Quantitative Determination of Short-Chain Free Fatty Acids in Milk Using Solid-Phase Microextraction and Gas Chromatography

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The objective was to establish a rapid, precise, and accurate methodology for the quantification of short-chain free fatty acids (FFA) (C_4−C_{12}) in milk by solid-phase microextraction and gas chromatography. Sampling conditions such as fiber type, pH, salt addition, temperature, volume, and time were investigated. FFA extraction consisted of placing 40 mL of milk containing 28% NaCl at pH 1.5 in a sealed vial and equilibrating for 30 min at 70 °C. A polyacrylate fiber was exposed to the sample headspace for 60 min and desorbed for 5 min into the gas chromatograph. Calibration curves for FFA followed linear relationships with highly significant ($p < 0.001$) correlation coefficients ($R^2 = 0.99$). Coefficients of variation of less than 7.7% for FFA concentrations indicated that the technique was reproducible. The limits of quantification for C_4−C_{10} were in the low parts per million level, which were below the concentration range found in fresh pasteurized milk (0.48−2.52 ppm) or rancid milk (4.73−32.31 ppm).

Keywords: *Solid-phase microextraction−gas chromatography; short-chain free fatty acids; milk; rancid flavor*

INTRODUCTION

Short-chain free fatty acids (FFA) result from triglyceride degradation of milk fat causing the appearance of rancid flavors. Therefore, the detection of short-chain FFA is very important in the quality evaluation of milk and milk products (*1*). Traditionally, the acid degree value (ADV) has been used as a measure of hydrolytic rancidity; however, the Standard Methods for the Examination of Dairy Products recognizes the limitations of the ADV (*2*). The ADV is not a good measure of rancid flavor development since it does not detect the short-chain-length FFA (C_4−C_{12}) responsible for the off-flavor (*3*−*5*).

Individual FFA determination in dairy products consists of lipid extraction, isolation of the FFA, and gas chromatographic quantification (*6*). The determination of FFA by gas chromatography enables the quantification of each fatty acid, but the quantitative recovery of short-chain FFA depends to a great extent on the method used for isolating them from the sample, and the procedure is generally complicated (*1*). Liquid chromatographic methods were also developed for the analysis of major FFA in milk fat by following a protocol that included FFA derivatization (*7*). Gas and liquid chromatographic techniques are widely applied for the analysis of FFA, but there is still a need to speed up the analysis by developing rapid techniques that allow quantitative recovery of the short-chain FFA (*8*). In recent years, capillary electrophoresis and indirect UV were used for the quantification of FFA in lipolyzed cream (*8*).

Solid-phase microextraction (SPME) is a fast, solventless alternative to conventional sample extraction techniques. In SPME, analytes establish equilibrium among the sample matrix, the headspace above the sample, and a stationary phase coated on a fused silica fiber and then are thermally desorbed from the fiber to a capillary GC column. Because no solvent is injected and the analytes are rapidly desorbed onto the column, minimum detection limits are improved and resolution is maintained. SPME is useful in many diverse analysis of a wide range of products, including characterization of flavor components in foods, beverages, and fragrance compounds (*9*, *10*).

The analysis of fatty acids (C_2−C_{10}) with SPME was accomplished in two different ways, by using direct fiber extraction or by derivatizing them on the fiber (*11*, *12*). The last approach was used to increase sensitivity of the method by lowering the limit of detection (LOD) from the high parts per billion to the low parts per billion level for the short-chain FFA (C_2−C_5) in water (*11*). Although derivatization of polar analytes is the best choice when low LOD are required, it is not necessary for the levels of FFA present in fresh or rancid milk. The FFA concentrations reported for milk either fresh or rancid were in the parts per million range (*6*, *13*). Thus, the objectives of this work were to establish SPME sampling conditions to extract underivatized short-chain FFA (C_4−C_{12}) directly from milk headspace and to quantify the individual FFA in fresh and laboratory-prepared rancid milks (LPRM).

MATERIALS AND METHODS

Reagents. Free fatty acids standards (butanoic, hexanoic, octanoic, decanoic, dodecanoic, tetradecanoic, and hexadecanoic) were purchased from PolyScience, Co. (Niles, IL).

