An Improved Liquid Chromatographic Method for the Quantitative Determination of Free Fatty Acids in Milk Products

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ABSTRACT

An existing HPLC method for the quantitative determination of the major FFA in milk fat was improved. Complete resolution of the *p*-bromophenacyl (PBP) ester derivatives of saturated and unsaturated FFA of acyl-chain length C_4 to C_{18} was accomplished by gradient elution (60 to 100% acetonitrile in water) when the temperature of the reverse-phase column was maintained at 10°C. This allowed the determination of all the major FFA in milk fat within a single chromatographic analysis. The improved method should save time in analysis of FFA in milk fat and also can be used to quantify the low concentrations (78 to 270 $\mu M \pm 17\%$) of the major FFA (myristic, palmitic, stearic, and oleic acids) in fresh pasteurized milk and follow the time course of milk fat hydrolysis where low amounts of FFA are liberated.

INTRODUCTION

Free fatty acids in dairy products can have both a positive or negative impact on flavor quality. Therefore, analysis of FFA is often used as one of several indicators of quality in various dairy products. Traditional methods of FFA analysis involve conversion of fatty acids into methylester derivatives before analysis by GLC (5). Due to the volatility of methylester derivatives of short-chain length fatty acids, methods for direct analysis of FFA in dairy products have been developed (3, 8). A preliminary preparative step of separating FFA from the lipid phase is required that involves the selective adsorption and sequential elution of FFA using materials such as silicic acid (8) and alumina (3). Although these methods yield accurate quantitation of the major FFA in dairy products, they are time-consuming. Excessive sample handling may also lead to losses in recovery of FFA.

Recently, HPLC methods using reverse-phase (C_{18}) columns for FFA analysis have been developed (2, 4, 6, 7). These methods are based on the separation of FFA-ester derivatives after reaction with *p*-bromophenacyl bromide (PBPB) (2, 6), *p*-phenylazophenacyl bromide
(7), and 2-nitrophenylhydrazine hydrochloride (4). However, these methods are currently unsuitable for providing a complete resolution and quantitative determination within a single chromatographic separation of the FFA ester derivatives containing the acyl chain lengths of FFA (C_4 and C_{18}) commonly found in dairy products. As a result, these methods are suitable only for quantifying either long-chain or short-chain length FFA in dairy products within a single chromatographic analysis (1, 6).

The method developed by Reed et al. (6) is of particular interest because it accurately quantifies FFA in dairy products. Recoveries by this method are nearly 100% for the major milk fat FFA. Furthermore, this method does not require separation of FFA from intact milk fat acylglycerides before FFA derivatization by PBPB and crown ether (18-crown-6; 1,4,7,10,13,16-hexaoxacyclooctadecane). However, two problems remain in the quantification of FFA in dairy products by this method. Two chromatographic runs are required for quantification of the PBP derivatives of FFA of acyl-chain lengths of C_4 to C_{18}. Gradient elution with 60% to 100% acetonitrile in water affords reso-

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lution of the short-chain length fatty acid derivatives (C_4 to C_{10}) but is limited by the coelution of the 14:0/18:2[2] and 16:0/18:1 PBP-FFA ester pairs. Isocratic elution with methanol:acetonitrile:water (82:9:9, vol/vol/vol) allows improved resolution and quantification of FFA derivatives of acyl chain length C_{12} to C_{18}, but the 16:0/18:1 PBP-FFA ester pair remains unresolved, and FFA of chain lengths C_{10} or shorter elute near the solvent front and cannot be quantified.

In adapting this method (6) to our laboratory, we investigated the possibility that the method could be modified to yield a complete resolution of all of the principle FFA (C_4 to C_{18}) in milk fat within a single chromatographic analysis. Herein we reported the results of these studies.

MATERIALS AND METHODS

Materials

Free fatty acids, PBPB, 1,4,7,10,13,16-hexaoxacyclooctadecane (18-crown-6), and formic acid were obtained from Sigma Chemical Co., St. Louis, MO). Acetonitrile was HPLC grade and all other chemicals were reagent grade or the best grade commercially available. Water was twice deionized and distilled. Fresh pasteurized and homogenized whole milk (3.5% fat, 8.0% SNF) and half-and-half (10.7% fat, 7.4% SNF) were obtained from the University of Wisconsin Dairy Plant.

