Development and Characterization of an Improved Silicic Acid-KOH Arrestant Column for Routine Quantitative Isolation of Free Fatty Acids[1]

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ABSTRACT

An improved silicic acid-KOH arrestant column method for the quantitative isolation of free fatty acids (FFA) is described. The method involves preparation of samples in acidified silicic acid caps that are placed directly above alkaline arrestant column portions in glass chromatographic tubes. Neutral lipids are eluted with solvents whereas FFA are stopped as K-salts. FFA are recovered by elution with formic acid-containing solvents. The accuracy and reproducibility of existing methods were greatly improved by minimizing and stabilizing the extent of alkali-induced glyceride hydrolysis. The improved method is suitable for adaptation to a wide variety of food products and biological systems.

INTRODUCTION

During the development of a method for the routine quantification of free fatty acids (FFA) for detection of lipase-induced rancidity in butter (1), it was necessary to select a means for isolating FFA from samples which was compatible with the routine analytical objective. Isolation of FFA on strong anion exchange resins as described by Hornstein et al. (2) and Bills et al. (3) was excluded because of criticisms of McCarthy and Duthie (4) about incomplete recovery of FFA, and those of Stark et al. (5) relating to induced glyceride hydrolysis. Additionally, anion exchange resin recovery of FFA was also time-consuming, and did not allow recovery of FFA from entire butter samples. Methods involving extraction of FFA into alkaline aqueous systems have been shown to induce hydrolysis even with Na_2CO_3 (6), and manipulations of fatty acid salts are difficult to carry out quantitatively (7).

The silicic acid-KOH arrestant column first described by McCarthy and Duthie (4) appeared to provide a suitable approach for isolation of FFA although its use has been strongly criticized by Stark et al. (5) because of induced hydrolysis of glycerides. Unfortunately, glyceride hydrolysis was not investigated as a part of the initial method development (4), and the column has been widely applied for the isolation of FFA from a variety of foods and biological materials with the assumption that the extent of induced hydrolysis was negligible (8-17).

This paper describes the development and characterization of an improved silicic acid-KOH arrestant column for routine isolation of FFA from butter which overcomes the inconsistent performance of the original column described by McCarthy and Duthie (4), and minimizes induced hydrolysis of glycerides. With small alterations in the procedure to compensate for larger amounts of water, the method is suitable for quantitative recovery of FFA from a wide range of food and biological materials.

EXPERIMENTAL PROCEDURES

Separation of FFA and Lipids from Entire Samples

Primary separations of FFA and lipids from entire samples were carried out by eluting these components from acidified caps (18) which were placed directly above alkaline arrestant sections in chromatographic tubes. Individual chromatographic caps were prepared by mixing 10 g of butter, 7 g of silicic acid, 15 g of anhydrous sodium sulfate, 3 g of Celite Analytical Filter Aid, 1 ml of C_7-fatty acid internal standard (0.12% in 10% acetonitrile in ethyl ether), and 0.2 ml of 1-N sulfuric acid. After mixing, caps were added through 40 ml of 20% petroleum ether in ethyl ether to the top of alkaline arrestant portions of columns contained in glass chromatographic tubes (32 cm x 2.5 cm id). Both lipids and FFA were eluted from the caps into the arrestant portions of the columns with 100 ml of 20% petroleum ether in ethyl ether followed by 50 ml of 10% acetonitrile in ethyl ether. During this elution, FFA were moved to and retained in the alkaline arrestant portion of the columns as potassium salts, and neutral lipids were carried through columns effecting the secondary separation of FFA from sample components.

FFA were subsequently released and eluted from the alkaline arrestant columns with 50 ml of 2% formic acid in ethyl ether followed by 100 ml of 0.5% formic acid/10% petroleum ether in ethyl ether under 2 psig nitrogen pressure. After careful removal of excess solvent, individual FFA were quantified gas chromatographically using a 10% neopentyl glycol adipate on a Chromosorb W column with formic-acid-saturated carrier gas (1,19).

Alkaline Arrestant Column Variations

Alkaline arrestant columns described by McCarthy and Duthie (4) were evaluated initially. These were prepared by mixing 4 g of silicic acid, 1 g of Celite Analytical Filter Aid, 30 ml of ethyl ether and 10 ml of isopropanolic-KOH (50 mg/ml). After standing 5 min, slurries were transferred with 30 ml of ethyl ether to chromatographic tubes plugged with small amounts of phosphoric acid-washed glass wool, and then were washed with 100 ml of ethyl ether before further usage. Modified arrestant column compositions were prepared in attempts to eliminate performance variability and induced glyceride hydrolysis. These included a reduction of the amount of the original isopropanolic-KOH (50 mg/ml) to 5 ml/column, and a substitution of 10 ml of a lesser concentrated isopropanolic-KOH (25 mg/ml) in the preparation of each column.