Collection of Samples. Raw milk was collected on two different occasions from a local dairy farm and stored at 4 °C until processed. Each of the raw milks were combined with pasteurized homogenized milk to prepare two different batches of LPRM. Fresh pasteurized homogenized milk was purchased from the local market and stored at 4 °C until used.

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Preparation of Rancid Milk Samples. Two different batches of LPRM were prepared as described by ref *14* with modifications. Raw and fresh pasteurized homogenized milk were mixed (1:1) and incubated at 5 °C for 24 h to allow rancidity to develop. LPRM was batch-pasteurized at 66 °C for 35 min and cooled rapidly. Subsamples of the LPRM were frozen in separate vials until use for analysis. FFA were tentatively identified by spiking samples with analytical standards. Acid degree values for milk samples were determined according to ref *15*.

SPME Procedure. The SPME device was purchased from Supelco Co. (Bellefonte, PA). Three phase coatings were tested: poly(dimethylsiloxane) (PDMS, 100 μm), poly(acrylate) (PA, 85 μm), and Carbowax−divinylbenzene (CW/DVB, 65 μm). The fibers were conditioned in a GC injection port at 250 °C for 1 h for PDMS, at 300 °C for 2 h for PA, and at 250 °C for 0.5 h for CW/DVB prior to use. Subsamples of the same batch of LPRM were used for establishing SPME conditions. The extraction procedure was as follows: a rancid milk sample with 28% NaCl (w/v, saturated solution) adjusted to pH 1.5 with sulfuric acid or left untreated (without NaCl or pH adjustment) was put in a 60-mL amber vial. When the treated sample (28% NaCl, pH 1.5) was compared to the untreated rancid milk, samples (40 mL) were maintained at 70 °C and exposed to the fiber for 60 min. Samples (40 mL) were equilibrated for 30 min and exposed to the fiber at different sample temperatures (60, 70, and 80 °C) in a shaker bath (Orbit Lab Line, Chicago, IL) maintained at 150 rpm. After the 30-min equilibration, the needle of the SPME device was pierced through the septum of the vial, and the plunger was depressed to expose the fiber to the headspace of the solution for 60 min. The fiber was withdrawn into the needle and transferred to the injection port of the GC. The needle of the SPME device penetrated the septum of the GC inlet, and the fiber was exposed for 5 min so that the analytes were thermally desorbed in the hot injection port and deposited onto the column where subsequent chromatographic analysis was performed. Similarly, extraction conditions with different sample volumes (20, 30, and 40 mL) maintained at 70 °C with a 60-min fiber exposure time were tested. Finally, fiber exposure times (30, 60, and 90 min) of 40-mL samples maintained at 70 °C were analyzed. All of the experiments were run five times, and the mean peak areas and coefficients of variation were calculated.

GC−FID Analysis Conditions. Gas chromatographic analysis was performed using a Hewlett-Packard 6890 gas chromatograph (Wilmington, DE) equipped with a flame ionization detector. The gas chromatograph injector was provided with a specific HP insert for SPME (0.75 mm i.d., Supelco Co., Bellefonte, PA). The target compounds were separated using a DB-FFAP column (30 m × 0.32 mm i.d., 0.25 μm; J&W Scientific, Folsom CA). Once the analytes were extracted, they were continuously desorbed at 250 °C for 5 min in the injection port of the GC by exposing the fiber immediately after the needle was introduced into the insert to avoid the peak splitting effect (*10*). A specific narrow bore insert for SPME allows rapid removal of volatiles from the injector and facilitates sharp SPME injection bands (*10*). The column was maintained at 35 °C for 2 min, ramped at 5 °C/min to 230 °C, and held for 20 min. The FID was maintained at 330 °C. Helium (UHP) at 0.7 mL/min was used as the carrier gas. Air (zero-gas) and hydrogen (UHP) made up the FID flame. The makeup gas was nitrogen (UHP).

