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COLUMN CHROMATOGRAPHY OF THE BROMO-MERCURI-METHOXY ADDUCTS OF FATTY ACID METHYL ESTERS AS A MEANS OF ISOLATING POLYENOIC ACIDS PRESENT IN LOW CONCENTRATIONS

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SUMMARY

Due to the extreme complexity of oils such as animal tallows, determination of fatty acid composition by direct gas chromatographic examination of their unfractionated methyl esters is of limited utility. A more detailed analysis of animal fats has been achieved using a silicic acid column chromatographic method to resolve into classes of differing degree of unsaturation, the bromo-mercuri-methoxy adducts of the methyl esters. Methyl esters were recovered from the separated adduct fractions by treatment with acid and analysed by gas chromatography.

This technique should be of general use in those cases where it is necessary to isolate small amounts of dienoic and polyenoic fatty acids from fats, as the required components are protected from autoxidative degradation during the isolation procedure.

INTRODUCTION

As part of an extensive study of animal tallows, a method was required for the determination of fatty acid composition, particular attention being directed towards the amount and nature of the small content of dienoic and more highly unsaturated acids.

Undoubtedly the most commonly used method for determination of the fatty acid composition of fats is gas chromatographic analysis of the methyl esters prepared therefrom. The technique is simple and quick and, in most instances, gives accurate results and excellent resolution of components of the mixture. However, direct analysis is inadequate in certain specialised cases, *e.g.*, determination of trace constituents and analysis of complex fats such as fish oils, animal tallows and hydrogenated oils. In such cases, it is usual first to effect a preliminary fractionation with the aim of enhancing the concentration of the trace components of interest, or

of producing several fractions of composition simpler than that of the starting material, which can then be analysed by gas chromatography. Ideally, the fractions submitted to gas chromatographic analysis should consist either of one unsaturation class (*i.e.*, saturated, monoenes or dienes etc.) or of one chain length.

Traditional methods for separating saturated from unsaturated acids, such as lead soap crystallisation[1] or low-temperature crystallisation of the acids or methyl esters from solvents[2], can no longer be considered as satisfactory techniques as they do not give clean-cut separations, nor are they easily applicable to small amounts of starting material.

IVERSON *et al.*[3,4] used urea clathration to fractionate olive and marine oils and to demonstrate the presence of trace concentrations of many acids not readily detectable in the gas chromatogram of the original oil. In a later paper, IVERSON AND WEIK[5] demonstrated that the ease with which the various methyl esters can form urea adducts depends upon the chain length, the degree of unsaturation and upon the degree of branching. Such methods, then, cannot be expected to effect a clean-cut class separation.

Two methods that do offer a specific separation on the basis of degree of unsaturation are chromatography on silver nitrate impregnated supports[6] and chromatography of the acetoxy–mercuri–methoxy adducts by paper[7], column[8–12] or thin-layer techniques[13].

For various reasons, these published methods were found to be unsuited to the task of quantitatively isolating the small concentration (2–4%) of polyunsaturated acids present in animal tallow.

Silver nitrate chromatography was abandoned because of autoxidation problems. As the polyunsaturated esters are last to be eluted from the column, as they are only present in small concentrations and, as they are fractionated as the underivatized ester with the double bond unprotected, it was found impracticable to prevent extensive autoxidative deterioration.

Chromatography of the mercury adducts offers the advantage that derivatization temporarily saturates the double bond and so inhibits autoxidation during fractionation. Due to the small content of polyunsaturated acids present in animal tallows, a column chromatographic rather than a paper or a thin-layer technique was indicated as a means of preparing manageable amounts of these minor components. A column technique also permits easy quantitation of the eluted classes. However, the techniques published to date require the use of an acidic solvent for the elution of the highly polar acetoxy adducts of the polyunsaturated esters and thus lead to recovery of the original unsaturated ester rather than the protected derivative.

WHITE[14] and WHITE AND POWELL[15] have shown that conversion of the acetoxy to the bromo derivative leads to an adduct of lower polarity, which is more amenable to separation by thin-layer chromatography. In the present paper it has been demonstrated that the reduced polarity of the bromo derivative can be used to advantage to allow elution of the intact derivative from a silicic acid column and so retain protection from autoxidation pending further analysis.

