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Optimization of Solid Phase Microextraction Analysis for the Headspace Volatile Compounds of Parmesan Cheese

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Optimum conditions of solid phase microextraction (SPME) analysis of the headspace volatile compounds of Parmesan cheese in airtightly sealed 100-mL bottles were developed. The coefficient of variation of SPME analysis on the headspace volatile compounds of Parmesan cheese was 2%. The reproducibility of SPME was improved by a combination of sampling at −10 °C, controlling the sample temperature, and uniform magnetic stirring of samples during equilibrium and isolation steps. The sensitivity of SPME increased by 125% in total peak areas by a combination of 40 min of sonication and 25% (w/v) sodium phosphate solution, compared with that of samples containing deionized water only ($P < 0.05$). The addition of salt solution or sonication treatment in samples increased the headspace volatile compounds of cheese quantitatively without producing any new volatile compounds.

KEYWORDS: Cheese; solid phase microextraction; volatile compound analysis; salt effects; sonication

INTRODUCTION

Flavor is one of the most important characteristics that determines the quality of cheese (*1*). Steam distillation, simultaneous distillation extraction, static headspace, and dynamic headspace have been used to study the volatile compounds in cheese (*2*−*5*). Steam distillation can cause the loss of heat sensitive volatile compounds, and solvents can provide undesirable artifacts during analysis (*6*). Although static headspace analysis is the simplest method for the determination of volatile compounds, it lacks sensitivity (*7*, *8*). Dynamic headspace analysis can overcome the sensitivity problem of static headspace analysis. Dynamic headspace analysis is an expensive, time-consuming, labor-intensive method and is less reproducible than static headspace analysis (*9*).

Solid phase microextraction (SPME) is an analytical technique for the isolation and concentration of volatile or nonvolatile compounds in foods; it integrates sampling, extraction, concentration, and sample introduction to gas chromatography (GC) or high-performance liquid chromatography (*10*). The principle of headspace SPME is an equilibrium partitioning of analytes among the coating of solid phase or fiber, sample matrix, and/or headspace (*11*). SPME has been used to analyze the volatile fraction of ewe's milk cheese (*12*) and the headspace volatile compounds in Cheddar cheese (*13*) and Swiss cheeses (*14*). Cryotrapping/SPME was designed to maximize the isolation and concentration of volatile compounds from cheese (*15*). Even though cryotrapping/SPME is a sensitive method, the reproducibility was not good enough due to the interference of trapped water in the SPME solid phase (*15*). Studies of SPME optimization on cheese volatile compounds have been focused on the selection of proper solid phases, exposure time, influence of absorption temperature, and the ratio of sample and headspace volume (*12*, *13*). However, few studies have been reported on the development of both reproducible and sensitive SPME conditions on the headspace volatile compounds due to the relatively low concentration and uneven distribution of volatile compounds in cheese and the continuous generation and transformation of volatile compounds during the ripening stage.

The objective of this study was to optimize the SPME conditions to increase reproducibility and sensitivity for the analysis of the headspace volatile compounds in Parmesan cheese.

MATERIALS AND METHODS

Materials. Parmesan cheese was donated from Antigo Cheese Co. (Antigo, WI) and stored in a −10 °C cold room. A manual SPME fiber holder unit, 75 μm (diameter) Carboxen/poly(dimethylsiloxane) (CAR/PDMS), serum bottles, Teflon-coated rubber septa, and aluminum caps were purchased from Supelco (Bellefonte, PA). Sodium chloride (NaCl), sodium phosphate (NaH_2PO_4), potassium chloride (KCl), sodium sulfate (Na_2SO_4), ethanol, acetic acid, butanoic acid, 2-heptanone, benzaldehyde, hexanoic acid, limonene, sorbic acid, 2-nonanone, undecane, octanoic acid, 2-undecanone, decanoic acid, and dodecanoic acid were purchased from Sigma (St. Louis, MO).

