ANALYSIS OF MIXTURES OF HIGHER SATURATED NORMAL FATTY ACIDS: A COMPARISON OF REVERSED-PHASE PARTITION CHROMATOGRAPHY AND ESTER FRACTIONATION

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INTRODUCTION

Investigations in this laboratory on lipids of rumen micro-organisms and on ruminant body and milk lipids demanded that, not only should analysis of milligram quantities of fatty acid mixtures be possible, but that odd-numbered, *n*-fatty acids should be separable and identifiable. It was also desirable that such analyses should be comparable with results obtained by large-scale ester-fractionation analysis.

The reversed-phase partition chromatographic method of HOWARD AND MARTIN[1] was selected for this purpose. The quantitative separation of milligram quantities of lauric, myristic, palmitic and stearic acids described by HOWARD AND MARTIN was extended by SILK AND HAHN[2] to include the even-numbered, *n*-fatty acids C_{16}–C_{24}. Whilst the present work was in progress HOUGEN[3] extended the method further to include the odd-numbered, *n*-fatty acids C_7, C_9 and C_{11}.

In this communication we report the quantitative chromatographic separation of all the odd-numbered, *n*-fatty acids C_9–C_{19} both from each other and from the even-numbered, *n*-fatty acids C_8–C_{20}. The method was applied to the analysis of the non-volatile saturated fatty acids of bovine milk fat and the results compared with those obtained by ester-fractionation analysis of the same fatty acid mixtures.

METHODS

The apparatus used was essentially that described by HOWARD AND MARTIN. Standard columns (30 × 1.5 cm) were each prepared from a mull of 20 g Hyflo Super-Cel treated with dichlorodimethylsilane and 16 ml liquid paraffin according to SILK AND HAHN. The mull was packed with a perforated plunger in the tube of a Liebig condenser; water at 37° was circulated through the condenser jacket. Each column was used several times, though the 'ageing' effect noted by CROMBIE, COMBER AND BOATMAN[4] sometimes became apparent after as few as four passages of fatty acids through the column. Owing to the time required for complete elution of some of the fatty acid mixtures it was necessary on occasion to store the partially-eluted column overnight in an incubator at 37°. Fatty acids were applied in the manner described by SILK AND HAHN and graded elution was carried out using acetone-water mixtures of successively increasing acetone content, *viz.* 35% (v/v), 40%, 45%, 50%, 53%, 55%, 58%, 60%, 63%, 65%, 68%, 70%, and 75%; the acetone used was of analytical reagent quality. The acetone-water mixtures, which contained 0.002% (w/v) bromothymol blue, were equilibrated with liquid paraffin at 37°. The eluate was collected in 1.5 or 2.5 ml portions and each was titrated against 0.01 *N* KOH. The column hold-up was 30 ml.

Reference fatty acids were purchased commercially and purified by vacuum fractional distillation of the acids or their methyl esters. Distillation was preceded in some cases by low-temperature crystallization of the acids from 10% (w/v) solution in ether at —40°. All the acids were homo-

References p. 195.

geneous when examined chromatographically (see below) with the exception of nonanoic acid from which traces of octanoic acid could not be completely removed by the procedures outlined above.

Ester-fractionation analysis of the mixed fatty acids from the milk fats was done according to the procedure described by HILDITCH[5].

RESULTS

1. *Chromatography of authentic fatty acids*

As reported by previous investigators[2,3] of the reversed-phase partition chromatographic method, recoveries of each of the even-numbered, *n*-fatty acids C_8–C_{20} (3–8 mg) alone and as a complete mixture were 95–98%. Sharp separations and 95–98% recoveries were obtained for each of the odd-numbered, *n*-fatty acids C_9–C_{19} (3–8 mg), alone or as a complete mixture or in mixture with the range C_8–C_{20} of even-numbered, *n*-fatty acids (3–8 mg of each acid). Nonanoic acid was eluted with 45% (v/v) aqueous acetone, undecanoic acid with 50–53%, tridecanoic acid with 55–58%, pentadecanoic acid with 60–63%, heptadecanoic acid with 68–70% and nonadecanoic acid with 73–75%. The titration curve for the complete mixture of *n*-fatty acids C_8–C_{20} is shown in Fig. 1.