RESULTS AND DISCUSSION

Recovery of total FFA in samples, as opposed to analyzing only that amount in the fat phase (3,5,9), was an important goal of the overall method development. Therefore, an acidic chromatographic cap system (18) was adapted for primary separation of lipids and FFA from entire samples. The placement of the cap system immediately above an alkaline arrestant column section in a chromatographic tube allowed both primary and secondary separation of FFA from samples from a single column passage. However, it was absolutely essential to incorporate adequate amounts

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of anhydrous sodium sulfate in the cap to prevent free water from entering the alkaline arrestant portion of the column where it would induce substantial glyceride hydrolysis. As a further precaution against permitting water to contact glycerides in the alkaline column medium, developing solvents were held over excess anhydrous sodium sulfate prior to use.

Mobilization of FFA from the cap material required acidification to pH 1.5 for uniform recovery which was slightly lower than that previously recommended (18,20). Otherwise, variable and incomplete recoveries were experienced. Acetonitrile additions to the second primary eluting solvent hastened mobilization of the more polar lipids and FFA from the cap materials. The original method (4) depended on gravity flow during the elution process, but this led to channeling and vapor pockets in the columns. As a result, extremely variable results were obtained because of incomplete recoveries and inconsistent extents of induced hydrolysis which were dependent on the time glycerides were in contact with the basic arrestant column portions. The use of about 2 psig nitrogen pressure during the entire elution period prevented occurrences of these problems.

Although the described procedural adjustments greatly improved repeatability of determinations, considerable variability was still observed. This was particularly notable for butyric acid which was a principal concern in the analysis. In view of the work by Stark et al. (5) which showed that substantial glyceride hydrolysis was induced by the basic conditions of the original column (4), attention was then directed toward minimizing this action. A reduction in the amount of isopropanolic-KOH solution (50 mg/ml) from 10 ml to 5 ml for arrestant columns appeared to minimize the extent of induced hydrolysis and overcome the variability associated with this action. However, when FFA were added to butter prior to analysis, this column variation yielded low recoveries for longer chain FFA, apparently from the reduced amount of isopropanol which suppressed ionization and/or partition of FFA in the arrestant column. The final version of the column (10 ml of 25 mg KOH/ml) gave very reproducible values in the analysis of butter (1), and the recovery of added FFA was reproducible and greater than 80% for each FFA.

The extent of hydrolysis occurring on the alkaline portions of columns was determined by sequential passages of lipids through new columns. Quantification of FFA in replicated passages through the new arrestant columns was achieved by additions of appropriate amounts of internal standard ($C_{7:0}$) to the eluate from the previous column. It was found that significant reductions in hydrolysis resulted from the revised column compared to the original column (Table I). It should be noted that the 2-fold reduction in hydrolysis shown for the C_{18} FFA was distorted slightly by passage of some C_{18} FFA through the first column (1).

The data reported by Stark et al. (5) concerning the degree of induced hydrolysis given by the original procedure provide support and insight into the interpretation of the current results. It can be seen in Table II that induced hydrolysis caused variable results, and the extent of hydrolysis of FFA analyzed (C_4-C_{12}) was of the same magnitude as that observed in this study using the original method. More accurate FFA levels in the butter oil were determined by a mild procedure involving short-path distillation which provides baseline amounts of the C_4-C_{12} FFA to be expected in good quality butter oil. The levels of FFA noted for the second passage of milk fat through the final modified column in this study (Table I) represent those from induced hydrolysis caused by the basic column. This point is especially clear for the C_4 and C_6 FFA which do not readily partition into the butter oil phase.

TABLE I

Comparison of the Extent of Milk Fat Hydrolysis during Sample Passage through Isopropanolic-KOH Arrestant Columns

Fatty acid	First passage: Final revised method	Second passage: Original method	Second passage: Final revised method	Reduction in hydrolysis achieved
	μg/g fat[a]			
$C_{4:0}$	81 ± 5	29 ± 1	5 ± 1	6x
$C_{6:0}$	25 ± 1	21 ± 1	4 ± 1	5x
$C_{8:0}$	16 ± 2	8 ± 1	3 ± 1	3x
$C_{10:0}$	159 ± 1	12 ± 1	1 ± 1	12x
$C_{12:0}$	399 ± 35	17 ± 1	4 ± 2	4x
$C_{14:0}$	1118 ± 10	55 ± 3	14 ± 2	4x
$C_{16:0}$	3100 ± 213	182 ± 15	36 ± 8	5x
Total-C_{18}	4337 ± 76	198 ± 5	119 ± 38	2x

[a]Mean of duplicate determinations ± standard deviation.