Quantitative Analysis. The analytical standards were short-chain FFA (C_4−C_{12}; PolyScience, Co., Niles, IL). Standard stock solutions (20 000 ppm) were prepared in methanol. Aliquots of stock solutions were used for spiking freshly pasteurized milk to final concentrations of 10, 20, 40, and 100 ppm. Spiked milks containing pentanoic acid (20 ppm) as an internal standard were analyzed. Dodecanoic acid (C_{12}) concentrations were calculated based on the calibration curve constructed for decanoic acid (C_{10}). Attempts to construct a calibration curve for dodecanoic acid failed since replicate analyses of spiked milk were highly irreproducible possibly due to problems with solubility of the acid in the milk sample. Blanks of freshly pasteurized milk were run, and FFA peak areas were subtracted for calculation of corrected FFA peak areas. Calibration curves for individual FFA were constructed by applying linear regression analysis using Systat/Sygraph (Systat, Inc., Evanston, IL) on concentrations (ppm) vs corrected FFA peak areas/area of internal standard. The precision of the method was determined by performing five consecutive analyses. The limit of quantification for each FFA was calculated to be the concentration that produced a signal-to-noise ratio of 10.

RESULTS AND DISCUSSION

Development of the SPME Method. Factors shown to determine quantitative differences in FFA concentrations by SPME were sample preparation, volume, temperature, fiber coating, and exposure time. The adsorption of free C_4−C_{12} acids on different fibers (PDMS, PA, and CBW/DVB) were compared when each of the three coatings was exposed for 60 min to 40 mL of LPRM containing 28% NaCl, pH 1.5, at 70 °C. PA was the fiber coating that adsorbed the most FFA and presented the lowest coefficients of variation of all three (Figure 1). The PDMS- and the CBW/DVB-coated fibers are relatively nonpolar, and the acids have less affinity toward them; thus, these fibers could not adsorb the acids effectively. Since PA is a polar coating, it extracted more of the analytes, thus PA-coated fibers were used for the remainder of this study. PA-coated fibers were also found to be most suitable for extracting FFA (C_6−C_{10}) from water (*11*).

Short-chain FFA were more efficiently carried to the headspace when 28% NaCl was added to milk and the pH was adjusted to 1.5 than when the milk was left untreated (Figure 2). Larger peak areas shown in the treated sample were due to short-chain FFA being less soluble in water under these conditions (Figure 2). The amounts of fatty acids extracted were increased by factors ranging from 13 for C_4 to 1 for C_{12} over those of untreated milk. The magnitude of the increase depends on the solubility of the acids. For dodecanoic acid, the addition of salt has no significant influence as compared to the untreated milk. This is due to the relatively low solubility of this acid in water. The "salting out" effect caused by the addition of salt into the sample matrix increases the amount of acids extracted with fibers. (*11*). Water molecules prefer to solvate the salt ions, thus the addition of salt into the sample matrix will decrease the solubility of the acids, which results in an increase in the amount extracted by the fiber (*11*). Since free C_4−C_{10} acids are polar at the pH of milk, all of them will exist to some degree in the ionic or salt form and therefore are more soluble in water than in the fiber coating. Upon lowering the pH of the sample matrix, the acid−base equilibrium shifts toward the neutral forms of the acids that have a greater affinity for the fiber, and the amount extracted increases (*11*). Thus, when both pH 1.5 and saturated salt conditions are used, optimum enhancement of the extraction can be achieved. It is believed that the lower the pH of the matrix, the larger the amount of acid that can be extracted by the fiber. However, the PA coating is not stable at pH < 1, thus the pH value of the sample matrixes cannot be lowered without destroying the fiber coating (*11*).

Temperature influences the vapor pressure of analytes; thus, this is the next factor that has to be tested in order to improve the sensitivity of the method. From the three temperatures tested for extraction of free FFA,

