Analysis of Free Fatty Acids in Whey Products by Solid-Phase Microextraction

Ross M. Tomaino, James D. Parker, and Duane K. Larick*

Department of Food Science, North Carolina State University, Raleigh, North Carolina 27695-7624

To evaluate the impact of Cheddar cheese starter cultures on the level of free fatty acids in liquid whey, a solid-phase microextraction (SPME) technique was utilized. The determination of response factors relative to an internal standard and the verification of linearity over a wide concentration range allowed for the quantitation of free fatty acids in experimentally produced liquid whey and in a wide range of dry whey ingredients. Results indicated that whey produced with a *Lactococcus lactis* subsp. *lactis* starter culture contained the highest level of total free fatty acids with significantly higher levels of lauric, myristic, and palmitic acids. Significant declines in oleic, linoleic, and palmitic acid occurred during storage. Commercial whey ingredients demonstrated a linear increase in free fatty acids with increasing percent protein, except for whey protein isolate, which had the lowest concentration. The use of SPME for the rapid analysis of free fatty acids in whey products should allow for further research to determine the importance of these compounds on the quality and stability of whey products.

Keywords: *Whey; solid-phase microextraction; SPME; Lactococcus lactis; starter culture; lipolysis; free fatty acids; response factors; standard curve; quantitation*

INTRODUCTION

Literature pertaining to the free fatty acid content of whey products is limited. Vaghela and Kilara (*1*, *2*) developed a modified Bligh and Dyer lipid extraction method (*3*) in combination with solid-phase extraction that allowed for the identification and quantification of individual and total free fatty acids in whey protein concentrate. Research conducted by de Wit et al. (*4*) investigated the free fatty acid content of whey protein concentrates, but only total free fatty acids were reported. The finiteness associated with the level of research conducted on the free fatty acids in whey products may be due to the difficulty associated with the extraction of lipid and subsequent separation of the free fatty acids prior to analysis. Theodet and Gandemer (*5*) stated that the low lipid content of whey products, the high protein content, and the presence of stable lipid−protein complexes impedes the complete extraction of lipids from whey products. The extraction method design of Vaghela and Kilara (*1*), although considered rapid, requires 12−14 h to produce a crude extract that is in need of further purification. The extract is subjected to gel filtration prior to the separation of lipid classes via solid-phase extraction at which time free fatty acids can be analyzed by gas chromatography. In the present study, a rapid technique for the analysis of free fatty acids was sought to detect the presence of lipolytic activity in liquid whey produced with different starter cultures. The extremely low lipid content of liquid whey, which typically contains less than 0.1% total lipids, along with the high percentage of moisture (93−94%) (*6*), combine to impede many of the common lipid-extraction techniques. The modified Bligh and Dyer technique of Vaghela and Kilara (*1*) would require a dry sample thereby necessitating the use of a drying technique that would extend preparation time. The solvent extraction methods such as those of Folch et al. (*7*) and Bligh and Dyer (*3*) would require large quantities of organic solvents to extract the lipid and the use of evaporation techniques to concentrate the sample enough for adequate analysis. In response to these concerns, the use of solid-phase microextraction (SPME) was investigated. The majority of the literature pertaining to SPME is related to environmental sampling, such as wastewater analysis, or the detection of flavor compounds in food, mostly beverages (*8*). Research conducted by Pan et al. (*9*) and Pan and Pawliszyn (*10*) demonstrated the ability of SPME fibers to bind fatty acids ranging from acetic acid (C2) to behenic acid (C22) through the use of direct emersion and headspace sampling in water and air matrixes. The application of SPME to free fatty acid analysis in cheese flavor has been explored by Chin et al. (*11*) and Wijesundra et al. (*12*). Because only the fatty acids ranging from acetic to lauric acid (C12) are considered to have influence on flavor, both studies only reported their identification. Although not pursued, Wijesundra et al. (*12*) reported that the SPME fiber became saturated with the fatty acids myristic (C14) through stearic acid (C18) during absorption times greater than 30 min. Given that the predominant fatty acids in dairy products range from butyric (C4) to oleic acid (C18:2), the use of SPME should allow for the determination of free fatty acids in liquid whey.

The objective of the present research was to monitor the lipolytic activity of different starter cultures in whey through the utilization of a solid-phase microextraction technique. A second objective pertained to evaluating the capability of the SPME procedure to analyze the free fatty acids in commercially available whey ingredients.

* To whom correspondence should be addressed (e-mail duane_larick@ncsu.edu; phone (919)-515-4390; fax (919)-515-2873).