Effects of Sample Size on Reproducibility. All samples were prepared at −10 °C in a cold room in the Department of Food Science at The Ohio State University. Cheese was cut with a cheese cutter into small pieces ~1 mm in diameter at −10 °C to increase the surface area. To study the effects of sample size, 10, 15, 20, 25, and 30 g of prepared Parmesan cheese with 10, 15, 20, 25, and 30 mL of deionized

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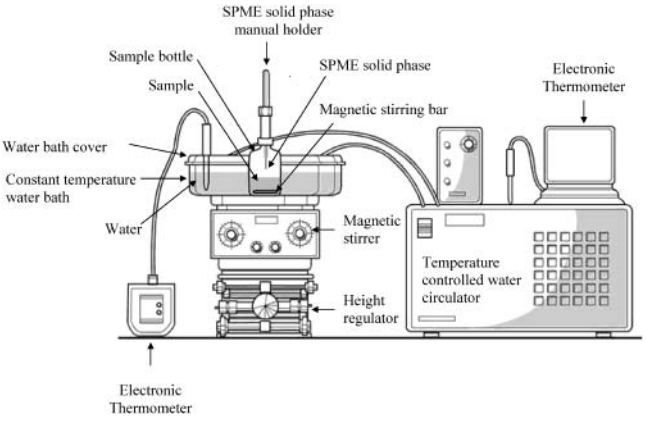

Figure 1. Flavor isolation apparatus, which can control temperature and magnetic stirring speed of a sample for SPME analysis.

water, respectively, were put in a 100-mL bottle with a magnetic stirring bar (8 × 25 mm). To study the ratio of cheese to water contents, samples of 25 g of prepared cheese with 20, 25, or 30 mL of deionized water, respectively, were put in a 100-mL bottle with a magnetic stirring bar (8 × 25 mm). Sample bottles were prepared in triplicate and sealed airtight with aluminum caps and Teflon-coated rubber septa. Sample bottles wrapped in aluminum foil were kept in a −10 °C cold room until use.

Headspace Volatile Compound Analysis by SPME. Sample bottles were put in a new flavor isolation apparatus that can regulate the temperature and stirring of a sample (**Figure 1**). The sample bottles were kept for 10 min at 50 °C in the new flavor isolation apparatus to melt the cheese. Sample was magnetically stirred in the new flavor isolation apparatus for 20 min at 50 °C to homogenize the sample and to accelerate equilibrium of headspace volatile compounds between the cheese matrix and the headspace. The 75 μm CAR/PDMS fiber trapped the headspace volatile compounds for 30 min at 40 °C during stirring. The volatile compounds isolated by CAR/PDMS were desorbed in the injector port of a GC (Hewlett-Packard 5890 GC).

Effects of Salt Concentration. NaCl was added into deionized water to obtain 5, 20, and 25% (w/v) and saturated salt solution. To study the effects of salt concentration on the sensitivity of SPME, headspace volatile compounds from Parmesan cheese samples with deionized water, 5, 20, and 25% (w/v), or saturated NaCl solution were analyzed by SPME-GC.

Effects of Salt Types. NaCl, NaH_2PO_4, KCl, or Na_2SO_4 was added into deionized water to obtain 25% (w/v) solutions. To study the effects of salt types on the sensitivity of SPME, headspace volatiles from Parmesan cheese samples with deionized water, 25% NaCl, NaH_2PO_4, KCl, or Na_2SO_4 solution were analyzed by SPME-GC.

Effects of Sonication. To determine the effects of sonication time on the sensitivity of SPME, sample bottles, which were kept for 10 min at 50 °C in the new flavor isolation apparatus without stirring and then magnetically stirred for 20 min at 50 °C in the new flavor isolation apparatus, were sonicated for 0, 10, 20, 30, 40, 50, and 60 min in a Fischer Scientific ultrasound water bath (Shelton, CT). A constant water level of 2000 mL was maintained for each sonication treatment. The water temperature in the ultrasound water bath was maintained at 40 ± 0.5 °C during sonication. Sample bottles in a 40 °C water bath without sonication were used as controls. After sonication, an equilibrium step was introduced for 10 min at 40 °C in the new flavor isolation apparatus with stirring.

Combination Effects of Sonication and Salt. To study the combination effects of sonication and salt on the sensitivity of SPME, Parmesan cheese samples with 25% (w/v) NaH_2PO_4 solution were sonicated for 40 min at 40 °C. The headspace volatile compounds in cheese samples with deionized water only, 25% NaH_2PO_4 solution only, or 25% NaH_2PO_4 solution with 40 min of sonication were isolated by SPME-GC.

