Recovery of Short, Medium, and Long Chain Fatty Acid Methyl Esters Using Wet Halogenated Hydrocarbons

JOSEPH MacGEE and **KENNETH G. ALLEN**, Basic Science Laboratory,
Veterans Administration Hospital, Cincinnati, OH 45220, and the Departments
of Biological Chemistry and Medicine, College of Medicine,
University of Cincinnati, Cincinnati, OH

ABSTRACT AND SUMMARY

Fatty acid methyl esters are prepared and analyzed by gas liquid chromatography using a simple procedure that does not involve evaporation steps or drying of the final extract. The esters of fatty acids down to caproate (C_6) are recovered quantitatively. The long chain fatty acid esters, including the polyunsaturated esters, are also recovered quantitatively and are at least as stable as esters isolated by conventional procedures. Preparation of the esters in methanolic reagents is followed by partition of the mixture between a small volume of ethylene chloride and a large volume of water. The lower halogenated hydrocarbon phase contains the methyl esters ready for analysis, and the aqueous phase contains the methanol, catalysts, and other water-soluble materials.

INTRODUCTION

In gas liquid chromatogrphic (GLC) analysis of fatty acids, the methyl esters are usually prepared by acid or alkali-catalyzed methanolysis (1,2) of the naturally-occurring esters, and the free acids are esterified with acidic methanol (1). The resultant esters are then extracted into a large volume of a nonpolar solvent, and the extract is then dried with a drying agent and reduced in volume by evaporation with a stream of inert gas. During evaporation, there is invariably a loss of the short chain esters. The degree of loss is inversely proportional to a function of the chain length and directly proportional to a function of the inert gas flow, the temperature, and the length of time of the evaporation. In addition, when very small amounts of esters are processed, losses caused by surface reactions or adsorption to the glassware become significant. Methyl esters of fatty acids can also be prepared with excellent recoveries of the short chain esters by reaction of the silver salts of the acids with methyl iodide (3,4). However, these procedures are considerably more complex than the one described in this report.

We have examined an alternate means of isolating the methyl esters, which is more rapid and convenient than other procedures. This method yields the concentrated esters in a stable medium, results in better recovery of the more volatile products, and allows for further concentration of the solution when necessary. The means we have examined involve addition of a very small volume of a halogenated hydrocarbon to the methanolic reaction mixture and washing out the methanol and catalysts with water. Injection of an aliquot of the extract into the GLC unit yields excellent elution patterns. Good recoveries of the shorter chain esters (acetate and butyrate) and quantitative recoveries of the longer chain esters are achieved. Adherence to predetermined proportions of solvent, methanolic reagents, and water allows for prediction of recoveries of the shorter chain esters. Thus, it is possible to apply correction factors for losses of those esters. The slight solubility of some of the halogenated hydrocarbons in water allows for concentration of the esters by washing out the solvent with water.

EXPERIMENTAL PROCEDURES

Reagents

The 0.4 *N* sodium methoxide in methanol used in these studies was prepared from metallic sodium and anhydrous methyl alcohol. The 14% boron trifluoride in methanol was purchased from Applied Science Laboratories, State College, PA. It was not necessary to redistill any of the solvents used.

Instrumentation

The GLC analyses were performed on an F-11 Mk II gas chromatograph (Perkin-Elmer Corporation, Norwalk, CT) with 12-ft x 1/4-in. (3 mm ID) glass columns. One column was packed with 10% pretested Silar 10C on 100 to 120 mesh Gas Chrom Q (Applied Science), and the other column contained 10% pretested EGSS-X on 100 to 120 mesh Gas Chrom P (Applied Science). The injection ports, which contained removable glass inserts, were held at 240 C; the column temperatures are given with the specific examples; the carrier gas was nitrogen at 38 ml per min; and flame ionization detectors were used. Peak areas were measured with an electronic digital integrator (Autolab System I, Spectra-Physics, Santa Clara, CA).