EXPERIMENTAL

Reagents

Methanol and dioxane were Merck "guaranteed reagents" and were used as received. Diethyl ether, chloroform and petroleum ether b.p. 40–70° were commercial solvents distilled to ensure removal of non-volatile residues. Silicic acid, from Mallinckrodt, SilicAR CC7, 200–235 mesh, was dried overnight at 110°, deactivated by addition of 20% (w/w) water and equilibrated by standing overnight.

Safflower oil was a commercial sample obtained from Unilever Australia Pty. Ltd. (iodine value, 145.0). Mutton tallow was purchased from James Barnes Pty. Ltd. (iodine value, 45.9). Beef perirenal and pericardial tissues were butcher's samples and the lipid was extracted with chloroform–methanol (3:1) (iodine value, 39.3 and 35.2, respectively).

Procedures

The acetoxy–mercuri–methoxy adducts are prepared by boiling, under reflux, methyl esters (weight = 90/iodine value), mercuric acetate (2.25 g) and methanol (15 ml). After cooling, the mixture is diluted with water (160 ml) and extracted five times with ether (35 ml) and twice with chloroform (30 ml). The ether and chloroform extracts are separately pooled. The combined ether extracts are washed three times with water (50 ml) and each water wash is separately re-extracted with

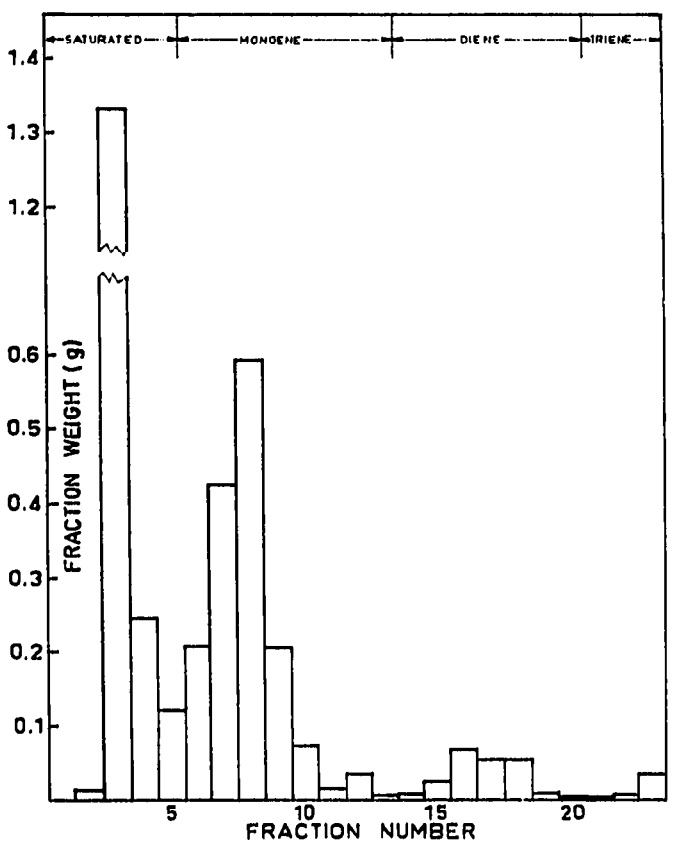

Fig. 1. Histogram showing silica gel chromatographic separation of the bromo–mercuri–methoxy adducts of beef pericardial lipids.

the chloroform extract. The combined ether and chloroform extracts are evaporated dry at low temperature using a rotary evaporator, taken up in chloroform (20 ml) and converted to the bromo derivative by mixing with 5% methanolic solution of sodium bromide (30 ml). The solution of bromo derivatives is diluted with water (60 ml), the chloroform layer removed and the aqueous layer re-extracted with chloroform (20 ml). The combined extracts are washed four times with water (50 ml) and each water wash is re-extracted with chloroform (10 ml). After removal of solvent at low temperature the bromo derivatives are dissolved (if the sample is relatively rich in saturated esters) or dispersed (if highly unsaturated esters predominate) in 1% dioxane in petroleum ether for application to the silicic acid column prepared from 30 g deactivated adsorbent and packed into a 2-cm-diameter column.