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MATERIALS AND METHODS

Whey Production. Liquid Cheddar cheese whey was produced as described by Tomaino (*13*). Starter cultures obtained from Rhodia (Madison, WI) or glucono-δ-lactone (GDL) obtained from Archer Daniels Midland (Decatur, IL) were inoculated into 68 L of whole milk. After a suitable pH decline, chymosin was added (chy-max ultra, Chr Hansen, Milwaukee, WI) to induce curd formation. The whey was then drained, filtered, clarified, pasteurized (77 °C for 16 s), and filled directly into 125-mL HDPE bottles (Nalge Company, Rochester, NY) and either immediately frozen (referred to as the fresh samples) or stored for 14 days at 4 °C then frozen (storage samples) until analysis. Four treatments were replicated three times in different lots of milk and were as follows: *Lactococcus lactis* subsp. *lactis* (MM380), *Lactococcus lactis* subsp. *cremoris* (MM170), a combination of MM380 and 170, and glucono-δ-lactone (GDL), the control.

SPME Procedure and Sample Preparation. Liquid whey (5 g) was added to a 10-mL screw cap vial (Supelco, Bellafonte, PA) along with 5 μL of an internal standard composed of 1 mg heptadecanoic acid (C17)/mL of methanol (1μg/g I. S. concentration) and a $^5/_8$ in. long × $^5/_{16}$ in. diam octagonal stirring bar (Fisher Scientific, Pittsburgh, PA). The vial was then sealed with a screw cap fitted with a PTFE/Neoprene septa (Supelco). The septa was punctured with the fiber sheath of a 30-μm thick poly(dimethylsiloxane) fiber (Supelco) attached to a fiber holder (Supelco) set to a depth of 1 cm, and the vial was then placed into a heating block (Pierce Chemical Co., Rockford, IL) adjusted to 110 °C with the stirring module set on high. Immediately upon placement in the heating block the fiber was exposed for 40 min during which time the block temperature was monitored with a thermocouple. After 40 min the fiber was retracted then removed from the vial. Prior to desorption the fiber holder was adjusted to a depth of 3 cm then placed into the injector (250 °C) of a Varian 3700 gas chromatogram (Walnut Creek, CA) equipped with a DB-FFAP column (30 m, 0.25 mm i.d., 0.25-μm film thickness; J&W Scientific, Folsom CA) operating with the split flow off. The temperature program was initially set at 100 °C and held for 2 min then increased at rate of 10 °C/min up to 245 °C and held for 10 min. The fiber remained in the injector for the entire GC run time, after which the fiber was retracted and removed and the GC was set at 200 °C for 30 min then returned to 100 °C for the next run. These steps were added to minimize any possible carryover effects of the fiber or on the column. The fatty acids were detected with a flame-ionized detector set at 250 °C.

Whey samples were defrosted in a room temperature (25 °C) water bath for 1 h. Each sample was adjusted to pH 2.0 with 3.3 M HCl then stored at refrigerated temperature until analysis, at which time they were equilibrated to room temperature in a water bath (25 °C). All samples were analyzed in duplicate with added internal standard and once without added internal standard. Four commercially available whey ingredients from three different companies were obtained, and included whey powder (12.5% protein), whey protein concentrate (34.3% protein), whey protein concentrate (86.5% protein), and whey protein isolate (92.6% protein). Each of the samples was reconstituted with distilled water to produce a final solution containing 6.3% solids. The pH was adjusted to 2.0 for each sample and the analysis was conducted as stated above for liquid whey, except samples were replicated four times with the addition of C17 internal standard and in duplicate without internal standard.

Response Factors. To quantitate the peak area for each fatty acid with the use of an internal standard, response factors were calculated. Four liquid whey samples without added fatty acids were analyzed, then the analysis was repeated in four different samples with the addition of 5 μL of a standard solution containing 1 mg of each of the following fatty acids (Nu-Chek-Prep, Elysian, MN) per 1 mL of methanol: caproic (C6), caprylic (C8), capric (C10), lauric (C12), myristic (C14), palmitoleic (C16:1), palmitic (C16), heptadecanoic (C17), oleic (C18:1), linoleic (C18:2), and stearic (C18). The peak area differences between the samples with and without added standard solution were used to calculate response factors relative to the internal standard (C17):

$$\text{response factor} = \frac{\text{PA C17 with SS} - \text{PA C17 w/o SS}}{\text{PA fatty acid with SS} - \text{PA of fatty acid w/o SS}}$$

where PA = peak area and SS = standard solution.