Conditions of Gas Chromatography. A Hewlett-Packard 5890 gas chromatograph was equipped with a 0.75 mm i.d. glass injection liner, a flame ionization detector, and a 30 m × 0.25 mm i.d., 1.0 μm film, DB-5 column, from J&W Scientific (Folsom, CA). The oven temperature was held at 40 °C for 2 min and increased from 40 to 160 °C at the rate of 6 °C/min and from 160 to 210 °C at 10 °C/min. The temperatures of the injector and detector were 250 and 300 °C, respectively. The flow rate of nitrogen carrier gas was 1.0 mL/min. The isolated volatile compounds in the solid phase of SPME were desorbed at 250 °C for 2 min.

Identification of Volatile Compounds in Parmesan Cheese. The headspace volatile compounds in samples of cheese only, cheese with deionized water, cheese with 25% NaH_2PO_4 solution, and cheese with deionized water and sonication treatment were isolated by CAR/PDMS, separated by GC, and identified by MS. A Hewlett-Packard 5971A mass selective detector equipped with a Hewlett-Packard 59822B ionization gauge controller was used. All mass spectra were obtained at 70 eV and an ion source temperature of 220 °C. Identification of compounds was made by the combination of NIST mass spectra and gas chromatographic retention times of standard compounds. Helium carrier gas at 0.9 mL/min and an HP-5 column (30 m × 0.25 mm i.d., 0.25 μm thick) from Agilent Technologies (Palo Alto, CA) were used. The GC conditions for GC-MS were the same as the gas chromatographic analysis conditions described previously.

Statistical Analysis. One-way analysis of variance and Tukey's multiple comparisons were used to analyze the data. A P value of ≤ 0.05 was considered to be significant. All statistical analyses were conducted with Minitab 12.1 (Minitab Inc., State College, PA).

RESULTS AND DISCUSSION

Effects of Sample Preparation Temperature and Sample Size on Reproducibility. The coefficient of variation of SPME analysis on the headspace volatile compounds in Parmesan cheese was 2% for total GC peak areas. The reproducibility of SPME could be achieved by a combination of sampling at −10 °C, controlling the sample temperature, and uniform magnetic stirring of samples during equilibrium and isolation steps.

A new flavor isolation apparatus, which can control the temperature and the magnetic stirring speed of a sample, was designed for this study to provide constant volatile compound isolation conditions (**Figure 1**). Water temperature, water level, and the magnetic stirring speed of a sample were kept constant in the new flavor isolation apparatus for each sample treatment. The water level in the apparatus can be maintained by minimizing the evaporation of water using a water bath cover. This new apparatus can be used to measure the headspace volatile compounds from other aqueous food samples and to analyze light-sensitive samples when the constant-temperature water bath and a sample bottle are wrapped with aluminum foil to make dark conditions.

Magnetic stirring plays an important role in increasing the reproducibility of SPME by accelerating the mass transfer between the sample and solid phase and shortening the equilibrium time (*11*, *16*). Preliminary study showed that the coefficient of variation from samples containing only cheese without magnetic stirring was >15% due to the uneven distribution of volatile compounds in cheese samples (data not shown).

Sample preparation at −10 °C also is important in achieving reproducibility of SPME. Preliminary study showed that the coefficients of variation of volatile compounds from samples prepared at 5 and 25 °C were 11 and 19%, respectively (data not shown).

Effects of sample size and the ratio of sample to headspace volume in a 100-mL sample bottle on the headspace volatile compounds in Parmesan cheese by SPME are shown in **Table 1**. As the amount of cheese increased from 10 to 15, 20, 25, and 30 g in 1:1 ratio of sample to headspace volume, total peak areas increased from 2.30 to 3.56, 5.21, 6.99, and 8.07 (1 ×

Table 1. Effects of Sample Size and the Ratio of Sample to Headspace Volume in a 100-mL Sample Bottle on the Headspace Volatile Compounds in Parmesan Cheese by SPME

cheese (g)/ water (mL)	sample vol/ headspace vol	total GC peak areas in electronic count[a] (1×10^5)	coefficient of variation (%)
10:10	20:80	2.30 ± 0.10a	4.3
15:15	30:70	3.56 ± 0.12b	3.4
20:20	40:60	5.21 ± 0.19c	3.6
25:20	45:55	6.85 ± 0.10e	1.4
25:25	50:50	6.99 ± 0.06e	0.9
25:30	55:45	5.67 ± 0.12d	2.1
30:30	60:40	8.07 ± 0.52f	6.4

[a] Mean ± SD ($n = 3$). Different letters indicate a significant difference ($P < 0.05$).