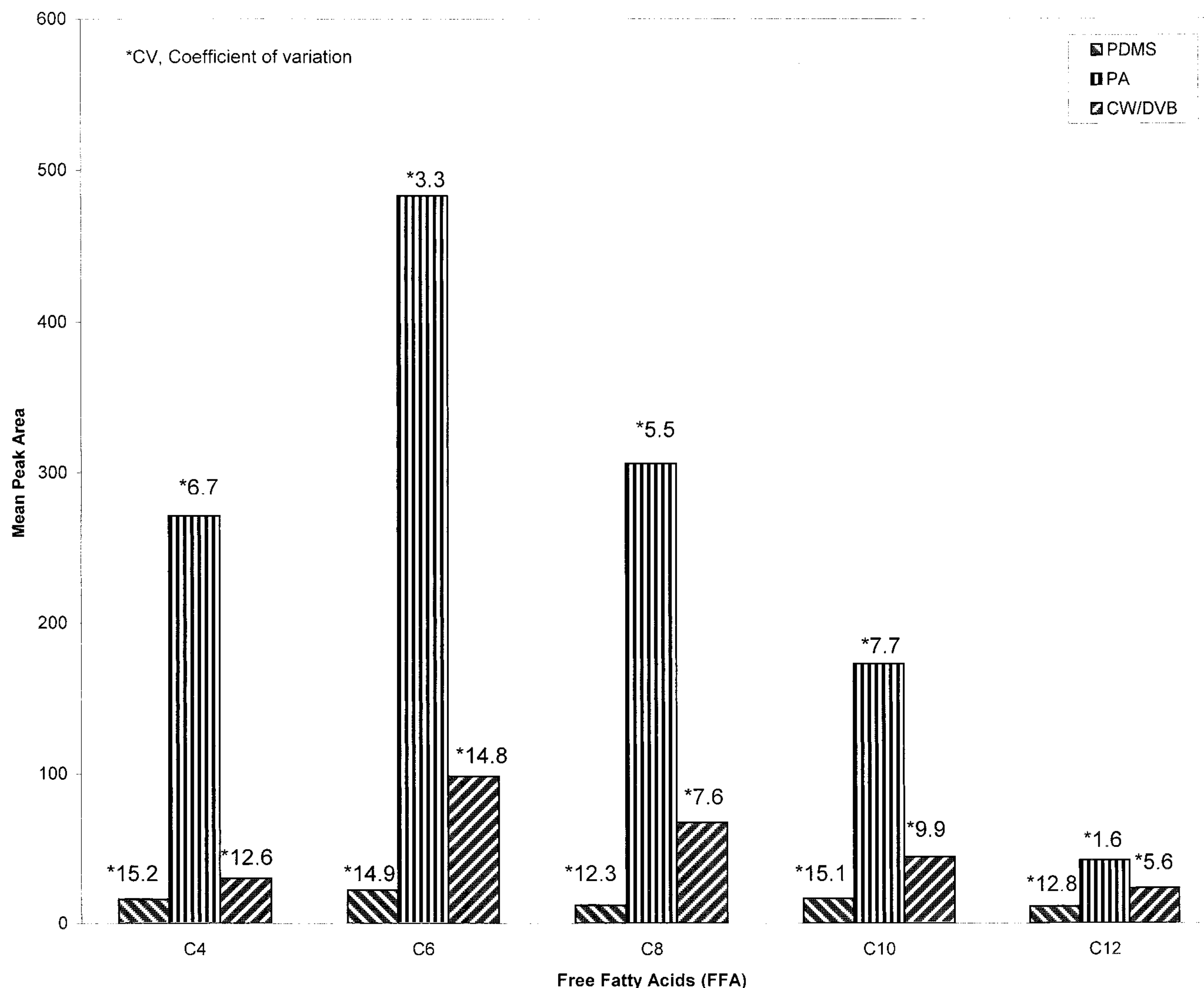

Figure 1. Adsorption efficiencies of different fiber coatings. Extraction conditions for FFA: a sample of rancid milk (40 mL) saturated with 28% NaCl (w/v), pH 1.5, at 70 °C and exposed to the different fibers for 60 min.

Table 1. Effect of Temperature on Extraction of Free Fatty Acids from Rancid Milk[a]

	temperature (°C)					
	60		70		80	
FFA	mean peak area	CV[b] (%)	mean peak area	CV[b] (%)	mean peak area	CV[b] (%)
C_4	353.60	12.0	271.33	6.7	278.28	19.8
C_6	488.18	4.4	482.95	3.3	498.38	1.9
C_8	286.55	9.1	305.94	5.5	357.66	1.3
C_{10}	112.97	13.3	172.80	7.7	298.72	3.2
C_{12}	24.09	12.4	42.16	1.6	93.50	12.5

[a] Rancid milk (40 mL) saturated with 28% NaCl (w/v), pH 1.5; fiber exposure time: 60 min. [b] CV, coefficient of variation, $n = 5$.