Table 1. Response Factors and Linear Relations of Individual Fatty Acids

fatty acid	response factor[a]	standard curve R^2 [b]
C6	19.18	.999
C8	6.67	.999
C10	3.20	.999
C12	1.29	.999
C14	0.82	.997
C16	0.86	.997
C16:1	0.75	.996
C17[c]	1.00	.999
C18	0.79	.993
C18:1	0.63	.999
C18:2	0.82	.996

[a] Response factors were calculated as presented in Materials and Methods. [b] R^2 was calculated from a seven point standard addition curve. [c] Internal standard.

Standard Curve and Quantitation. To demonstrate that all the fatty acids being quantitated were responding in a linear fashion a standard addition curve was constructed. Aliquots of 0 μL, 5 μL, 10 μL, and 15 μL of the 1 mg fatty acid/mL methanol standard solution, were added to liquid whey samples and replicated four times for each quantity. Then a second standard solution containing the same fatty acids at a concentration of 5 mg/mL methanol was added in the same manner at 5-μL, 10-μL, and 20-μL volumes. Average peak areas were used to construct a seven point standard addition curve for each fatty acid that ranged from zero added fatty acid to the addition of 20 μg each fatty acid/g liquid whey.

Quantitation of peak responses in all the samples was calculated based on the internal standard, response factors, and a correction factor. The correction factor was necessary to account for the small but inherent level of heptadecanoic acid in each sample and was calculated as follows:

$$\text{correction factor} = \frac{\text{PA IS (C17)}}{\text{PA of C17 w/IS} - \text{PA C17 w/o IS}}$$

where IS = internal standard.

A concentration value for each fatty acid peak was calculated by dividing its peak area by that of the internal standard and then multiplying by the response and correction factors.

Statistical Analysis. All the data were analyzed for statistical differences using SAS statistical software (*14*; SAS Institute, Cary, NC). Analysis of variance and mean separations were performed by PROC ANOVA and Duncan's procedure, respectively. All the significant differences reported are at least at the $p < 0.05$ level.

RESULTS AND DISCUSSION

Response Factors and Standard Curve. The use of standard addition curves to quantitate is often recommended for SPME procedures (*15*). If the purpose of a study is to analyze for differences among a variety of samples, standard addition curves need to be constructed within each sample, which may be a cumbersome process. The reporting of peak areas for SPME analyses is commonplace, but may be misleading as the binding affinity of compounds to SPME fibers can vary greatly. In the present study, instead of using standard addition curves or reporting peak areas an attempt was made to calculate response factors for each fatty acid in relation to the internal standard. Table 1 illustrates

Table 2. Effect of Treatment on the Free Fatty Acid Concentration of Liquid Whey

	μg free fatty acid/g liquid whey			
fatty acid	MM380/170	GDL (glucono-δ-lactone; control)	MM380 (*Lactococcus lactis* subsp. *lactis*)	MM170 (*Lactococcus lactis* subsp. *cremoris*)
C6	1.08[a]	0.99[a]	1.06[a]	1.14[a]
C8	0.83[a]	0.83[a]	0.86[a]	0.86[a]
C10	0.65[a]	0.67[a]	0.70[a]	0.64[a]
C12	0.44[a]	0.43[a]	0.48[b]	0.44[a]
C14	1.19[a]	1.15[a]	1.29[b]	1.19[a]
C16	3.91[a]	3.83[ab]	4.09[c]	4.00[bc]
C16:1	0.23[a]	0.21[a]	0.22[a]	0.23[a]
C18	2.27[a]	2.39[a]	2.31[a]	2.28[a]
C18:1	3.15[a]	3.06[a]	3.35[a]	3.11[a]
C18:2	0.85[b]	0.81[ab]	0.86[b]	0.71[a]
total	14.61	14.37	15.23	14.60

[a–c] Means within rows with different letters are statistically different ($p < 0.05$).

that the internal standard (C17) responded to the PDMS fiber in a manner similar to that of the C12 − C18:2 fatty acids, but clearly a progressive and rapid loss of sensitivity occurred with decreasing fatty acid chain length. The validity of response factors depends on the assumption that the analytical method, in this case a conjunction of the affinity of each compound to the SPME fiber and to the GC detector, responds in a linear fashion to the internal standard and all the analytes of interest. To address the concern of linearity, and therefore validity of the utilization of response factors to quantitate fatty acid concentration, a seven point standard addition curve was constructed. All the fatty acids responded linearly over a wide concentration range with all R^2 values greater than 0.993 (Table 1), thereby justifying the quantitation method for the experimental whey. The standard addition curve constructed for the experimental whey encompassed all the peak areas found in the commercial samples and supported the use of the response factors for their quantitation. The only assumption made to quantitate the commercial samples was that all the fatty acids responded to the internal standard just as it was found in the experimental whey. Another potential factor that limits SPME data is analyte competition, which has been shown to be a problem with some SPME data (*16*). The linear responses determined are a strong indicator that analyte competition was not detected within the range of standard addition curve.