The components of the mixture are eluted with petroleum ether containing 1% (saturated), 5% (monoenes), 10% (dienes) and 20% dioxane (trienes) and collected in 25-ml fractions. Appropriate fractions are pooled (Fig. 1) and the esters recovered by boiling under reflux for 5 min with methanol (90 ml) and hydrochloric acid (10 ml), then diluting with water and extracting into petroleum ether. To ensure that all the extract is in the ester form, ethereal diazomethane is added to the ester solution until a permanent yellow colour remains.

RESULTS AND DISCUSSION

Fig. 1 illustrates a typical separation obtained when adducts prepared from beef pericardial lipid were submitted to chromatographic fractionation. In Tables I–IV, results are shown of analyses carried out on samples of safflower oil, mutton tallow and the lipids obtained from beef pericardial and perirenal tissue.

It would not normally be of interest to submit safflower oil or any other oil

TABLE I

ANALYSIS OF SAFFLOWER OIL

	Saturated (Fraction No. 8)	*Monoene (Fraction Nos. 9–13)*	*Diene (Fraction Nos. 14–23)*	*Safflower oil*	
				Calc.[a]	*Detn.*[b]
12:0	0.4	1.1	—	0.2	0.3
14:0	1.8	0.9	tr	0.3	0.2
15:0	0.2	tr	—	tr	—
16:0	62.3	14.2	0.2	6.0	6.5
16:1	—	0.8	—	0.1	—
17:0	0.3	—	0.1	0.1	—
18:0	28.8	3.9	0.1	2.5	2.3
18:1	0.4	74.0	2.0	11.0	12.6
18:2	tr	4.0	97.7	79.4	78.1
20:0	3.4	—	—	0.2	—
20:1	—	1.0	—	0.1	—
22:0	2.2	—	—	0.2	—
% recovered	6.7	13.0	82.4	102.1	—

[a] Algebraic summation of the compositions of the separated fractions, normalised to 100%.
[b] Determined by direct GC analysis of the unfractionated methyl esters.

TABLE II

ANALYSIS OF MUTTON TALLOW

	Relative retention (18:0 = 1)	*Saturated (Fraction Nos. 1–4)*	*Monoene (Fraction Nos. 5–13)*	*Diene (Fraction Nos. 14–21)*	*Triene (Fraction Nos. 22 and 23)*	*Tallow*	
						Calc.[a]	*Detn.*[b]
10:0	0.061	tr	0.1	—	—	0.1	0.1
12:0	0.121	0.1	tr	—	—	0.1	0.1
13:0	0.163	tr	—	—	—	tr	tr
iso-14:0	0.206	0.1	—	—	—	0.1	0.1
14:0	0.242	4.2	1.0	0.1	0.4	2.5	2.6
iso-15:0	0.294	0.5	—	—	—	0.3	
14:1	0.307	—	0.3	—	—	0.1	0.8
anteiso-15:0	0.312	0.5	—	—	—	0.3	
15:0	0.344	1.2	0.3	0.1	0.3	0.7	0.7
15:1	0.392	—	0.2	—	—	0.1	
?	0.414	—	—	0.1	—	tr	0.3
iso-16:0	0.418	0.4	—	—	0.1	0.2	
16:0	0.488	37.8	5.4	0.8	3.1	21.2	20.4
?	0.559	0.3	—	—	—	0.1	—
16:1	0.584	—	4.3	0.8	0.9	2.0	2.5
iso-17:0	0.592	1.0	—	—		0.5	
anteiso-17:0	0.628	1.5	—	—	—	0.7	0.7
17:0	0.694	3.4	—	—	0.4	1.7	
16:2	0.70–0.81[c]	—	—	2.0	0.4	0.1	2.2
iso-17:1?	0.703	—	0.4	—	—	0.2	
17:1	0.817	—	1.4	—	—	0.7	1.0
iso-18:0	0.848	0.3	—	—	—	0.2	
18:0	1.000	47.4	4.4	1.0	3.6	25.5	24.6
18:1	1.14	0.3	80.3	9.3	3.6	37.3	37.7
iso-19:0	1.24	0.1	—	—	—	0.1	
iso-19:1?	1.35	—	1.7	—	—	0.8	
18:2	1.24–1.71[d]	—	—	68.2	5.8	2.7	4.3
19:0	1.42	0.6	—	—	—	0.3	
19:1	1.60	—	0.2	—	—	0.1	—
iso-20:0	1.80	tr	—	—	—	tr	—
18:3	1.95	—	—	—	74.0	0.3	
20:0	2.03	0.3	—	—	—	0.2	
19:2?	2.07	—	—	6.7	—	0.3	1.5
?	2.27	—	—	1.5	—	0.1	
?	2.52	—	—	7.5	—	0.3	0.4
?	2.69	—	—	0.6	4.2	tr	—
20:2?	2.89	—	—	0.9		tr	—
?	3.29	—	—	0.4	2.3	tr	—
?	3.73	—	—	—	0.9	tr	—
% recovered		49.4	45.9	3.9	0.4	99.6	—