The HPLC equipment included Isco (Lincoln, ME) model 2350 pumps, an Isco V4 detector (254 nm), and an Isco ODS-2 (C_{18}) column (5μ packing, 4.6 mm × 50 mm guard column plus a 4.6 mm × 250 mm separating column). Detector response (.005 to .10 AUFS) was outputted to an IBM PC and analyzed by the Isco ChemResearch™ data management software package. The gradient profile used for these studies was a linear increase from 60% to 100% acetonitrile in water over the first 10 min, followed by isocratic elution with 100% acetonitrile for an additional 20 min. All chromatograms were subject to manual baseline review to ensure proper integration of peaks. Chromatographic conditions were varied (see text), except that the flow rate was held constant (1.6 ml/min). Column temperature was maintained by immersing the column in a closed styrofoam container through which water was circulated from a controlled-temperature water bath. Temperatures were maintained to ± .5°C.

Derivatization of Free Fatty Acids

The FFA standards were dissolved in methanol:chloroform (1:1, vol/vol) containing 1 g 17:0/L (internal standard). Derivatization was achieved by the method of Reed et al. (6). A .25-ml portion of the fatty acid standards (or extracts from whole milk or lipase-hydrolyzed half-and-half) was combined with .5 ml of 5 g PBPB/ml, .05 ml of 5 g 18-crown-6/ml (both dissolved in acetonitrile), and .2 g potassium carbonate and brought to 5 ml with acetonitrile. The PBPB:FFA ratio for the derivatization of standards was always above 1.6:1, as previously suggested (6). After heating for 30 min at 80°C, 10 μl formic acid (4%, wt/vol) was added, and the contents were heated for an additional 5 min. The mixture was cooled for at least 1 h at 0 to 4°C, and 10 μl of the filtrate passing through a .45-μ nylon membrane were injected onto the HPLC.

Hydrolysis Experiments

Hydrolysis was achieved by incubating 100 ml of half-and-half with .25 g Enzyco lipase XX (Enzyme Development Corporation), pH 6.4, at 5 or 20°C in a closed, jacketed reaction vessel. The contents were magnetically stirred and the temperature was controlled with a circulating water bath. The pH was kept constant by automatic titration with 5 N KOH. At selected intervals for up to 3 h, a .5-ml portion of the reaction mixture was removed and quenched with 5 ml methanol:chloroform (1:1, vol/vol) containing 1 g 17:0/L, and dried with 7 g sodium sulfate. The organic layer was filtered through a .45-μ nylon membrane, and the FFA were derivatized and analyzed by HPLC as described for the FFA standards, except that the PBPB:FFA ratio was at least 8:1 (8). Estimates of the FFA in the hydrolyzed samples were calculated from the amount of titrant required at any given time interval of the reaction.

[2]Fatty acid designation is acyl chain length and number of double bonds (all *cis*), if any.

Analysis of Free Fatty Acids in Fresh Whole Milk

Extracts of whole milk were prepared as described for the hydrolysis experiments. Derivatization of FFA in the extracts was similar to the methods described except that the reaction mixture was not brought to 5 ml with acetonitrile (final volume of reaction mixture was .80 ml) and a PBPB:FFA ratio of 1.6:1 was used. These modifications were necessary to increase the sensitivity of FFA measurement and prevent interference in the chromatographic separation of the PBP-FFA esters by excess PBPB.

RESULTS AND DISCUSSION

Chromatographic separation of PBP esters of FFA at 20°C by the 60 to 100% acetonitrile gradient developed by Reed et al. (6) is shown in Figure 1b. Adequate resolution of the 14:0/18:2 and 16:0/18:1 pairs of PBP-FFA esters, especially the former, was not achieved, as reported by Reed et al. (6). Our attempts to resolve these pairs by modifying the gradient profile by employing a step-gradient elution or changing flow rate of the eluting solvent were unsuccessful (data not shown).

Because column temperature is often used as a variable in developing chromatographic separations, we explored the effect of this parameters on the separation of the PBP-FFA esters. Conventional wisdom is to elevate the column temperature to enhance peak resolution and reduce the time requirement of analysis. Although we did find that increasing column temperature to 30°C reduced the time to analyze the PBP-FFA esters, no improved resolution of the 14:0/18:2 and 16:0/18:1 pairs of PBP-FFA esters was evident (Figure 2). Results were similar when the column temperature was controlled at temperatures up to 80°C (Figures 1a and 2).