Analysis of Liquid Whey Samples. Lactic acid bacteria are considered to be weakly lipolytic and to generally hydrolyze triacylglycerides in a nonspecific manner (*17*). The concentration of free fatty acids indicates that the *Lactococcus lactis* subsp. *lactis* culture (MM380) had significantly greater levels of C12 and C14 compared to those of the other treatments and significantly greater levels of C16 in relation to the control and the MM380/170 treatment (Table 2). Although the data are statistically different, the levels of each fatty acid and the total fatty acids are only minimally greater in the MM380 treatment. The presence of continued hydrolysis during storage was not detected, indicating that if lipases were present they were not active following pasteurization. In contrast, the storage data indicated a slight decrease in all the free fatty acids with significant losses of C16, C18:1, and C18:2 (Table 3).

The SPME analysis was not an exhaustive extraction, and therefore the detection of fatty acids was dependent upon a series of equilibrations between the whey proteins (especially β-lactoglobulin and serum albumin), the whey solution, the air, and the fiber (protein ↔ solution ↔ air ↔ fiber). Concurrent research (*13*) and other storage studies (*18, 19*) pertaining to whey ingredients have identified large increases in volatile oxidation products, especially aliphatic aldehydes, which typically arise from the oxidation of unsaturated fatty acids. Increases in aldehydes such as hexanal and octanal strongly support an oxidative mechanism for the decreases in linoleic and oleic acids seen in this study, but may not sufficiently explain the decrease in palmitic acid. Research by Hidalgo and Kinsella (*20*) studied the interactions of linoleic acid 13-hydroperoxide with β-lactoglobulin and found that the hydroperoxide and many of the resulting secondary oxidation products, such as hexanal, were readily able to react with the protein and influence structural changes such as polymerization. Similar research by Stapelfeldt and Skibsted (*21*) found that in the presence of the aldehydes pentanal, hexanal, and heptanal, polymers of up to 12 β-lactoglobulin monomers could be detected with size-exclusion chromatography. Changes in the conformational state of a protein due to oxidation, or matrix shifts due to lipid degradation, may alter the equilibrium balances between the protein, solution, air, and the SPME fiber, thereby influencing detection. It is doubtful that the internal standard would be able to account for the matrix change, therefore if alterations did occurr they could not be accounted for in the present study.

Table 3. Effect of Time on the Concentration of Free Fatty Acids in Liquid Whey

	μg free fatty acid/g liquid whey	
fatty acid	fresh	storage
C6	1.12[a]	1.01[a]
C8	0.87[a]	0.83[a]
C10	0.67[a]	0.66[a]
C12	0.45[a]	0.45[a]
C14	1.22[a]	1.20[a]
C16	4.01[a]	3.90[b]
C16:1	0.23[a]	0.21[a]
C18	2.38[a]	2.25[a]
C18:1	3.33[a]	3.00[b]
C18:2	0.86[a]	0.75[b]
total	15.13	14.28

[a–b] Means within rows with different letters are statistically different ($p < 0.05$).

Analysis of Commercial Samples. Four commercially available whey samples with varying protein concentrations were analyzed for free fatty acids. The data demonstrate a linear relationship ($R^2 = 0.999$) between protein concentration and total free fatty acids in the whey powder (WP), whey protein concentrate (WPC34), and another whey protein concentrate (WPC86.5)

Table 4. Concentrations of Free Fatty Acids in Commercially Available Whey Ingredients

	whey powder 12.5% protein		whey protein concentrate 34.3% protein		whey protein concentrate 86.5% protein		
fatty acid	concentration	SD[a]	concentration	SD	concentration	SD	R^2 value[b]
C8	0.16	0.01	1.35	0.12	0.88	0.07	.156
C10	0.28	0.03	3.78	0.18	9.37	0.66	.990
C12	0.47	0.03	3.82	0.15	16.77	1.18	.991
C14	2.00	0.05	8.56	0.33	35.96	2.37	.989
C16	6.76	0.06	21.61	0.65	75.61	4.09	.993
C16:1	0.39	0.05	1.25	0.07	4.99	0.16	.987
C18	2.74	0.05	7.76	0.18	13.71	0.47	.966
C18:1	4.74	0.10	13.87	0.60	31.48	1.67	.997
C18:2	1.43	0.05	4.06	0.22	6.75	0.30	.950
total (wet basis)[c]	18.96		66.06		195.51		.999
total (dry basis)	300.90		1048.37		3102.74		

[a] SD, standard deviation. [b] Correlation between percent protein concentration and free fatty acid concentration. [c] Wet basis, ug free fatty acid/g of reconstituted (6.3% solids) liquid whey; dry basis, ug free fatty acid/g of dry whey.