[a] Algebraic summation of the compositions of the separated fractions normalised to 100%.
[b] Determined by direct GC analysis of the unfractionated methyl esters.
[c] Unresolved group of peaks containing at least three components.
[d] Unresolved group of peaks containing at least five components. Probably isomeric C_{18} dienes.

of simple composition to mercury adduct fractionation. Safflower oil was selected as a simple model mixture to demonstrate the potential of the technique. However, it is of interest to note that a number of acids present in trace concentrations, and not visible in the chromatogram of the original oil, are readily detected in the

TABLE III

ANALYSIS OF BEEF PERICARDIAL TISSUE LIPID

	Relative retention (18:0 = 1)	*Saturated (Fraction Nos. 1–5)*	*Monoene (Fraction Nos. 6–13)*	*Diene (Fraction Nos. 14–20)*	*Triene (Fraction Nos. 21–23)*	*Pericardial lipid*	
						Calc.[a]	*Detn.*[b]
10:0	0.059	0.1	—	—	—	0.1	0.1
12:0	0.120	0.2	—	—	0.3	0.1	0.1
iso-14:0	0.206	0.1	—	—	0.2	0.1	0.1
14:0	0.241	6.1	1.0	0.1	1.4	4.2	4.3
iso-15:0	0.292	0.4	—	—		0.3	
14:1	0.307	—	0.6	0.1	0.8	0.2	0.9
anteiso-15:0	0.310	0.7	—	—		0.4	
15:0	0.343	1.2	0.1	—	0.5	0.8	0.9
iso-16:0	0.417	0.4	—	—	0.5	0.3	0.3
16:0	0.491	43.5	5.4	0.6	10.4	29.8	30.1
16:1	0.580	—	4.9	1.6		1.7	2.1
iso-17:0	0.596	0.7	—	—	1.1	0.4	
anteiso-17:0	0.630	1.0	0.2	—		0.8	0.9
17:0	0.695	2.3	—	—	0.7	1.5	1.6
iso-17:1?	0.700	—	0.1	—		tr	
16:2	0.64–0.80[c]	—	—	1.9		0.1	0.4
?	0.754	0.6	—	—	0.7	0.4	
17:1	0.804	—	1.2	—		0.4	0.4
iso-18:0	0.853	0.1	—	—	—	tr	
18:0	1.000	40.8	4.0	0.8	9.5	27.6	27.7
18:1	1.14	1.7	81.5	19.3	10.4	28.0	27.6
iso-19:0	1.22	tr	—	—	—	tr	
iso-19:1?	1.34	—	0.9	—	—	0.3	
19:0	1.41	0.1	—	—	—	tr	2.1
18:2	1.29–1.65[d]	—	—	62.0	11.0	1.9	
19:1	1.58	—	0.1	—	—	tr	
18:3	1.94	—	—	—	39.8	0.3	
19:2	2.00	—	—	4.8	—	0.1	0.4
20:0	2.01	0.1	—	—	—	0.1	
?	2.18	—	—	2.3	—	tr	—
?	2.44	—	—	4.6	1.2	tr	—
?	2.61	—	—	0.8	1.4	tr	—
20:2?	2.81	—	—	1.0	—	tr	—
?	2.97	—	—	—	1.7	tr	—
?	3.27	—	—	—	5.8	0.1	—
?	3.75	—	—	—	1.9	tr	—
?	4.09	—	—	—	0.4	tr	—
?	4.51	—	—	—	0.2	tr	—
% recovered		64.1	32.2	2.3	0.8	99.4	—