70 °C was the optimum. Although higher recovery of C_8–C_{12} was obtained as temperature increased from 60 to 80 °C, this trend was not observed for butanoic (C_4) and hexanoic acids (C_6) (Table 1). At 80 °C, the coefficient of variation (CV) for butanoic acid was the highest (19.8%); thus, these temperatures were not further considered. On the contrary, at 70 °C, the CV for this acid was the lowest; therefore, this temperature was chosen for further work. Butanoic acid is one of the most important short-chain FFA responsible for the development of rancid flavor (*2, 3*); thus, the sample temperature chosen was based on the performance of the technique for butanoic acid.

In general, at 60 °C the coefficients of variation were higher since it may be more difficult to release the FFA at the lowest temperature. Heating a sample to elevated temperature provides energy for molecules to overcome energy barriers that tie them to the matrix. However, the absorption of analytes by the fiber coating is an exothermic process, which means that while the high temperature is good for the release of analytes from their matrix, it can adversely affect the absorption by the coating due to the decrease of the partition coefficient. As a result, there is always an optimum temperature for headspace sampling (*16*).

The effect of sample volume and fiber exposure time while maintaining all other factors constant were tested. Using a sample volume of 40 mL resulted in lower coefficients of variation than 7.7% for all FFA under study (Table 2). This may be due to the fact that using sample volumes larger than the limiting value, which is given by fiber capacity, results in better precision since the variation of the sample volume does not affect the results (*10*). A 60-min fiber exposure to the sample was more reproducible since it presented coefficients of variation lower than 7.7% for all FFA (Table 3). In these experiments, equilibrium was not reached even at 90-