Experimental Samples

A mixture of fatty acid methyl esters was prepared in ethylene chloride (1,2-dichloroethane) by adding the individual esters for acetate to laurate and Fat and Oil Reference Mix No. 6 (Applied Science) for myristate to linolenate to yield the concentrations listed in Table I. GLC analysis of the prepared mixture on the Silar 10C column was performed with a temperature program from 50 C to 175 C at 3 C per min, and the final temperature was held until baseline was reestablished after elution of methyl linolenate ($C_{18:3}$).

An aliquot (0.3 ml) of the prepared mixture was analyzed after evaporation just to dryness with a gentle stream of dried and filtered nitrogen at 50 C and redissolving in 0.3 ml of ethylene chloride. After removal of a 5-μl aliquot for analysis, the evaporation, redissolving, and analysis were again performed.

Another 0.3-ml aliquot of the prepared mixture was mixed with 0.3 ml of methanol and shaken vigorously by hand for 1 min with 3 ml of water. After centrifugation for 1 min, a 5-μl aliquot was subjected to GLC analysis as above. The aqueous phase was removed, and the solvent extract was washed with four more 3-ml portions of water with centrifugation and GLC analysis after each wash. Recoveries were calculated relative to the methyl oleate peak area and by using the area of each ester peak in the original mixture as 100% recovery. The degree of concentration resulting from the water washout of the solvent was based on the increase of the methyl oleate peak area when compared with the original sample.

The above experiments were repeated using Freon 113 (1,1,2-trichloro-1,2,2-trifluoroethane) in place of the ethylene chloride.

Two studies on the precision of the recoveries of the

TABLE I

Recovery of Fatty Acid Methyl Esters, Percent[a]

Fatty acid C number	concentration mg/ml	Evaporations		Water washes (ethylene chloride)					Water washes (Freon 113)				
		1	2	1	2	3	4	5	1	2	3	4	5
2	1.0	0	0	35	14	6	3	2	4	0	0	0	0
4	1.0	3	0	89	80	67	55	42	71	46	31	20	12
6	1.0	38	15	99	100	100	99	97	99	93	91	87	84
8	1.0	61	34	100	99	100	100	100	103	100	100	99	99
10	1.0	71	55	100	100	100	100	100	104	100	101	100	100
12	1.0	76	69	99	99	100	100	100	104	100	101	100	100
14	0.2	96	93	101	101	100	100	101	103	100	102	100	100
16	3.0	99	99	99	99	100	100	99	100	100	98	99	99
16:1	0.3	98	98	98	99	99	99	99	102	100	101	101	101
18	1.4	101	101	101	101	101	101	101	100	101	102	101	101
18:1	4.1	(100)	(100)	(100)	(100)	(100)	(100)	(100)	(100)	(100)	(100)	(100)	(100)
18:2	0.7	102	102	101	101	102	102	101	101	101	101	101	102
18:3	0.3	103	104	102	103	101	103	102	101	101	102	102	103
Concentration factor				1.1	1.3	1.4	1.6	1.7	1.0	1.0	1.0	1.0	1.0

[a]Recoveries calculated relative to recovery of methyl oleate (18:1).

TABLE II

Precision of Recoveries of Short Chain Fatty Acid Methyl Esters

Fatty acid C number	Percent recovery, $\bar{x} \pm s$				
	1st Wash n = 11	2nd Wash n = 5	3rd Wash n = 5	4th Wash n = 5	5th Wash n = 5
2	34.7 ± 2.0	14.2 ± 0.4	5.9 ± 0.3	2.9 ± 0.3	1.8 ± 0.3
4	89.3 ± 2.1	80.0 ± 1.9	66.5 ± 1.7	54.9 ± 1.7	42.1 ± 1.9
6	9.0 ± 3.0	100.4 ± 2.2	99.7 ± 2.6	98.5 ± 2.4	96.9 ± 2.3
8	100.5 ± 1.8	---	---	---	---
10	100.4 ± 1.1	---	---	---	---