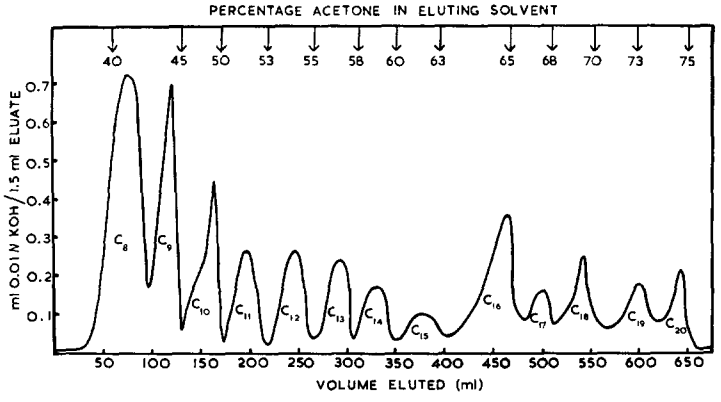

Fig. 1. Reversed-phase partition chromatographic separation of a complete mixture of *n*-fatty acids C_8–C_{20}. The curve joins titration points for every 1.5 ml eluate. Changes in eluting solvent indicated by arrows.

2. *Analysis of the non-volatile, saturated fatty acids of bovine milk fat*

Four such groups of acids were examined. These acids were prepared by GARTON AND DUNCAN[6] in the course of another investigation; steam-volatile fatty acids were removed from the mixed acids of four milk fats and the saturated, non-volatile acids prepared by crystallization at —40° from a 10% (w/v) solution in ether of the total non-volatile acids. The composition of the saturated acids was determined by ester fractionation; the low residual iodine value of each group of acids was calculated, following the usual practice, as oleic acid since this acid accounts for most of the unsaturation of milk fats.

When monoethenoid fatty acids such as oleic acid are chromatographed they are eluted along with the saturated acids having a chain length shorter by two carbon atoms[4]. Accordingly, in the results of the chromatographic analyses presented in Table I, the amounts of palmitic acid recorded are 'corrected' for the small amount of

oleic acid which was concurrently eluted. In Table I is also recorded the results of the analyses by the ester-fractionation procedure.

It is evident from Table I that the two methods of analysis gave results in general accord. The mean molecular weight of each group of acids calculated from the chromatographic or ester-fractionation data agreed quite well with that determined directly on the fatty acids themselves (Table II), though the chromatographic analyses consistently revealed more myristic and stearic acids and less palmitic and arachidic acids than did the ester-fractionation procedure.

TABLE I

ANALYSIS OF NON-VOLATILE SATURATED FATTY ACIDS FROM BOVINE MILK FAT

Acids from milk fat	*Iodine value (Wijs)*	*Method of analysis*	*Weight of acid or esters used*	*Acids present (% w/w)*				
				Myristic	*Palmitic*	*Stearic*	*Arachidic*	*Oleic**
A	3.5	Chromatography	44.8 mg	4.2	62.7**	27.6	1.8	3.8
		Ester fractionation	30.3 g (11 fractions)	2.2	63.5	25.6	4.9	
B	4.3	Chromatography	49.4 mg	11.1	59.3**	24.9	None detected	4.7
		Ester fractionation	28.5 g (11 fractions)	7.4	67.8	16.8	3.3	
C	7.3	Chromatography	17.8 mg	10.6	62.6**	18.8	None detected	8.0
		Ester fractionation	28.8 g (11 fractions)	8.5	66.1	14.4	3.0	
D	2.5	Chromatography	15.6 mg	11.6	65.4**	18.0	2.1	2.9
		Ester fractionation	29.6 g (10 fractions)	9.3	72.0	12.2	3.6	

* All unsaturation calculated as oleic acid from iodine values of ester fractions.
** After allowing for oleic acid which was eluted with palmitic acid.