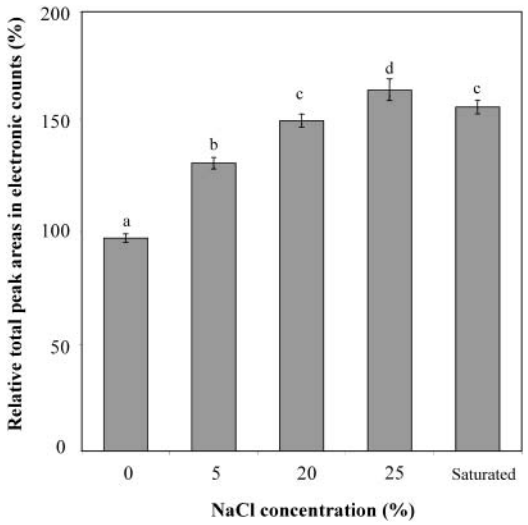

Figure 2. Effects of 0, 5, 20, 25% (w/v), and saturated NaCl solution on the headspace volatile compounds in Parmesan cheese by SPME. Bars with different superscripts are significantly different ($P < 0.05$).

10^5), respectivelt, and the coefficient of variation in total peak areas ranged from 0.9 to 6.4%.

The total peak areas of samples containing 25 g of cheese with 20, 25, and 30 mL were 6.85, 6.99, and 5.67 (1×10^5), respectively. The 20% decrease in total peak areas in the samples of 25 g of cheese with 30 mL of water may be due to the increase of water vapor pressure at the current analysis conditions. Water vapor competes with volatile compounds toward the active sites of SPME solid phases and decreases the total peak areas in aqueous foods such as orange juice (*17*). The mixture of 25 g of cheese and 25 g of water was chosen as the optimum sample size for this study, considering the best reproducibility. The optimum ratio of sample to headspace volume for SPME varies depending on the sample's characteristics. The ratio of orange juice to headspace was 1:5 (*17*), and that of soybean oil to headspace was 2:1 (*18*).

Effects of Salt Concentration. Relative total peak areas from Parmesan cheese samples with 0, 5, 20, 25%, and saturated NaCl solution are shown in **Figure 2**. As salt concentration increased from 0 to 25%, relative total peak areas increased significantly by 70% ($P < 0.05$). The amount of headspace volatiles produced by samples with saturated NaCl was 15% less than that produced by samples with 25% NaCl.

Electrolytes, such as salt, in an aqueous system can influence the phase boundary properties and decrease the solubility of hydrophobic compounds by competing water molecules, which

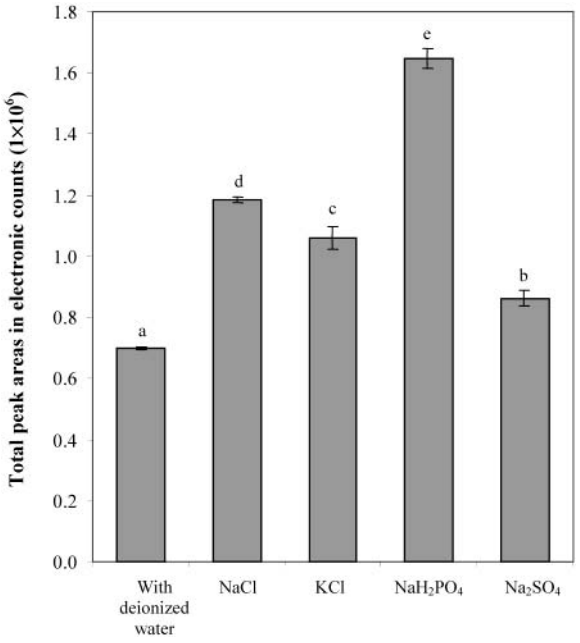

Figure 3. Effects of sodium chloride, potassium chloride, sodium phosphate, and sodium sulfate solution on the headspace volatile compounds in Parmesan cheese by SPME. Bars with different superscripts are significantly different ($P < 0.05$).

is called "salting out" (*19*, *20*). Although the efficiency of volatile compound extraction depends on the concentration of salt solution, a 20−30% (w/v) salt concentration was reported to be sufficient to give the best sensitivity for most volatile compounds (*20*). A salt concentration of 25% was chosen as the optimum level for this study.