(Table 4). The individual free fatty acids also increased in a linear fashion ($R^2 > 0.950$), except for C8. All three samples were produced by different manufacturers; therefore, obtaining strong linear increases for each fatty acid would be difficult because of the inherent variability in the cheese milk and processing used in their manufacture. The linear increase in the higher chain fatty acids is related to the association of these apolar compounds with β-lactoglobulin and serum albumin. One of the physiological roles of serum albumin (*22*), and possibly β-lactoglobulin (*23*), is as a fatty acid carrier protein. The processing of the WP, WPC34, and WPC86.5 seemingly was not able to break the protein−lipid complexes, and therefore the fatty acids were concentrated along with the protein, while the more polar fatty acids, which are not known to strongly associate with proteins, were removed. It is therefore plausible to believe that all of the fatty acids detected in this study were associated with the proteins. β-lactoglobulin comprises approximately 45% of the whey proteins (*24*) and is able to carry approximately 0.5−0.71 mol of fatty acid per mole of monomer in milk (*25*, *26*), whereas serum albumin constitutes only 5% of whey proteins (*24*), but carries approximately 4.8 mol of fatty acid per mole of protein in milk (*26*). A whey protein concentrate of 86.5% protein would then be able to carry an estimated range of fatty acids between 0.87 and 1.15 μmol. The molar concentration found in this study for the WPC86.5, using a molar weight average of 245 for the fatty acids, was 0.80 μmol, which lies just outside the calculated range, but supports the concept that the fatty acids detected are predominately associated with the proteins in WPC. The whey protein isolate (WPI) had the lowest concentration of free fatty acids of all the whey products analyzed (Table 5). WPIs are commonly produced through the use of an ion exchange process that utilizes either cation or anion resin beads, in acid or alkaline environments respectively, that bind the whey proteins and allow for the removal of other components prior to ultrafiltration, diafiltration, and spray drying (*27*). Only fatty acids of chain lengths greater than 10 were detected in WPI. Serum albumin (*26*) and β-lactoglobulin (*28*) have been shown to contain fatty acids with chain lengths of 10 and greater, and presumably are responsible for those detected in the WPI. The greater than 20-fold decrease in the level of free fatty acids in WPI as compared to that of the WPC86.5 can have many implications relating to flavor and protein stability and functionality. Morr and Foegeding (*29*) analyzed 3 ion exchange WPIs and 8 ultrafiltrated WPCs and reported that solutions of WPI's were either totally bland or exhibited a slight old whey flavor with intensities lower than 2 on a 5 point scale, whereas all the WPC solutions had stale old whey flavor scores of 4 or greater. Lipid oxidation reactions are thought to initiate the deterioration of flavor in whey products through the formation of lipid oxidation products that contribute to off flavors and the promotion of Maillard reactions (*30*). Studies have also shown that fatty acid binding to purified β-lactoglobulin can increase its resistance to thermal (*31*) and enzymatic denaturation (*32*), whereas others have reported the modifications of gelation behavior induced through the addition of fatty acids (*33*, *34*). Alterations in the properties of serum albumin have also been shown. Bernal and Jelen (*35*) reported an increased thermal resisitance of serum albumin in the presence of fatty acids. It can be inferred that there exists a balance between the poor oxidative stability of whey protein concentrates reported by Morr and Foegeding (*29*) and the enhancement of physiochemical properties observed in studies with β-lactoglobulin and serum albumin. Research is lacking in the understanding of the oxidative instability of whey ingredients as well as the role of fatty acids in the functionality of whey protein ingredients. The high content of unsaturated fatty acids in whey protein concentrates observed herein and by Vaghela and Kilara (*2*) constitute a risk factor for oxidation and therefore quality.

Table 5. Concentration of Free Fatty Acids in a Commercially Available Whey Protein Isolate (92.6% protein)

fatty acid	concentration	SD[a]
C8	ND[a]	-
C10	0.76	0.07
C12	0.66	0.05
C14	1.38	0.14
C16	2.80	0.27
C16:1	0.22	0.02
C18	1.06	0.15
C18:1	2.01	0.29
C18:2	0.51	0.08
total (wet basis)[b]	9.39	
total (dry basis)	149.02	

[a] SD, standard deviation; ND, not detected. [b] Wet basis, μg free fatty acid/g of reconstituted (6.3% solids) liquid whey; dry basis, μg free fatty acid/g of dry whey.