[a–d] See footnotes to Table II.

chromatograms of the fractions. Thus saturated acids containing 15, 17, 20 and 22 carbon atoms and monoenes of chain lengths 16 and 20 were demonstrated. Whilst the fractions obtained are essentially true to class, they do contain small amounts of atypical acids. In general, there is a more pronounced tendency for components to tail into the following fraction than for the converse phenomenon of leading to occur. It has been found that chromatography of the bromo adducts allows good separation of the monoene from the polyene fraction, but that it is more difficult to effect absolutely clean separation of the saturated acids from the monoenes. The converse situation applies when the acetoxy adducts are submitted

TABLE IV

ANALYSIS OF BEEF PERIRENAL TISSUE LIPID

	Relative retention (18:0 = 1)	*Saturated (Fraction Nos. 1–4)*	*Monoene (Fraction Nos. 5–13)*	*Diene (Fraction Nos. 14–20)*	*Triene (Fraction Nos. 21–23)*	*Perirenal lipid*	
						Calc.[a]	*Detn.*[b]
10:0	0.059	tr	—	—	—	tr	0.1
12:0	0.121	0.6	0.1	—	0.2	0.4	0.4
13:0	0.171	tr	0.1	—	—	0.1	0.1
iso-14:0	0.208	0.3	—	—	—	0.2	0.2
14:0	0.242	12.2	0.7	0.2	1.1	7.3	7.3
iso-15:0	0.293	0.5	—	—	—	0.3	1.5
14:1	0.308	—	1.6	0.3	0.7	0.6	
anteiso-15:0	0.311	1.0	—	—	—	0.6	
15:0	0.343	1.8	0.2	0.1	0.5	1.1	1.1
iso-16:0	0.418	0.6	—	—	—	0.4	0.4
16:0	0.487	44.0	2.1	0.7	11.1	26.3	26.2
16:1	0.580	—	6.6	1.0	1.8	2.6	2.8
iso-17:0	0.597	0.8	—	—	—	0.4	
anteiso-17:0	0.630	1.1	—	—	—	0.6	0.8
17:0	0.695	2.2	—	—	0.6	1.3	1.4
16:2	0.64–0.81[c]	—	—	2.1	—	0.1	0.1
?	0.765	tr	—	—	—	tr	
17:1	0.818	—	1.2	—	—	0.5	0.4
iso-18:0	0.860	tr	—	—	—	tr	
18:0	1.000	34.20	1.6	1.5	12.0	20.5	20.6
18:1	1.14	0.7	82.0	9.8	13.0	32.4	33.4
iso-19:1?	1.35	—	1.2	—	—	0.5	1.9
19:0	1.42	0.1	—	—	—	tr	
anteiso-19:1?	1.46	—	0.2	—	—	0.1	
18:2	1.26–1.65[d]	—	—	47.1	11.0	1.6	
19:1	1.60	—	0.5	—	—	0.2	—
?	1.70	—	—	—	1.1	tr	1.5
18:3	1.94	—	—	—	44.8	tr	
19:2	1.97	—	—	22.3	—	0.8	
iso-20:1	2.02	—	0.8	—	—	0.3	
20:0	2.04	tr	—	—	—	tr	
20:1	2.25	—	1.0	—	—	0.4	0.1
?	2.42	—	—	11.0	2.3	0.4	—
20:2?	2.81	—	—	3.0	—	0.1	tr
?	3.19	—	—	0.8	—	tr	—
% recovered		58.0	38.6	3.4	0.04	100.0	—

[a–d] See footnotes to Table II.

to chromatography, for, in this case, the larger polarity difference between the saturated esters and the monoene adducts leads to facile resolution. The trace concentration of linoleic acid present in the saturated fraction is thought to be due to incomplete reaction or to hydrolysis before chromatographic separation. Cross-contamination from all sources is sufficiently limited to be of no significant consequence.