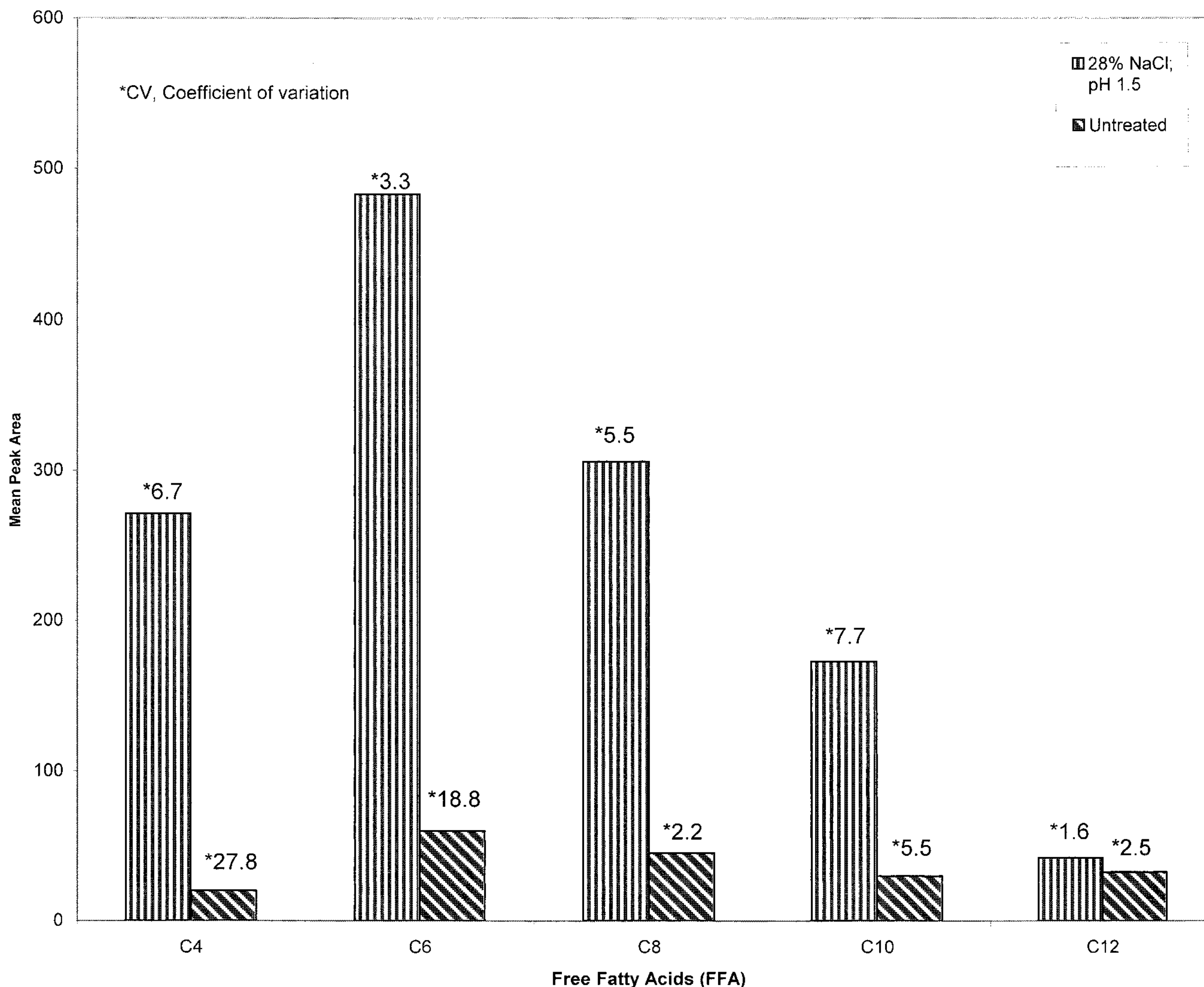

Figure 2. Effect of sodium chloride and pH in the release of free fatty acids into the headspace. Extraction conditions for FFA: a sample of rancid milk (40 mL) saturated with 28% NaCl (w/v), pH 1.5 or left untreated, at 70 °C and exposed to a PA fiber for 60 min.

Table 2. Effect of Sample Volume on Extraction of Free Fatty Acids from Rancid Milk[a]

	volume (mL)					
	20		30		40	
FFA	mean peak area	CV[b] (%)	mean peak area	CV[b] (%)	mean peak area	CV[b] (%)
C_4	421.64	18.9	506.16	25.2	271.33	6.7
C_6	609.03	3.0	684.03	6.5	482.95	3.3
C_8	342.21	11.0	371.99	18.1	305.94	5.5
C_{10}	199.71	10.0	224.86	7.7	172.80	7.7
C_{12}	55.81	15.7	49.38	29.1	42.16	1.6

[a] Rancid milk saturated with 28% NaCl (w/v), pH 1.5 at 70 °C; fiber exposure time: 60 min. [b] CV, coefficient of variation, $n = 5$.

Table 3. Effect of Fiber Exposure Time on Extraction of Free Fatty Acids from Rancid Milk[a]

	fiber exposure time (min)					
	30		60		90	
FFA	mean peak area	CV[b] (%)	mean peak area	CV[b] (%)	mean peak area	CV[b] (%)
C_4	269.01	5.8	271.33	6.7	345.81	13.6
C_6	508.68	3.7	482.95	3.3	556.88	4.5
C_8	266.85	6.5	305.94	5.5	379.54	2.1
C_{10}	115.73	23.4	172.80	7.7	260.27	5.0
C_{12}	31.55	42.2	42.16	1.6	78.73	21.3

[a] Rancid milk (40 mL) saturated with 28% NaCl (w/v) at 70 °C and pH 1.5. [b] CV, coefficient of variation, $n = 5$.

min exposure time since peak areas did not seem to reach a plateau. The objective of the SPME experiments, in principle, is to reach distribution equilibrium in the system since at this condition a variation of mass transfer does not affect the final results; however, it has been recommended that, when equilibration times are too long, shorter extraction times can be chosen for quantification (*10*). When using shorter extraction time as compared to equilibration time, care should be taken to control the exposure time (*10*). Thus, final selected sampling conditions consisted of placing 40 mL of milk containing 28% NaCl at pH 1.5 in a sealed vial, equilibrating for 30 min at 70 °C, and exposing a PA fiber for 60 min to the vial headspace.

Quantitative Determination of FFA in Fresh and Rancid Milks. Calibration curves constructed for the individual short-chain free fatty acids (C_4-C_{10}) spiked into milk followed linear relationships with highly significant ($p < 0.001$) correlations coefficients (Table 4). Dodecanoic acid (C_{12}) concentrations were calculated based on the calibration curve constructed for decanoic acid (C_{10}). CVs <7.7% for FFA concentrations indicated that the technique was reproducible.