Evaluation of Method. Using the data presented by Vaghela and Kilara (*1*), the concentrations of free

fatty acids in a whey powder (12% protein) and two whey protein concentrates (34% and 75% protein) were calculated. The results show that the WP, WPC34, and WPC75 that Vaghela and Kilara (*1*) analyzed had 440.6, 1213, and 1802 μg free fatty acids/g dry whey, respectively. These values are similar to those calculated in the present study (Table 4), but it is difficult to make definite comparisons because the composition and processing histories of the whey ingredients were not known or presented in either study. The compositions of individual fatty acids were similar except that the very high levels of butyric acid reported by Vaghela and Kilara (*2*) were not found in this study. They hypothesized that the high levels of butyric acid were the result of residual lipase activity, which was not detected in this research. The inability to detect C6 occurred with all the whey samples except the experimental whey, while the loss of detection of C8 within the WPI supports the loss of these free fatty acids during processing. These free fatty acids in whey products might not be important unless residual lipase activity does exist and results in levels high enough to impart off flavors. The PDMS fiber is a nonpolar stationary phase, and therefore it may be possible to detect the short chain fatty acids with the use of a more polar fiber.

The advantages of the SPME procedure include rapid evaluation with little sample preparation, the absence of organic solvents, and good reproducibility. The only sample preparation required involved the reconstitution of the dry ingredients to 6.3% solids and the adjustment of pH to 2.0. The adjustment of all the samples to 6.3% solids allowed for easier comparisons between the commercial samples (especially the whey powder) and the liquid whey produced in this study. The pH adjustment was necessary to greatly increase the peak area response for each fatty acid and is commonly practiced in SPME procedures. It has been recommended that the pH of solutions be adjusted to a value at least 2 pK_a values below that of free fatty acids because the fiber can bind only the associated form of each fatty acid (*36*). A concurrent affect of the pH decline involves alteration of protein conformations that lowers the affinity of free fatty acids to the protein and allows for an increased availability to the fiber. At the pH of 2.0 β-lactoglobulin exists as an expanded monomer (*37*), but serum albumin becomes fully uncoiled. (*38*). Both proteins would be positively charged resulting in a high level of electrostatic repulsion between proteins that greatly increases their resistance to thermal aggregation (*35*), while also decreasing their association to free fatty acids. The only concern with the adjustment of the pH to 2.0 was the possible hydrolysis and release of fatty acids from other lipids such as triacylglycerides, but no increase in free fatty acids could be detected during several days of storage. Addition of salt, in conjunction with acid adjustment, has been shown to act synergistically in the improvement of analyte response to SPME fibers (*9*). Salt addition was not pursued as addition to whey samples at pH 2.0 resulted in the coagulation of proteins, and therefore difficulty in obtaining a homogeneous sample.

The reproducibility of the SPME procedure used in this study varied with each fatty acid in relation to the internal standard. All the fatty acids in the laboratory-produced whey had average duplicate standard deviations between 3 and 10.2% except for C6 which was 14.1%. One problem with this study that became apparent upon data analysis was the use of only one internal standard. The C17 internal standard presumably was not capable of accounting for the variability in responses of C6 that occurred, possibly due to temperature fluctuations ($\sim\pm$ 4 °C) in the heating block. The incorporation of a second internal standard to account for the short chain fatty acids would decrease the variability in the data, and allow for a more representative level of the C17 internal standard to be used to quantitate the long chain fatty acids in samples with higher levels of free fatty acids, such as the WPC86.5. The use of crimp-seal vials instead of those with screw caps may also help decrease the potential for leakage, and therefore variability of volatile components at the high sampling temperature. Another disadvantage of using C17 as the internal standard is that small but detectable levels were observed in all the samples, therefore making it necessary to account for that level with a correction factor for more accurate quantitation. The difficulty of choosing a proper internal standard when analyzing the free fatty acids in dairy products, such as whey, is the wide range of free fatty acids present (*39*). It may be possible to use an unsaturated fatty acid such as pentadecenoic (15:1) or heptadecenoic acid (17:1) and eliminate the need to account for inherent levels.

The standard deviations in the commercial samples were also less than 10%, except for the whey protein isolate samples, which had a range from 8.1 to 15.6%. Unlike the other samples, the WPI formed into a thick liquid that restricted the function of the stir bar resulting in a lack of circulation throughout the sample. Considering reproducibility was much greater in all the other samples, it was assumed that the change in the matrix resulted in the inconsistent release of each fatty acid including the internal standard.

