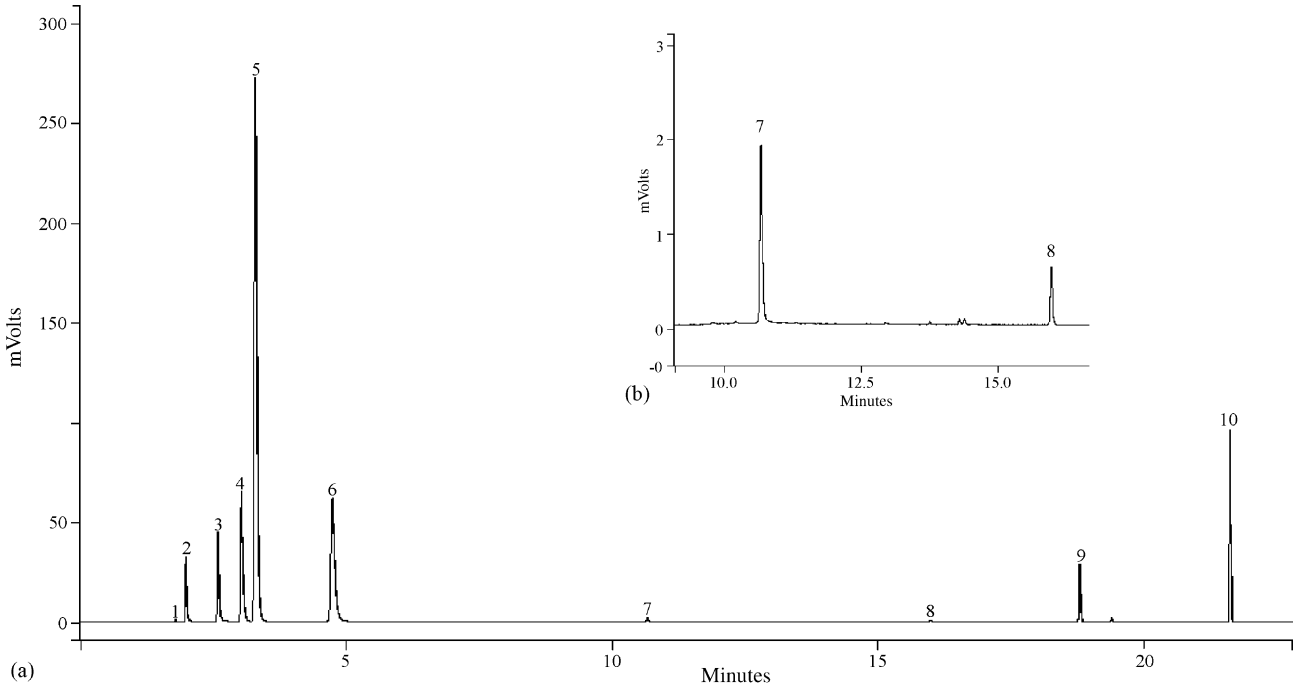


Fig. 4. Chromatogram of sulfur profile for Cheddar cheese obtained using developed SPME–GC–PFPD technique (2 g sample extracted for 30 min at 50 °C): (a) overall sulfur profile where (1) = carbonyl sulfide, (2) = hydrogen sulfide, (3) = methanethiol, (4) = carbon disulfide, (5) = dimethyl sulfide, (6) = ethyl methyl sulfide [internal standard, 6.5 ppb (μg/L)], (7) = dimethyl disulfide, (8) = dimethyl trisulfide, (9) = dimethyl sulfoxide, (10) = dimethyl sulfone and (b) magnified portion of chromatogram.