short and medium chain fatty acid esters were performed. In one study, a mixture of the esters from acetate (C_2) to laurate (C_{12}) in ethylene chloride was analyzed before and after a single partition with added methanol and water. The partitions and analyses were performed eleven times, as was the analysis of the original solution. Chromatography was performed on the Silar 10C column with temperature programming, and the peak areas were related to methyl laurate (C_{12}). In the second study, the esters from acetate (C_2) to caprylate (C_8) were analyzed isothermally at 80 C before partition and after the second through the fifth washes with water. In this second study, the partitions and analyses were performed five times each and the peak areas were compared with the methyl octanoate (C_8) peak area. The results of these two studies are combined in Table II.

Butter fatty acid methyl esters were prepared by a procedure involving evaporation and by our new procedure not involving evaporation. Forty mg of butter oil, obtained by centrifuging melted butter, was incubated at 50 C for 1 hr with 0.5 ml of 0.4 *N* sodium methoxide in methanol (2) and then for an additional 20 min after adding 0.5 ml of 14% boron trifluoride in methanol (1). The incubations were carried out in a 12-ml screw-capped centrifuge tube with a Teflon-lined cap, and the tube was chilled in an ice bath before each opening of the tube. One-half of the reaction mixture was mixed with 0.5 ml of saturated aqueous sodium chloride, and the esters were extracted into 5 ml of hexane. After drying the hexane over anhydrous sodium sulfate, the hexane phase was evaporated just to dryness at 50 C with a gentle stream of filtered and dried nitrogen. The esters were redissolved in 0.5 ml of ethylene chloride, and 5 μl of the solution was analyzed with the same column and temperature program as used with the prepared methyl ester mixture.

The second half of the butter ester reaction mixture was mixed briefly with 0.5 ml of ethylene chloride and then shaken vigorously by hand for 1 min with 5 ml of water. After centrifugation for 1 min, a 5-μl aliquot of the lower ethylene chloride phase was analyzed in the same fashion as the evaporated sample.

Fatty acid methyl esters were prepared from pooled human blood serum as follows. The lipids obtained by the procedure of Folch et al. (5) from 2 ml of serum were methanolyzed with 2 ml of 0.4 *M* sodium methoxide in methanol for 30 min at 65 C (2). Any free fatty acids in the specimen were next methylated by heating for 10 min at 65 C after the addition of 2 ml of 14% boron trifluoride in methanol (1).

Four ml of saturated aqueous sodium chloride was added to half of the methanolic solution of the serum methyl esters, and the esters were extracted into 5 ml of hexane. The hexane phase was dried over anhydrous sodium sulfate and evaporated just to dryness with a gentle stream of filtered and dried nitrogen at 50 C. The esters were dissolved in 0.3 ml of ethylene chloride and a 5-μl aliquot was immediately analyzed on the EGSS-X column isothermally at 175 C. After removal of this and subsequent samples for analysis, the tube was flushed briefly with a gentle stream of filtered and dried nitrogen, stoppered, and stored at room temperature. Analyses were repeated after 1, 4, 7, and 14 days.

The second half of the methanolic reaction mixture of the serum methyl esters was mixed with 0.3 ml of ethylene chloride and 3 ml of water. After shaking vigorously by hand for 1 min, the sample was centrifuged and a 5-μl aliquot of the lower phase was subjected to GLC analysis on the EGSS-X column at 175 C. This sample was not flushed with nitrogen, and it too was stored at room temperature. The lower phase was reanalyzed on days 1, 4, 7, and 14.

RESULTS AND DISCUSSION

Halogenated hydrocarbons were chosen for this study in

order to obtain the esters in very small volumes of the lower phase in conical centrifuge tubes. This allows the analyst to withdraw a large portion of the extract into an injection syringe, primed with the same solvent, and to subject it to GLC analysis. In this way, good analyses are readily obtained with microscale lipid specimens when necessary. An additional advantage is realized in that large suppiles of solvents are not necessary. The economic and safety considerations of this fact can be significant in those laboratories where large numbers of analyses are performed.