Effects of Salt Types. Effects of 25% NaCl, KCl, NaH_2PO_4, or Na_2SO_4 solution on the volatile compounds in Parmesan cheese are shown in **Figure 3**. Addition of Na_2SO_4, KCl, NaCl, and NaH_2PO_4 solution increased the total peak areas by 23, 51, 70, and 126%, respectively, compared with samples with deionized water. NaH_2PO_4 showed the highest increases in the headspace volatile compounds. The phosphate ion from NaH_2PO_4 can chelate the calcium ion in cheese and change the ratio of calcium to phosphate concentration, which can solubilize the coagulated milk proteins and loosen the cheese matrix (*21*). The loosened structure would accelerate the release of volatile compounds trapped inside the matrix of the cheese, whereas Na_2SO_4, KCl, and NaCl may just compete for water with the volatile compounds without changing the structure of the cheese matrix.

Effects of Sonication. Effects of sonication times of 0, 10, 20, 30, 40, 50, and 60 min on the headspace volatile compounds in Parmesan cheese are shown in **Figure 4**. Total peak areas of Parmesan cheese with sonication were significantly greater than those of samples in a water bath without sonication from 10 to 40 min ($P < 0.05$). Total peak areas in Parmesan cheese increased by 70% up to 40 min of sonication and decreased significantly after 40 min ($P < 0.05$), which may be due to the increase of water vapor pressure in the headspace of sample bottles. Sonication can loosen the structures of sample matrix and release the volatile compounds physically trapped in the matrix. Sonication treatment has been reported to increase the amount of polyaromatic hydrocarbons in headspace from water samples (*11*). Forty minutes of sonication was chosen for further experiments in this study.

Combination Effects of Sonication and Salt. Effects of the combination of sonication and NaH_2PO_4 on the total peak areas

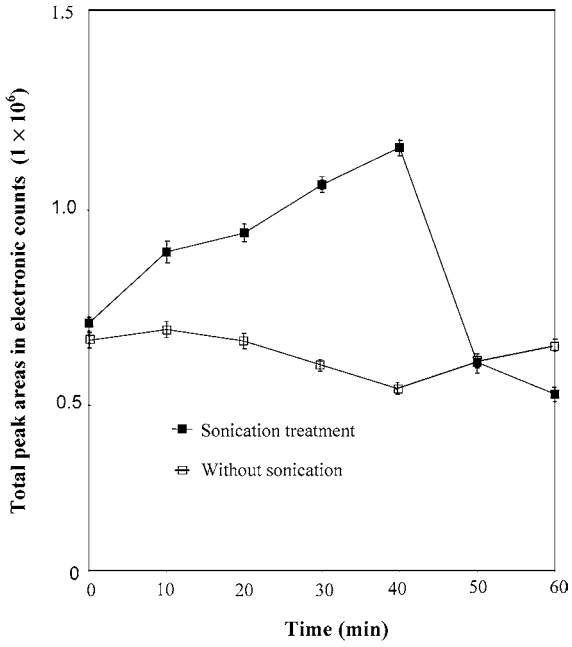

Figure 4. Effects of sonication on the headspace volatile compounds in Parmesan cheese by SPME.

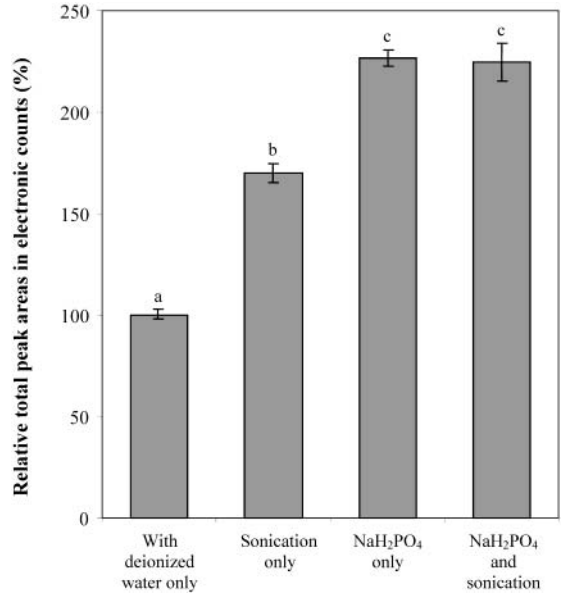

Figure 5. Combination effects of salt solution and sonication on the headspace volatile compounds in Parmesan cheese by SPME. Bars with different superscripts are significantly different ($P < 0.05$).

in cheese are shown in **Figure 5**. Total peak areas in cheese treated with sonication only, 25% NaH_2PO_4 solution only, and the combination of sonication and NaH_2PO_4 solution increased by 70, 126, and 125%, respectively, compared with samples with deionized water only. Effects of a combination of sonication and salt were not significantly different from those of samples with salt solution only ($P > 0.05$). Thus, for time efficiency reasons, salting with 25% NaH_2PO_4 solution only is recommended.