The results of Tables II–IV illustrate the extreme complexity of animal tallows and demonstrate that a large amount of additional information on their compositions can be obtained following adduct fractionation. This is further illustrated in Fig. 2, where it can be seen that even after isolation of the diene and triene

fractions, their compositions are still too complex to be completely interpreted. This arises from the fact that, in ruminant animals, the lipids in the food ingested are partially hydrogenated in the rumen and give rise to a large number of isomeric acids. The triene fraction is further complicated because 20% dioxane elutes any traces of free acid that might have been present in the original ester. On esterification with diazomethane, this gives rise to a chromatogram of the original fat superimposed on the triene chromatogram. In the case of animal tallows, which contain only a

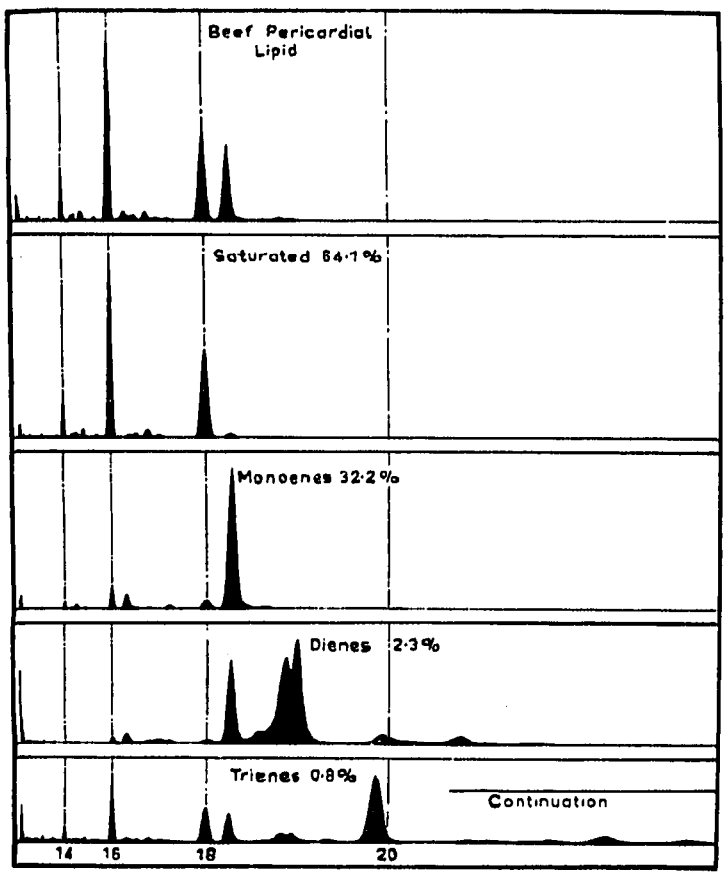

Fig. 2. GLC analysis of beef pericardial lipid methyl esters and of the saturated monoenoic, dienoic and trienoic esters recovered from fractions obtained by silica gel chromatographic fractionation of bromo–mercuri–methoxy adducts. GC parameters: column 5 ft. × 4 mm I.D.; 15% diethylene glycol succinate on acid-washed Chromosorb W; 30 ml N_2/min; 160°.

very small amount of trienoic acids, it only needs a minute concentration of free acid in the original sample significantly to distort the triene chromatogram. Interpretation can still be effected, as the trienes of animal tallow elute later than the majority of the peaks of the unfractionated tallow.

It is evident that further fractionation will be necessary to clarify this situation and this work will form the subject of a subsequent publication. However, it is pertinent to note that the method does allow isolation of trienes when present in concentrations below 1%, in a form protected from oxidation and suitable for further analysis.

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