Table I shows the recoveries of the fatty acid methyl esters of the prepared mixture after one and two evaporations and also after each washing of the halogenated hydrocarbon solution with five separate changes of the aqueous phase. After a single evaporation, all of the methyl acetate (C_2) was lost. Significant losses of the lower methyl esters up to myristate (C_{14}) were noted after one evaporation and these losses were even greater after two evaporations. It can be seen that the losses are inversely proportional to a function of the chain length or the boiling point. Recoveries of the longer chain esters, palmitate (C_{16}) to linolenate ($C_{18:3}$) were quantitative, within experimental error.

Losses of the esters in the water-washed solutions were significantly lower. No loss of methyl caprylate (C_8) or any of the longer esters was seen from either of the halogenated hydrocarbons after up to five water washes. Indeed, for a short chain fatty acid-containing specimen, such as butter, after a single water wash, a small correction for loss need only be applied to butyrate (C_4).

Ethylene chloride has a significant solubility in water as can be seen from the concentration factors given for the water-washed ethylene chloride solution of the esters. The use of five aliquots of water, each equal to ten volumes of the solvent phase, results in nearly a doubling of concentration of the fatty acid esters with no loss of caprylate (C_8) or higher fatty acid methyl esters. Since a very large number of fatty acid GLC analyses never report data below myristate, this suggests that water washout of the halogenated hydrocarbons can offer a convenient means for concentrating specimens for GLC analysis without corrections down to caprylate (C_8).

Washing of the Freon 113 solution with water did not concentrate the esters due to Freon 113's extreme insolubility in water.

Application of the procedure described in this report to the analysis of the fatty acid content of a sample of butter yielded the elution pattern shown in Figure 1, and the data from this chromatogram are compared in Table III with the data obtained when the same methyl ester preparation was subjected to an evaporation step. The differences in the normalized peak areas are due to the loss of the short chain fatty acids in the evaporation step.

The stability of fatty acid methyl esters, including those of the biomedically-important polyunsaturated fatty acids is demonstrated in Table IV. In this table are compared the analyses of the same mixture of esters obtained from pooled human blood serum when those esters were treated in a conventional manner (the control), which included drying the solvent extract before evaporation and storage under dry nitrogen, with the specimen treated by the simplified procedure described in this report (the experimental). These data show that the esters in the ethylene chloride extract stored under water are at least as stable as the dry sample stored under nitrogen. There is a small, but statistically significant ($p < 0.01$ by Student's *t*-test), difference between the analyses for myristate (C_{14}) in the two samples. This is in agreement with the data in Table I where it was shown that evaporation results in a small loss of this ester while the water wash does not result in a loss. The other small differences, e.g., between the values for palmitoleate ($C_{16:1}$) and the values for linolenate ($C_{18:3}$) are not statistically significant by the same criterion.

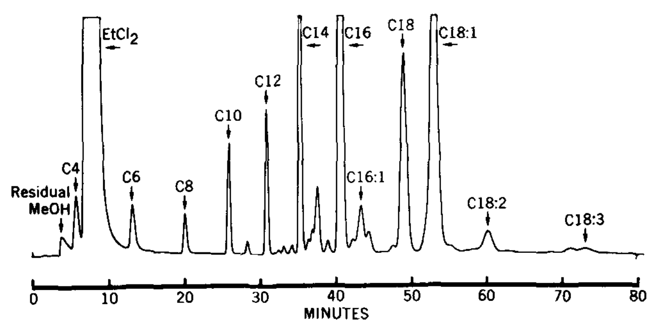

FIG. 1. Fatty acid methyl esters prepared from butter. Five µl of the esters in wet ethylene chloride were chromatographed on the Silar-10C column. Temperature program; 50 C to 175 C at 3 C per min; 175 C to the end of the analysis. Only the major peaks are labeled – see Table III. The residual methanol is from the methylation reagents.