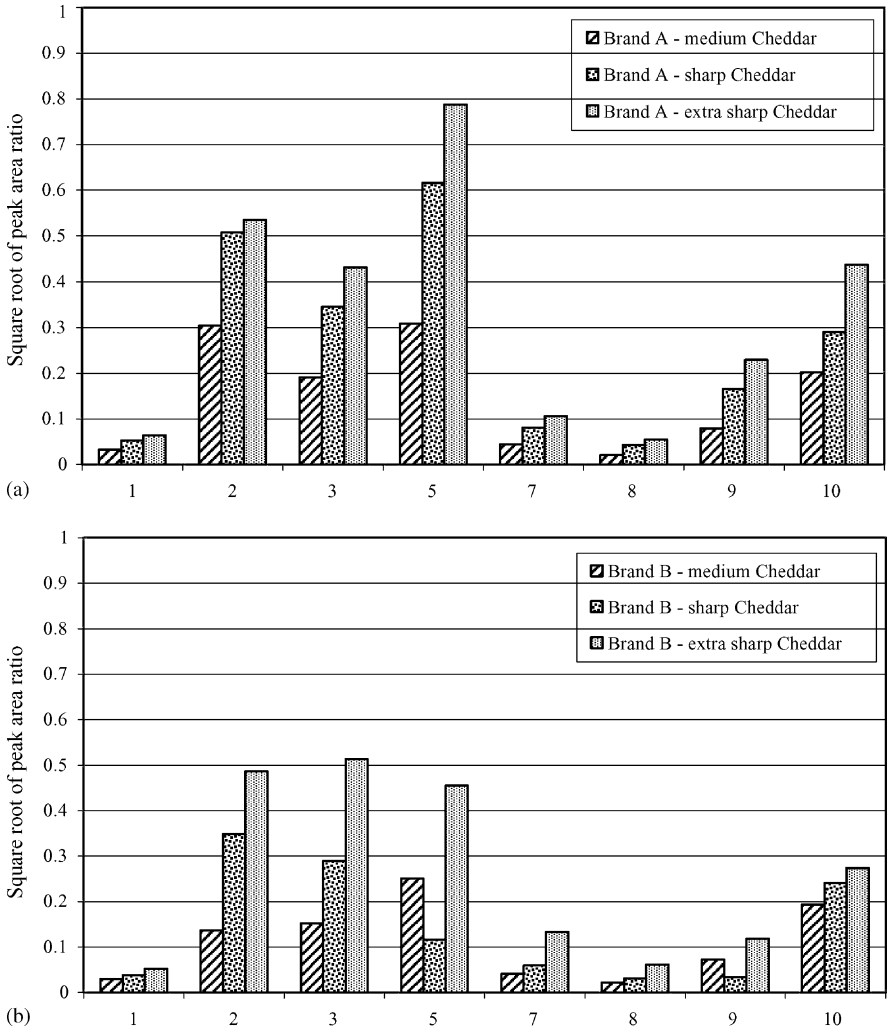


Fig. 5. Representation of sulfur content of two commercial Cheddar cheese brands, each with three levels of aging. Number (*x*-axis) corresponds to VSCs as listed in Fig. 4 and height of bar is equal to the square root of area ratio (peak area of VSC/peak area of internal standard, ethyl methyl sulfide at 6.5 ppb).

as used in the time and temperature trials to improve stirring, facilitated the generation of carbon disulfide in some cheese samples. However, when the water was replaced with vegetable oil, the amount of carbon disulfide detected was greatly reduced therefore, vegetable oil was used for the remainder of the experiment as a means to minimize artifacts. Also, numerous GC–PFPD chromatograms for "blanks" that were run between actual extractions (to ensure complete desorption of analytes) confirmed that this compound was almost always present in the fiber to some degree, i.e. "carryover". While the possibility of this compound, carbon disulfide, being a result of artifact formation is highly likely due to supporting evidence, further studies are still necessary to determine its source of origin.

### *3.3. Sulfur profile for aged cheddar cheese*

The developed headspace SPME extraction and GC–PFPD methodology was used to analyze the sulfur content of Cheddar cheeses. An example chromatogram for the sulfur profile of Cheddar cheese is shown in Fig. 4. The main sulfur compounds were identified, in order of retention time, as: carbonyl sulfide, hydrogen sulfide, methanethiol, carbon disulfide, dimethyl sulfide, dimethyl disulfide, dimethyl trisulfide, methional, dimethyl sulfoxide, and dimethyl sulfone. The lower molecular weight VSCs (namely hydrogen sulfide, methanethiol, dimethyl sulfide) constituted the majority of total peak area while the contribution from the later-eluting VSCs to total peak area was minimal, with the exception of the peak for dimethyl sulfone which can be relatively large in some aged cheeses. However, since dimethyl sulfone is essentially odorless, its presence does not directly affect the aroma or flavor of aged Cheddar cheese.

Two different commercial Cheddar cheese samples (labeled A and B, each with three levels of aging: medium, sharp, and extra-sharp) were analyzed and the overall sulfur content of each is represented in Fig. 5. Since the presence of carbon disulfide was highly variable in all samples and

may largely be a result of artifact formation as discussed above, this compound was excluded from further discussion. The results show that the amount of sulfur-containing compounds present in Cheddar cheese can indeed be related to the age of the cheese although the specific sulfur content can be different. When the medium-aged cheeses were compared against the extra sharp cheeses, the amount of increase in VSC levels varied. It was observed that some sulfur compounds in Sample B, such as hydrogen sulfide, methanethiol, dimethyl disulfide, and dimethyl trisulfide increased three-fold while others (dimethyl sulfide, dimethyl sulfoxide and dimethyl sulfone) showed only a two-fold increase. For comparison, all VSCs in Sample A were found to increase roughly two-fold. In addition, it was observed that the sharp-aged sample B had lower levels of dimethyl sulfide and dimethyl sulfoxide than found in the younger, medium-aged cheese but because these samples were obtained commercially, slight deviations were expected to occur. Since many manufacturing parameters, including quality of milk, types of culture, and aging temperature, can contribute to different flavor developments during the cheese aging process, the developed sulfur profile will be distinct.

## 4. Conclusions

SPME fiber extraction parameters, including time, temperature, and sample size all play important roles in the successful extraction of VSCs in cheese. When SPME is combined with PFPD, the overall sensitivity is greatly enhanced allowing for compounds present at very low levels to be easily analyzed. The developed headspace SPME–GC–PFPD method is quite sensitive to analyze the volatile sulfur compounds in Cheddar cheese. When this technique was employed to analyze commercial Cheddar cheese samples, it was found that the sulfur contents were related to the aging process. While a generally consistent increase was seen for the VSC content in the different ages of Sample A, the same behavior was not noted in Sample B where some VSCs increased more than others. This indicates that manufacturing parameters, as mentioned, can have noteworthy effects on the amount of VSCs present in aged Cheddar cheese. The technique developed in this study will be applied toward the quantification of VSCs so that a better understanding can be gained of how VSCs contribute to the overall flavor and aroma of aged Cheddar cheese.

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