Identified Headspace Volatile Compounds in Parmesan. Identified headspace volatile compounds from Parmesan cheese without water, with deionized water, with 25% NaH_2PO_4 solution, or with deionized water and sonication are shown in **Table 2**. Addition of deionized water, 25% NaH_2PO_4 solution, and sonication treatment changed the headspace volatile compounds in Parmesan cheese quantitatively but did not produce any new volatile compounds, which were not present in cheese samples. Low molecular weight and water-soluble compounds including ethanol, acetic acid, and butanoic acid decreased in the samples of deionized water, compared with cheese-only samples due to the solubility of these compounds in water. Addition of salt or sonication treatment to cheese samples increased some of the headspace volatiles quantitatively but not qualitatively.

Table 2. Identified Headspace Volatile Compounds in Parmesan Cheese without Water, with Deionized Water, with 25% NaH_2PO_4 Solution, and with Sonication

volatile compound[a]	cheese only	with deionized water	with 25% NaH_2PO_4 solution	with sonication
ethanol[GC,MS]	27.2[b]	19.6	29.5	28.6
1,3-pentadiene[MS]	12.8	10.2	13.2	12.1
2-methylfuran[MS]	3.2	2.1	3.9	3.7
acetic acid[GC, MS]	32.6	21.5	36.4	30.5
2-pentanone[MS]	0.7	1.1	1.2	1.1
butanoic acid ethyl ester[MS]	0.8	1.2	1.6	1.3
butanoic acid[GC,MS]	7.5	3.9	9.5	8.7
1,2-dimethylbenzene[MS]	0.3	0.3	0.5	0.3
2-heptanone[GC,MS]	6.9	4.2	7.5	6.8
benzaldehyde[GC,MS]	0.4	0.5	0.7	0.5
hexanoic acid[GC,MS]	18.5	18.1	21.2	20.5
limonene[GC,MS]	0.4	0.4	0.5	0.4
benzenacetaldehyde[MS]	0.6	0.7	0.9	0.8
sorbic acid[GC,MS]	4.7	4.5	6.2	4.2
2-nonanone[GC,MS]	7.8	8.2	10.3	9.4
undecane[GC,MS]	0.3	0.4	0.6	0.5
octanoic acid[GC,MS]	3.8	4.2	4.8	4.3
octanoic acid ethyl ester[MS]	1.8	0.9	2.3	2.1
octanoic acid isopropyl ester[MS]	0.2	0.3	0.4	0.3
2-undecanone[GC,MS]	0.3	0.3	0.4	0.3
decanoic acid[GC,MS]	0.7	0.7	0.9	0.8
dodecanoic acid[GC,MS]	0.2	0.3	0.4	0.3

[a] Superscript GC,MS represents a volatile identified by both GC retention time of a standard compound and GC-MS. Superscript MS represents a volatile identified by GC-MS only. [b] Mean value of peak area of a compound in ion count (1×10^7) by SPME-GC-MS ($n = 3$).

Most of the identified volatile compounds were reported previously (*2*, *22*). High molecular weight and relatively nonpolar compounds including decanoic and dodecanoic acids, which were reported by simultaneous distillation and extraction method (*2*) but not by purge-and-trap technique (*2*, *22*), can be detected by CAR/PDMS SPME-GC analysis.

In conclusion, a reproducible and sensitive SPME condition for the analysis of headspace volatiles in cheese was developed. Reproducibility of SPME was achieved by a combination of sampling at −10 °C, using 25 g of cheese with 25 mL of water in a 100-mL sample bottle, controlling the sample temperature, and uniform magnetic stirring of samples during equilibrium and isolation steps using a flavor isolation apparatus. Addition of NaH_2PO_4 solution increased the sensitivity of analysis of volatile compounds in cheese significantly. The reproducible and sensitive conditions of SPME analysis in this study can be used for the analysis of the cheese volatile compounds during cheese manufacturing and aging to enhance the flavor quality of cheese.

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