TABLE II

Extent of Glyceride Hydrolysis Induced in Butter Oil by the Original Arrestant Column[a]

Fatty acid	FFA by cold-finger molecular distillation	FFA from Sequential column passages (1)	(2)	(3)	(4)
	μg/g fat				
$C_{4:0}$	0.07	30.3	43.0	24.0	19.6
$C_{6:0}$	0.75	14.6	22.4	15.6	15.8
$C_{8:0}$	4.5	10.5	10.2	5.6	6.1
$C_{10:0}$	40.1	48.5	22.3	13.2	13.6
$C_{12:0}$	89.1	93.4	31.8	18.8	19.9

[a]From Stark et al. (5) using Iyer et al. (9) method adaption.

Recovery studies had shown that the C_{18} FFA were not completely recovered in the initial column passage using the modified procedure (82%). Therefore, the data for these acids in Table I from the second column passage includes contributions from both recovery inefficiency and hydrolysis. In order to determine suitable correction factors to compensate for method-induced hydrolysis, FFA in a butter sample were determined after each of 3 consecutive passages through modified arrestant columns (Table III). The data for the second and third passages show the same magnitude of FFA hydrolysis as indicated in Table III, except that the third passage gave the expected lower figure for C_{18} FFA. Therefore, hydrolysis correction factors based on the third passage of milk fat (Table III) have been adopted for routine calculations.

The standard deviations for each set of fatty acid column hydrolysis data along with standard deviations observed in the sample analyses roughly define the lower limits of detection of individual fatty acids by the method. Generally, it can be concluded that the lower accurate estimation limit for the C_4-C_{12} acids is ca. 2 ppm on a fat basis. Longer chain acids (C_{14}-C_{18}) can be quantified down to ca. 30 ppm on a fat basis because the column trapping efficiency for C_{18} increases when the concentration is greatly decreased.

The data of Stark et al. (5) show that substantially less than 2 ppm of C_4 and C_6 FFA occurs in butter oil, and it is appropriate to conclude that the currently described

TABLE III

Extent of Milk Fat Hydrolysis Occurring during Isolation of FFA with Final Revised Arrestant Columns

Fatty acid	FFA from Sequential column passages (1)	(2)	(3)
	μg/g fat[a]		
$C_{4:0}$	15 ± 2	6 ± 1	9 ± 2
$C_{6:0}$	9 ± 1	7 ± 1	9 ± 1
$C_{8:0}$	16 ± 1	3 ± 1	3 ± 1
$C_{10:0}$	81 ± 5	8 ± 1	6 ± 2
$C_{12:0}$	158 ± 11	12 ± 2	10 ± 2
$C_{14:0}$	387 ± 2	24 ± 1	22 ± 1
$C_{16:0}$	1207 ± 76	45 ± 3	42 ± 3
Total-C_{18}	1993 ± 97	51 ± 4	31 ± 4

[a]Mean ± standard deviation.

TABLE IV

Influence of Residence Time in Final Revised Arrestant Column upon Induced Hydrolysis of Milk Fat from a Butter Sample

Fatty acid	Time in contact with alkaline section: Transient flow	Held 30 min	Held 60 min
	μg/g fat[a]		
$C_{4:0}$	3 ± 1	10 ± 1	21 ± 6
$C_{6:0}$	4 ± 1	8 ± 1	16 ± 3
$C_{8:0}$	15 ± 4	21 ± 4	19 ± 4
$C_{10:0}$	81 ± 8	88 ± 14	103 ± 4
$C_{12:0}$	118 ± 23	128 ± 28	120 ± 21
$C_{14:0}$	229 ± 38	250 ± 44	239 ± 40
$C_{16:0}$	871 ± 90	935 ± 116	926 ± 143
Total-C_{18}	1655 ± 188	1575 ± 26	1545 ± 161

[a]Mean ± standard deviation.

method would not be suitable for measuring short chain FFA in butter oil. However, Stark et al. (5) concluded that short chain FFA did not contribute to the flavor of high-quality butter oil because they were present below threshold levels (21). On the other hand, it has been established that biphasic intact butter contains substantially higher amounts of the short chain FFA which can be expected to contribute to butter flavor (5,6,21,22), and the method is quite suitable for this analysis.

Experiences with the original arrestant column had revealed that glyceride contact time with the alkaline portion of the column influenced the extent of induced hydrolysis observed. Since routine laboratory activities could develop patterns where samples were not immediately passed through columns, inadvertent errors could result. The data in Table IV indicate the extent of increased glyceride hydrolysis induced after washing lipids from cap materials into alkaline portions of columns with 50 ml of solvent, and then holding for the indicated times. The short chain FFA are particularly influenced by the increased contact times, and these data support the suggestion of Stark et al. (5) that certain glycerides appear more prone to hydrolysis than others. In the current alkaline arrestant column, glycerides containing the short chain acids may more readily associate with the alkaline medium than those containing long chain acids because of higher polarities and lesser steric hindrances. In any event, it is necessary to minimize contact time in the alkaline arrestant column to limit hydrolysis and stabilize the data for expected hydrolysis.

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