Conclusion. The analysis of the experimental whey samples indicated that starter cultures may have an impact on the level of free fatty acids in whey products produced from Cheddar cheese. The use of SPME should allow for similar research to be conducted on cheese varieties that utilize cultures with greater lipolytic activity or incorporate lipase enzymes for flavor development, as is done in many Italian varieties. The impact of free fatty acid on the quality of whey ingredients may be related to oxidative stability. The inability to remove free fatty acids, particularly the unsaturated fatty acids, from whey products such as whey protein concentrate, as was demonstrated in this research, may result in flavor and functional property deterioration due to oxidation. Starter cultures or added enzymes may have an impact through the release of free fatty acids during cheese production, but only with greater investigation will their definitive impact on the oxidative stability of whey products be determined. The SPME procedure that was utilized for this research allows for the qualitative and quantitative analysis of free fatty acids in whey products. Traditional techniques involve time-consuming procedures to extract, separate, and concentrate the free fatty acid fraction and the use of large quantities of organic solvents. This SPME procedure allows for the detection of fatty acids at low concentrations with little sample preparation. More research will be required to investigate the full potential of SPME for the analysis of free fatty acids in other whey products.

LITERATURE CITED

(1) Vaghela, M, N.; Kilara, A. A rapid method for extraction of total lipids from whey protein concentrates and separation of lipid classes with solid-phase extraction. *J. Am. Oil Chem. Soc.* **1995**, *72*, 1117−1121.
(2) Vaghela, M.; Kilara, A. Lipid composition of whey protein concentrates manufactured commercially and in the laboratory. *J. Dairy Sci.* **1996**, *79*, 1172−1183.
(3) Bligh, E. G.; Dyer, W. J. A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* **1959**, *37*, 911−917.
(4) deWitt, J. N.; Klarenbeek, A. M. Evaluation of functional properties of whey protein concentrates and whey protein isolates. 2. Effects of processing history and composition. *Neth. Milk Dairy J.* **1986**, *40*, 41−56.
(5) Theodet, C.; Gandemer, G. Comparaison de cinq methodes pour extraire les lipides du lactoserum et de ses derives. *Lait* **1990**, *71*, 41−54.
(6) Jelen, P. Whey: Composition, properties, processing, and uses. In *Wiley Encyclopedia of Food Science and Technology*, 2nd edition; Francis, F. J., Ed.; Wiley: New York, 2000; pp 2652−2661.
(7) Folch, J.; Lees, M.; Sloane-Stanley, G. H. A simple method for the isolation and purification of total lipids from animal tissues. *J. Biol. Chem.* **1957**, *226*, 497−509.
(8) Harmon, A. D. Solid-phase microextraction for the analysis of flavors. In *Techniques for Analyzing Food Aroma*; Marsili, R., Ed.; Marcel Dekker: New York, 1997; pp 81−112.
(9) Pan, L.; Adams, M.; Pawliszyn, J. Determination of fatty acids using solid-phase microextraction. *Anal. Chem.* **1995**, *67*, 4396−4403.
(10) Pan, L.; Pawliszyn, J. Derivatization/solid-phase microextraction: New approach to polar analytes. *Anal. Chem.* **1997**, *69*, 196−205.
(11) Chin, H. W.; Bernhard, R. A.; Rosenberg, M. Solid-phase microextraction for cheese volatile compound analysis. *J. Food Sci.* **1996**, *61*, 1118−1122, 1128.
(12) Wijesundera, C.; Drury, L.; Walsh, T. Determination of free fatty acids and lactones in cheese by solid-phase microextraction (SPME). *Aust. J. Dairy Tech.* **1998**, *53*, 146.
(13) Tomaino, R. M. The effect of *Lactococcus lactis* starter cultures on the flavor and stability of liquid Cheddar cheese whey; MS Thesis, North Carolina State University, 2000; pp 72−100.
(14) SAS. *Statistical Analysis System User's Guide: Statistics*; SAS Institute: Cary, NC, 1998.
(15) Penton, Z. Method development with solid-phase microextraction. In *Solid-Phase Microextraction: A Practical Guide*; Wercinski, S. A. S., Ed.; Marcel Dekker: New York, 1999; pp 27−57.