TABLE III

Fatty Acid Methyl Esters Prepared From Butter

Fatty acid C number	Normalized peak areas: Evaporated	Normalized peak areas: Wet extract
4	0.0	2.1
6	0.0	1.8
8	0.2	1.2
10	1.5	2.7
10:1	0.2	0.3
12	3.0	3.3
13(i- + a-)[a]	0.1	0.1
13	0.2	0.2
14(i-)	0.2	0.2
14	12.1	11.6
15(i-)	0.5	0.4
15(a-) + 14:1	0.7	0.7
15	2.4	2.4
16(i-)	0.5	0.4
16	33.9	30.5
17(i-)	0.6	0.6
16:1 + 17(a-)	2.5	2.4
17	1.0	0.9
18(i-)	0.0	0.4
18	11.8	10.7
18:1	25.3	23.9
18:2 + 19	2.3	2.3
19:1	0.2	0.2
18:3 + 20	0.5	0.4
18:3	0.4	0.3

[a](i-) = iso-; (a-) = ante iso-.

Samples of serum fatty acid methyl esters have also been subjected to experiments as those just discussed but with different halogenated hydrocarbons. Similar results were obtained when chloroform, carbon tetrachloride, and Freon 113 were used in place of the ethylene chloride but with some limitations.

The halogenated hydrocarbon to be used will most likely be one of personal choice unless certain short chain esters are to be measured or if concentration by water washout of the solvent is desired. On the Silar 10C column, the elution of the solvent could interfere with the analysis of certain esters. Esters with elution times near methyl valerate (C_5) could be lost under the ethylene chloride peak. Freon 113 elutes before methyl formate (C_1) and would be the solvent of choice in cases where concentration by water washout of the solvent is not desired, except that the losses of the short chain esters are greater for this solvent. When butter oil fatty acid methyl esters were analyzed by a procedure like that described except for the use of Freon 113 in place of the ethylene chloride, the expected significant losses of the shorter chain esters were obvious, but there were no significant peaks under the region of the pattern usually occupied

TABLE IV

Recovery and Stability of Methyl Esters Prepared from Human Blood Serum Lipids

Fatty acid C number	Days stored 0	1	4	7	14	$\bar{x} \pm s$
	Normalized peak area					
	Control (dry)					
14:0	0.84	0.89	0.90	0.87	0.89	0.88 ± 0.02
16:0	23.49	23.45	23.60	23.71	23.44	23.54 ± 0.12
16:1	3.73	3.79	3.65	4.44	4.09	3.74 ± 0.33
18:0	7.15	7.14	7.12	7.10	7.80	7.26 ± 0.30
18:1	30.45	30.43	30.45	30.22	30.22	30.35 ± 0.12
18:2	24.30	24.35	24.37	24.09	23.82	24.19 ± 0.23
18:3	0.36	0.36	0.37	0.36	0.37	0.36 ± 0.00
18:3	0.86	0.83	0.81	0.50	0.84	0.77 ± 0.15
20:3	1.28	1.26	1.27	1.26	1.22	1.26 ± 0.02
20:4	7.57	7.53	7.49	7.44	7.32	7.47 ± 0.10
	Experimental (Wet)					
14:0	0.94	0.99	0.93	0.99	0.96	0.96 ± 0.03
16:0	23.53	23.68	23.65	23.67	23.46	23.60 ± 0.10
16:1	4.18	4.28	4.18	4.27	4.21	4.22 ± 0.05
18:0	7.05	6.83	7.06	7.08	7.10	7.02 ± 0.11
18:1	30.06	29.87	29.96	29.97	30.01	29.97 ± 0.07
18:2	24.24	24.39	24.36	24.16	24.27	24.28 ± 0.09
18:3	0.36	0.35	0.31	0.35	0.36	0.35 ± 0.02
18:3	0.90	0.89	0.89	0.87	0.89	0.89 ± 0.01
20:3	1.27	1.25	1.25	1.26	1.27	1.26 ± 0.10
20:4	7.51	7.47	7.45	7.41	7.46	7.46 ± 0.04