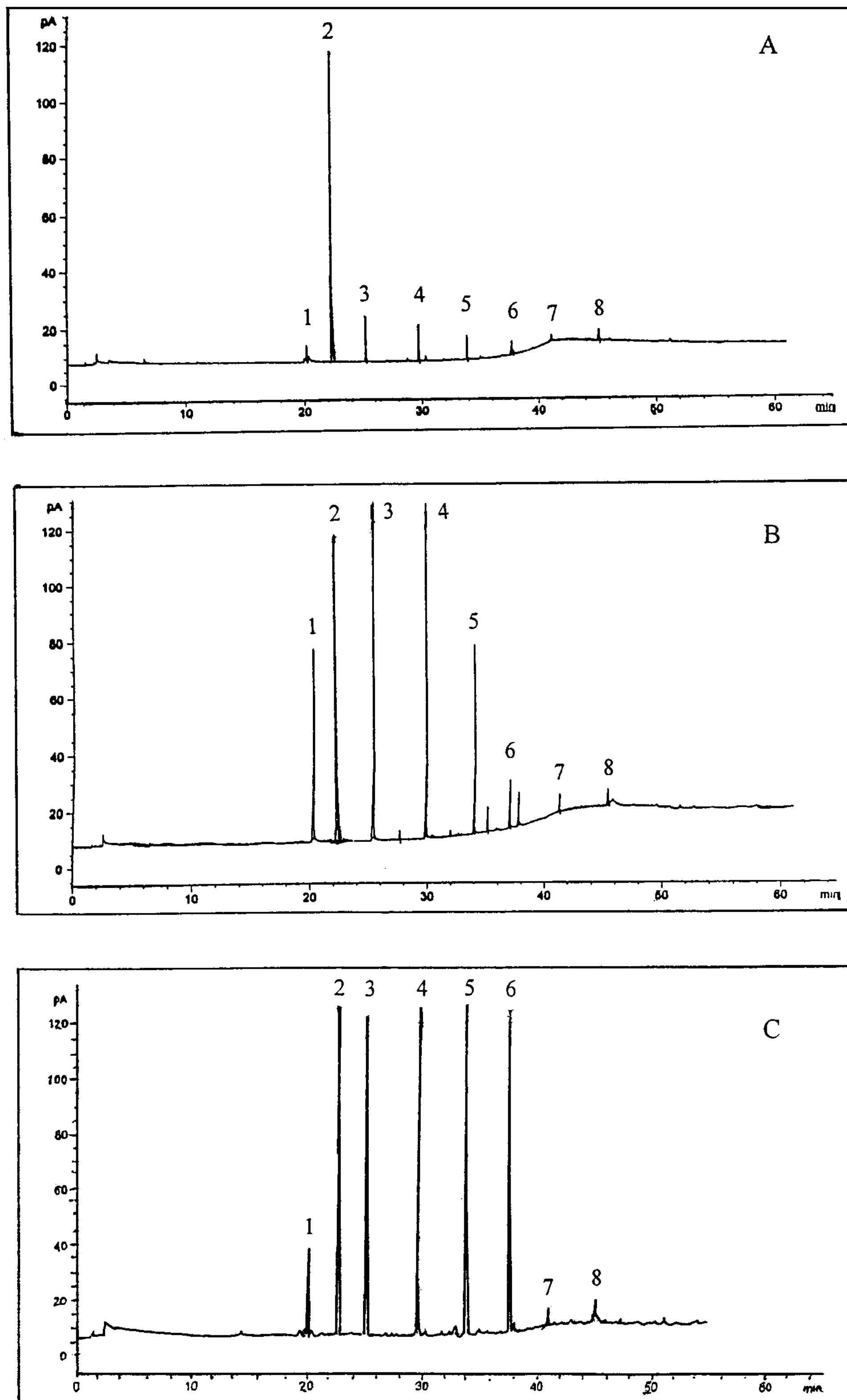

Figure 3. Free fatty acid gas chromatographic profiles of (A) commercially pasteurized homogenized fresh milk, (B) laboratory-prepared rancid milk, and (C) commercially pasteurized homogenized fresh milk spiked with analytical standards: (1) butanoic acid, (2) pentanoic acid (internal standard), (3) hexanoic acid, (4) octanoic acid, (5) decanoic acid, (6) dodecanoic acid, (7) tetradecanoic acid, and (8) hexadecanoic acid.