When column temperature was reduced to below 20°C, resolution was improved (Figure 2). When column temperature was reduced to 10°C (Figure 1c), the 14:0/18:2 and 16:0/18:1 pairs of PBP-FFA esters became completely resolved. Reducing the temperature further to 4°C failed to improve resolution (data not shown). The time required for separation was only marginally increased from 22 min at 20°C (Figure 1b) to 29 min at 10°C (Figure 1c). The advantage of quantifying all PBP-FFA esters in a single chromatographic run at 10°C clearly outweighs the 32% increase in time required. The advantage becomes even more evident if one considers that two separate chromatographic separations at 20°C, using two different solvent systems, are required to obtain quantification of all of these PBP-FFA esters (6).

Standard curves were prepared for each fatty acid (C_4 to C_{18}, $C_{18:1}$, and $C_{18:2}$) from about 150 to 950 pmol. The detector response [at .05 absorbance units full scale (AUFS)] in this range was linear, and regression equations were calculated for each FFA. The slopes of these lines averaged 11.8 (x = pmol FFA; y = detector response) and ranged between 9.84 and 13.8 ($r^2 \geq .9396$). Because the slopes of these lines are similar in value, a regression line was calculated for the collective response for this set of PBP- FFA esters (Figure 3). Therefore, analysis for total FFA in dairy products, based on the total integrator response for the PBP-FFA ester peaks, should yield a fairly accurate estimation of the extent of acylglyceride hydrolysis.

We also evaluated the capability of using this method to follow the course of lipase action on milk fat. Data are provided for the hydrolysis of butyrate, laurate, and stearate at 5 and 20°C (Figure 4). The CV for the internal standard (17:0) in these trials (n = 10) was 4.4%, indicating that the extraction procedure used afforded a quantitative recovery of FFA in hydrolyzed samples. Reed et al. (6) reported a similar CV of 5.83% in their studies.

Finally, we evaluated the ability of the improved method to quantify the major FFA at the low concentrations expected in fresh dairy products using whole milk as an example. Triplicate analysis (detector sensitivity at .005 AUFS) of a sample of whole milk yielded the following estimates and standard deviations: $C_{14:0}$, 78 (± 12) μM; $C_{18:1}$, 257 (± 37) μM; $C_{16:0}$, 270 (± 44) μM; and $C_{18:0}$, 146 (± 31) μM. Although the error in the analysis of FFA at the concentrations found in fresh milk is somewhat high (SD of 14.5 to 21.2%), the improved method yields reasonable estimates of the major FFA in milk. Furthermore, the improved method allowed the individual analysis of the 16:0 and 18:1, which was not possible in the original method (6).

In conclusion, an existing HPLC method for the analysis of FFA in dairy products has been