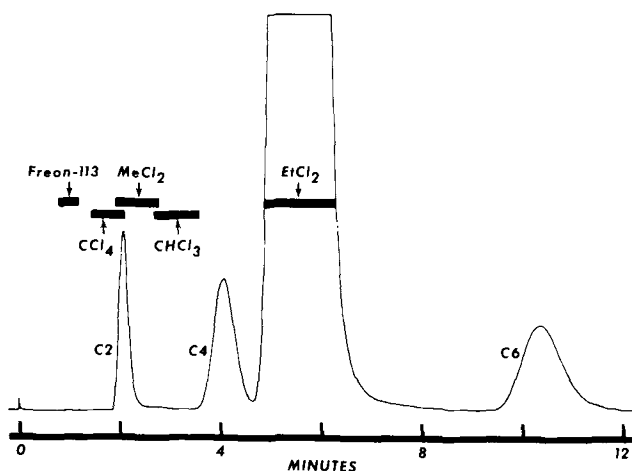

FIG. 2. Positions occupied by halogenated hydrocarbons relative to short chain fatty acid methyl esters. Five μl of a mixture containing 10 mg of each of the esters per ml of ethylene chloride was chromatographed isothermally at 80 C on the Silar-10C column. The horizontal bars indicate the positions and the widths of the solvents at 50% full scale on the chart when 5 μl of the solvents are injected at the same conditions used to obtain the chromatogram.

by ethylene chloride. Therefore, analyses of butter oil fatty acid esters using ehtylene chloride are satisfactory with the specimen of butter used since there are no fatty acid esters hidden by this solvent. Had carbon tetrachloride been used, any peak from butter after formate could have been measured. The use of dichloromethane could have resulted in a loss of any acetate or propionate under the solvent peak, and any propionate and butyrate could have been hidden if chloroform had been the solvent. The positions occupied by these solvents are demonstrated in Figure 2.

Luddy et al. (6) described simple and rapid procedures for the preparation of short, medium, and long chain fatty acid methyl esters from a variety of fats. In their procedures, they recovered the methyl esters by adding carbon disulfide to the methanolic reaction mixtures and removed all the methanol and catalysts with a mixture of calcium chloride and silica gel. The volatility, toxicity, unpleasant odor, and fire and explosion hazards of carbon disulfide are disadvantages that are greatly diminished by our use of halogenated hydrocarbons. The toxicity hazard still remains however. The final specimen in the carbon disulfide is found in the upper layer over the added calcium chloride and silica gel. This limits the proportion of the final extract that can be withdrawn for GLC analysis. Our procedure allows for minaturization to the extent that essentially all the specimen can be recovered for analysis. Since carbon disulfide, like the halogenated hydrocarbons, has a density greater than water, it can be used in our procedure, and the recoveries are comparable to those found with ethylene chloride, but we feel that the halogenated hydrocarbons are more satisfactory because of the hazards and disadvantages of carbon disulfide listed above.

The esterification procedure used in these studies, sodium methoxide-catalyzed methanolysis of the naturally-occurring esters followed by boron trifluoride-catalyzed methylation of any free acids, was chosen over either of the single reactions because the double reaction is more complete than the alkaline reaction and more rapid than the acidic reaction. We find that cholesteryl esters are methanolyzed about seven times as rapidly and glyceride esters are methanolyzed over twenty times as rapidly with sodium methoxide as with boron trifluoride. The alkaline reaction must be followed by an acidic methylation, however, because any traces of water in the system will result in hydrolytic losses of stoichiometric amounts of esters as their sodium salts. These losses can be significant when minute quantities of esters are involved. In addition, any free fatty acids in the specimen will not be esterified but will be lost as their sodium salts with the sodium methoxide reagent alone. Addition of methanolic boron trifluoride in excess of the alkaline reagent and reincubation results in rapid methylation of the free acids. In this acid-catalyzed methylation, any traces of moisture only result in insignificant losses of esters due to the equilibrium of the reaction.