TABLE II

SAPONIFICATION EQUIVALENTS (MEAN MOLECULAR WEIGHTS) OF GROUPS OF FATTY ACIDS ANALYSED BY CHROMATOGRAPHY AND BY ESTER FRACTIONATION

Acids from milk fat	*Saponification equivalent*		
	Determined on fatty acids	*Derived by calculation from ester-fractionation data*	*Derived by calculation from chromatographic data*
A	263.0	265.4	263.3
B	263.9	260.6	259.9
C	259.8	260.1	259.1
D	257.7	258.5	258.3

3. *Analysis of the saturated fatty acids from individual ester fractions*

No odd-numbered, *n*-fatty acids were detected chromatographically in any of the whole groups of ether-insoluble, non-volatile fatty acids, thus justifying the use of the mean equivalent in calculating the composition of each ester fraction and hence of the whole groups of fatty acids. However, in view of the reports of SHORLAND and co-workers[7-9] that extreme traces of *n*-undecanoic, *n*-tridecanoic and *n*-pentadedanoic acids occur in bovine milk fat it was decided to investigate chromatographically the fatty acid composition of a number of selected ester fractions.

References p. 195.

These analyses showed, as indeed was to be expected from the overall analyses, that in nearly every ester fraction examined only two saturated components were present in proportions similar to those deduced by calculation from the mean equivalents and that no odd-numbered *n*-fatty acid, if present, was detectable. The noteworthy exception was the first ester fraction to be distilled from each group of acids which, in addition to containing myristic and palmitic acids (as deduced from the saponification equivalent), also contained some lauric acid and traces of tridecanoic acid, neither of which was of course detected in the chromatographic analysis of the whole mixed acids (see Table I). This was shown to best advantage in the first distilled ester fraction of the acids of milk fat C; the titration curve of the acids recovered from the methyl esters (Sap. equiv. 245.8, Iodine value 1.1) is shown in Fig. 2. It should be emphasized that the amount of lauric acid detected accounts for only 0.34% (w/w) of the total non-volatile, saturated acids of the milk fat, whilst the amount of tridecanoic acid accounts for only 0.1% (0.05% of the total fatty acids of the whole fat) and that the finding of these 'trace' constituents does not invalidate ester-fractionation analysis as a general analytical procedure.

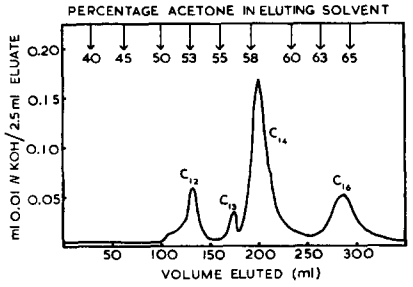

Fig. 2. Reversed-phase partition chromatographic separation of the fatty acids (9.2 mg) from the first distilled ester fraction of the fatty acids of milk fat C. The curve joins titration points for every 2.5 ml eluate. Changes in eluting solvent indicated by arrows.

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SUMMARY

The reversed-phase partition chromatographic method of HOWARD AND MARTIN for higher fatty acid analysis has been extended to include the odd-numbered, *n*-fatty acids C_9–C_{19}. The method was applied to the analysis of the saturated, non-volatile fatty acids from four samples of bovine milk fat and the results compared with the analyses obtained by the ester-fractionation procedure; good agreement between the two methods was found. No odd-numbered *n*-fatty acids were detected chromatographically in the whole groups of mixed saturated fatty acids, though traces of tridecanoic acid were detected when the fatty acids from individual ester fractions were analysed chromatographically.

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