They are comparable to those usually obtained in other SPME procedures developed for volatile organic compounds (*11, 17, 18*). The limits of quantification calculated for FFA (C_4–C_{10}) were in the low parts per million level (Table 4), which are below the concentration range found in fresh pasteurized milk (0.48–2.52 ppm) or LPRM (4.73–32.31 ppm) (Table 5). A typical chromatogram of fresh pasteurized milk showed the presence of short-chain FFA (C_4–C_{12}) at very low concentrations (Figure 3A); whereas rancid milk showed a pronounced increase in their concentrations (Figure 3B). Short-chain FFA (C_4–C_{12}), tetradecanoic (C_{14}), and hexadecanoic

Table 4. Calibration Curves for Free Fatty Acid Standards Spiked in Fresh Pasteurized Homogenized Milk

FFA	regression line[a]	R^2	LOQ[b] (ppm)	CV[c] (%)
C_4	$y = 0.0688 + 0.018x$	0.99	0.10	<6.7
C_6	$y = 0.363 + 0.068x$	0.99	0.10	<3.3
C_8	$y = 0.547 + 0.099x$	0.99	0.10	<5.5
C_{10}	$y = -0.204 + 0.142x$	0.99	1.60	<7.7

[a] y = concentration, ppm; x = corrected FFA GC peak area/GC internal standard area. [b] The limit of quantification was estimated to be the concentration of the analytes that produces a signal 10 times that of noise. [c] CV, coefficient of variation, $n = 5$.

Table 5. Free Fatty Acid Concentrations in Fresh Pasteurized Homogenized and Rancid Milks

	mean free fatty acids (ppm)							
	fresh pasteurized homogenized[a]				laboratory-prepared rancid milk[a]			
FFA	sample 1	CV[b] (%)	sample 2	CV[b] (%)	sample 3	CV[b] (%)	sample 4	CV[b] (%)
C_4	0.65	6.6	0.48	6.4	32.31	6.3	30.47	6.2
C_6	nq[c]	3.2	nq	3.5	16.53	3.2	17.85	3.3
C_8	nq	5.4	nq	5.1	4.73	5.5	8.13	5.4
C_{10}	2.28	7.3	2.52	7.6	13.34	7.7	15.50	7.1
C_{12}	1.92	1.5	1.97	1.4	10.20	1.2	11.13	1.5

[a] Extraction conditions for FFA: milk (40 mL) saturated with 28% NaCl (w/v) at 70 °C, pH 1.5, and exposed to a PA fiber for 60 min. [b] CV, coefficient of variation, $n = 5$. [c] nq, not quantifiable, $n = 5$.

acids (C_{16}) were tentatively identified by spiking milk with analytical standards, with the last two not being quantified. A marked increase in peak areas was observed when fresh milk was spiked with the analytical standards (Figure 3C). It has been reported that short-chain FFA (C_4–C_{12}) are associated with rancid or lipolized flavor, but FFA C_{14} and C_{16} are not associated with this flavor defect (*2*). LPRM showed higher FFA concentrations than those found in fresh milk, with this effect being more pronounced for butanoic acid (Table 5). FFA concentration ranges found in fresh milk were very close to those reported earlier (*6*). Similarly, FFA concentrations ranges found in rancid milks were very similar to those reported by ref *13*. ADVs for fresh milk (samples 1 and 2) were 0.6, which are within the range reported for normal milk (ADV < 0.7) (*15*). ADVs for LPRM, samples 3 and 4, were 6.5 and 7.2, respectively, which are within the range reported for extremely rancid milk (ADV > 1.4) (*15*). In conclusion, the extraction of FFA from milk by SPME prior to gas chromatographic determination resulted in a method of good precision, accuracy, and sensitivity. The SPME headspace sampling technique provided quantitative data that truly represented the short-chain FFA profile associated to milk rancidity since solvent artifacts or losses during FFA extraction of traditional procedures were eliminated. The FFA SPME procedure took 90 min, 30 min for sample equilibration and 60 min for fiber exposure with minimum sample manipulation. On the other hand, traditional methods involve steps that are time-consuming and require more sample manipulation such as lipid extraction and FFA isolation on alumina or an anion exchanger. The SPME–GC technique presented could be used to establish relationships between free fatty acids in milk and the presence of rancid flavors or to monitor the development of lipolysis in the generation of dairy flavors.

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