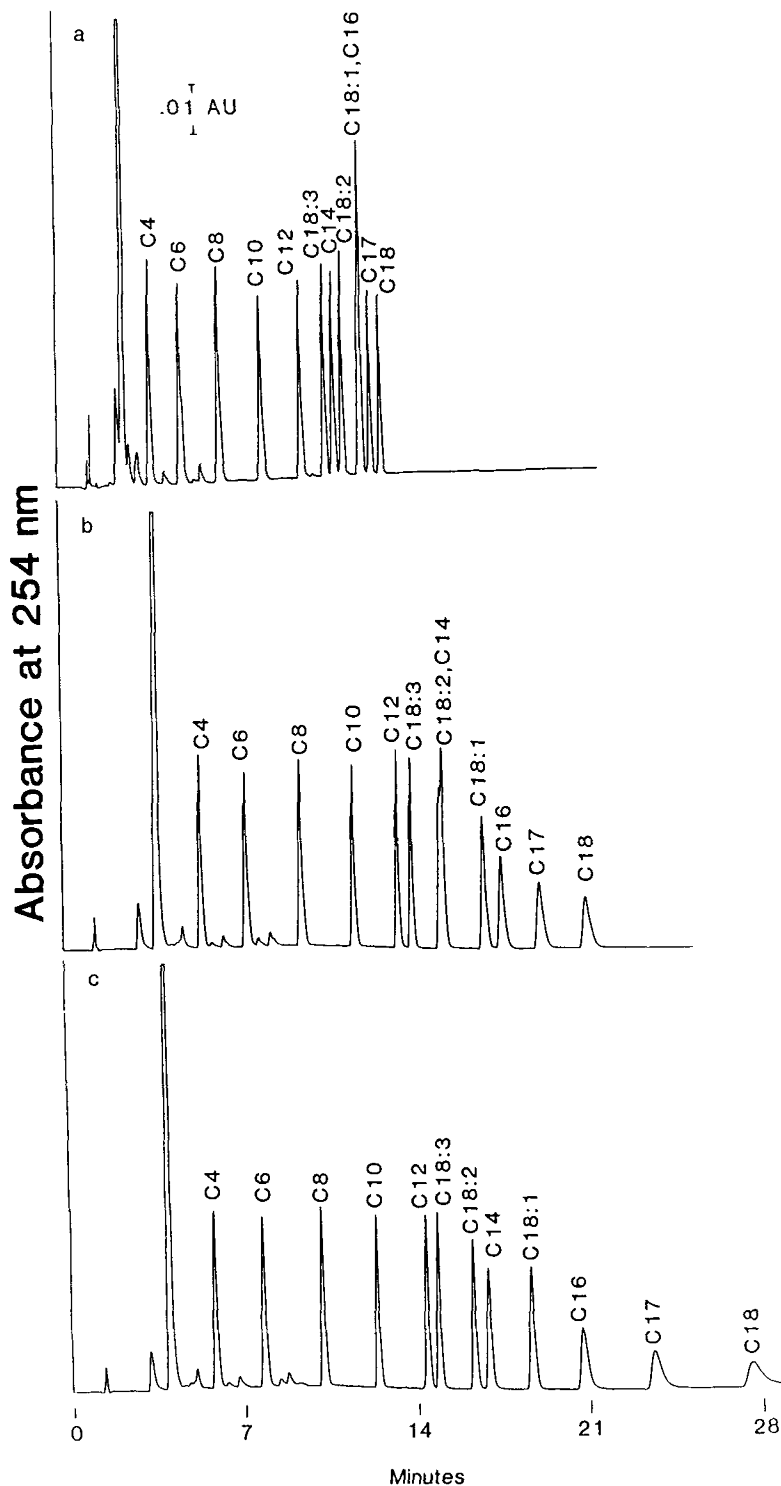

Figure 1. The HPLC separation of *p*-bromophenacyl-FFA derivatives at a) 80°C, b) 20°C, and c) 10°C.

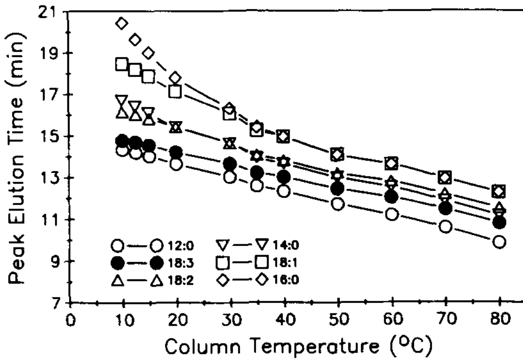

Figure 2. Retention time of *p*-bromophenacyl-FFA derivatives as a function of column temperature.

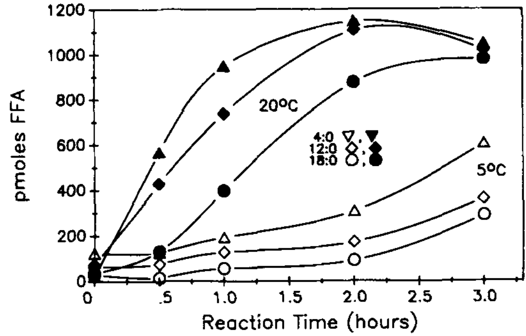

Figure 4. Time course of lipase deesterification of butyrate, laurate and stearate from milk fat (half-and-half) at 5 and 20°C. Concentrations of FFA are representative of about 100 μg of sample.

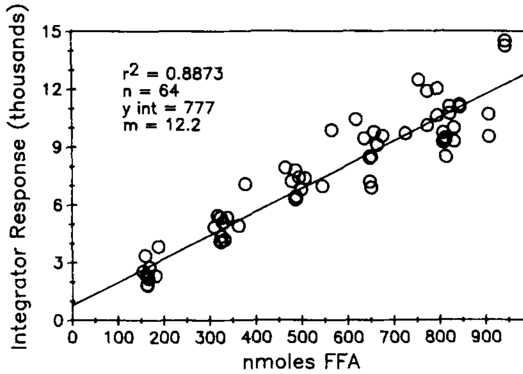

Figure 3. Regression plot of integrator response as a function of quantity of *p*-bromophenacyl-FFA injected for all fatty acid standards.

improved. The PBP-esters of the major FFA in milk fat could be completely resolved and quantified within a single chromatographic analysis rather than in two runs formerly reported necessary (6). Low temperature (10°C) chromatography allowed the complete resolution of saturated C_4 to C_{18} and unsaturated C_{18} acyl chain length FFA as their PBP ester derivatives by HPLC on a reverse-phase column.

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