(16) Coleman, W. M. A study of the behavior of Maillard reaction products analyzed by solid-phase microextraction−gas chromatography−mass selective detection. *J. Chrom. Sci.* **1996**, *34*, 213-218.
(17) Meyers, S. A.; Cuppett, S. L.; Hutkins, R. W. Lipase production by lactic acid bacteria and activity on butter oil. *Food Microbiol.* **1996**, *13*, 383−389.
(18) Lee, Y. B.; Morr, C. V. Changes of headspace volatile compounds due to oxidation of milk fat during storage of dried dairy products. In *Lipids in Food Flavors*; Ho, C. T., Hartman, T. G., Eds.; American Chemical Society: Washington, DC, 1994; pp 98−107.
(19) Lee, Y. B.; Laye, I.; Kim, Y. D.; Morr, C. V. Formation of volatile compounds in whey protein concentrate during elevated storage as a function of water activity. *Int. Dairy J.* **1996**, *6*, 485−496.
(20) Hidalgo, F. J.; Kinsella, J. E. Changes induced in β-lactoglobulin B following interaction with linoleic acid 13-hydroperoxide. *J. Agric. Food Chem.* **1989**, *37*, 860−866.
(21) Stapelfeldt, H.; Skibsted, L. H. Modification of β-lactoglobulin by aliphatic aldehydes in aqueous solution. *J. Dairy Res.* **1994**, *61*, 209−219.
(22) Peters, T. Serum Albumin. *Adv. Protein Chem.* **1985**, *37*, 161−245.
(23) Sawyer, L.; Brownlow, S.; Polikarpov, I.; Wu, S. Y. β-lactoglobulin: Structural studies, biological clues. *Int. Dairy J.* **1998**, *8*, 65−72.
(24) Swaisgood, H. E. Characteristics of edible fluids of animal origin: Milk. In *Food Chemistry*; Fennema, O. R., Ed.; Marcel Dekker: New York, 1985; pp 791−827.
(25) Perez, M, D.; Calvo, M. Interaction of β-lactoglobulin with retinol and fatty acids and its role as a possible biological function for this protein: A review. *J. Dairy Sci.* **1995**, *78*, 978−988.
(26) Perez, M. D.; Diaz de Villegas, C.; Sanchez, L.; Aranda, P.; Ena, J. M.; Calvo, M. Interaction of fatty acids with β-lactoglobulin and albumin from ruminant milk. *J. Biochem.* **1989**, *106*, 1094−1097.
(27) Huffman, L. M. Processing whey protein for use as a food ingredient. *Food Technol.* **1996**, *50*, 49−52.
(28) Diaz De Villegas, M. C.; Oria, R.; Sala, F. J.; Calvo, M. Lipid binding by β-lactoglobulin of cow milk. *Milchwissenschaft* **1987**, *42*, 357−358.
(29) Morr, C. V.; Foegeding, E. A. Composition and functionality of commercial whey and milk protein concentrates and isolates: A status report. *Food Technol.* **1990**, *44*, 100−111.
(30) Morr, C. V.; Ha, E. Y. W. Off-flavors of whey protein concentrate: A literature review. *Int. Dairy J.* **1991**, *1*, 1−11.
(31) Puyol, P.; Perez, M. D.; Ena, J. M.; Calvo, M. Effect of retinol and fatty acid binding by bovine β-lactoglobulin on its resistance to trypsin digestion. *Int. Dairy J.* **1993**, *3*, 589−597.
(32) Puyol, P.; Perez, M. D.; Peiro, J. M.; Calvo, M. Effect of binding of retinol and palmitic acid to bovine β-lactoglobulin on its resistance to thermal denaturation. *J. Dairy Sci.* **1994**, *77*, 1494−1502.
(33) Ikeda, S.; Foegeding, E. A.; Hardin, C. C. Phospholipid/fatty acid-induced secondary structural changes in β-lactoglobulin during heat-induced gelation. *J. Agric. Food Chem.* **2000**, *48*, 605−610.
(34) Puyol, P.; Perez, M. D.; Burgos, J.; Calvo, M. Effect of the binding of palmitic acid to β-lactoglobulin on its gelation properties. *Int. Dairy J.* **1998**, *8*, 119−123.
(35) Bernal, V.; Jelen, P. Thermal stability of whey protein - A calorimetric study. *J. Dairy Sci.* **1985**, *68*, 2847−2852.
(36) Pawliszyn, J. *Solid-Phase Microextraction: Theory and Practice*; Wiley-VCH: New York, 1997; pp 43−95.
(37) Wong, D. W.; Camirand, W. M.; Pavlath, A. E. Structures and functionalities of milk proteins. *Crit. Rev. Food Sci. Nutr.* **1996**, *36*, 807−844.
(38) Relkin, P. Thermal unfolding of β-lactoglobulin, α-lactalbumin, and bovine serum albumin. A thermodynamic approach. *Crit Rev. Food Sci. Nutr.* **1996**, *36*, 565−601.
(39) Jensen, R. G.; Ferris, A. M.; Lammi-Keefe, C. J. Symposium: Milk fat − composition, function, and potential for change. *J. Dairy Sci.* **1991**, *74*, 3228−3243.

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