In the preparation of methyl esters from butter, we used a lower incubation temperature (50 C) than we used with the serum lipids (65 C) in order to minimize losses of the

volatile esters. Because of this lower temperature, longer incubation periods were employed to assure completion of the reactions.

To determine whether the concentration of the esters influences their recoveries, three specimens over a 16-fold range of concentration were analyzed. The first specimen contained 1 mg of each of the esters from acetate (C_2) to laurate (C_{12}) per ml of ethylene chloride; the second contained 4 mg of each ester per ml; and the third contained 16 mg of each ester per ml. Each specimen was analyzed before partition and after adding one volume of methanol and ten volumes of water, i.e., one wash, as described earlier. Methyl laurate was the internal standard in each case. Only slight differences in the recoveries of acetate and butyrate, and essentially no differences in the recoveries of the longer chain esters, were noted. The recovery of acetate was 35%, 34%, and 40% for the 1-, 4-, and 16-mg per ml specimens, respectively. Butyrate was recovered 89%, 91%, and 97% for the 1-, 4-, and 16-mg per ml specimens, respectively.

To test whether temperature has an influence on the recoveries, one aliquot of the 1 mg per ml mixture of the methyl esters from C_2 to C_{12} was subjected to one water wash at room temperature (ca. 22 C) and another aliquot was washed at 0 C. Only the acetate recovery was different. At 0 C, the recovery of acetate was 25%, while at room temperature, the acetate recovery was 35%. This suggests that the relatively small variations in room temperature in different laboratories should not have a significant influence on recoveries of the esters.

The methyl alcohol from the reagents is nearly completely removed by a single water wash as can be seen in Figure 1. After the second and subsequent washes, no methyl alcohol can be detected on the GLC elution patterns.

We have described an approach for preparing fatty acid methyl esters for GLC analysis which is more rapid and convenient than the procedures that involve evaporation steps. In addition, the new technique yields better recoveries of the short chain fatty acid esters than are recovered when the ester solution is subjected to evaporation. In the past, we described procedures without evaporation which used extraction of the acids into very small volumes of quaternary ammonium hydroxide reagents. Injection of the quaternary ammonium slats of the fatty acids into the GLC unit yielded the alkylated fatty acids, but in the case of samples containing long chain fatty acids, quantitative data could not be obtained on the shorter ($<C_{12}$) fatty acids because of by-products of the flash alkylation of the reagent (7). In a procedure for acetate, in which we used propionate as an internal standard (8), the benzyl esters were generated in the instrument. In this case, the major by-products of this reaction eluted both before and after the two compounds of interest – essentially limiting the process to the measurement of these two compounds.

We feel that the procedure described in this report should be a satisfactory addition to the techniques available to investigators interested in fatty acid analysis and should be especially useful to those investigators concerned about measuring the short and medium chain fatty acids.

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REFERENCES

1. Metcalfe, L.D., A.A. Schmitz, and J.R. Pelka, Anal. Chem. 38:514 (1966).
2. Luddy, F.E., R.A. Barford, and R.W. Riemenschneider, JAOCS 37:447 (1960).
3. Gehrke, C.W., and D.F. Goerlitz, Anal. Chem. 35:76 (1963).
4. Johnson, C.B., and E. Wong, J. Chromatogr. 109:403 (1975).
5. Folch, J., M. Lees, and G.H. Sloane Stanley, J. Biol. Chem. 226:497 (1957).
6. Luddy, F.E., R.A. Barford, S.F. Herb, and P. Magidman, JAOCS 45:549 (1968).
7. MacGee, J., and K.G., Allen, J. Chromatogr. 100:35 (1974).
8. Richards, R.G., C.L. Mendenhall, and J. MacGee, J. Lipid Res. 16:395 (1975).

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