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PREPARATION AND THIN-LAYER CHROMATOGRAPHY OF BROMO-DERIVATIVES OF UNSATURATED FATTY ACID ESTERS

A SIMPLE AND RAPID PROCEDURE FOR FATTY ACID ANALYSIS

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SUMMARY

Bromo-addition products of unsaturated long-chain fatty acid esters have been prepared and chromatographed on thin layers of unmodified silica gel. The polarity of these derivatives was found to be directly related to the number of double bonds of the parent fatty acid from which they were derived. This has been made the basis of a simple method for assessing the relative proportions of the main fatty acid classes in a mixture.

INTRODUCTION

Bromine addition to the double bonds of unsaturated fatty acids has been widely used in the past as a means of achieving the separation of various fatty acid classes[1,2]; the method, however, has been little used since the inception of gas–liquid chromatographic techniques. Although reversed-phase partition thin-layer chromatography (TLC), as well as argentation TLC, have become popular tools for fatty acids analysis, few attempts have been made to separate the bromo-derivatives of unsaturated fatty acids by conventional TLC. Nevertheless, the work of Kaufmann *et al.*[3] demonstrated that addition of bromine to the double bonds of unsaturated fatty acids can be used advantageously to improve the resolution of certain critical pairs of fatty acids on a thin layer of kieselguhr impregnated with undecane.

Recently, we have reported that mild treatment of long-chain fatty acids with bromine in chloroform solution results in the production of non-polar derivatives and that the polarity of these derivatives is directly related to the number of double bonds in the fatty acids from which they are derived[4]. As will be shown here, these non-polar fatty acid derivatives are, in fact, bromine-containing ethyl esters of the acids. The observed excellent chromatographic resolution of the bromo-derivatives on layers of unmodified silica gel prompted us to devise the simple method for fatty acid analysis that will be detailed here.

EXPERIMENTAL

Preparation of fatty ethyl (or methyl) esters

Ethylation (or methylation) of the free fatty acids and trans-esterification of the glycerolipids were performed in 2–3 ml of 2% sulphuric acid in ethanol (or methanol)–light petroleum (4:1) at 70° for 1 h (for fatty acids) or 4 h (for glycerolipids).

Thin-layer chromatography

Ready-coated plates of silica gel (0.25 mm thick) supplied by Merck were used throughout this work, without prior activation. The substances were applied with a micropipette as a line 1 cm long and 1.5 cm from the lower edge of the plate. The chromatoplates were developed in unequilibrated tanks to a height of 12.5 cm, at room temperature, with light petroleum (60–80°)–diethyl ether (9:1, v/v) as the solvent system. Detection was achieved by using copper acetate reagent as described by Fewster *et al.*[5].

Thin-layer densitometry

The optical density of the charred spots was measured with a Vitatron TLD-100 flying-spot photodensitometer (Vitatron, Dieren, The Netherlands); peak areas were determined by scanning in the direction of the solvent flow and calculated by triangulation.

Gas–liquid chromatography (GLC)

Analysis of the fatty acid methyl esters was carried out on a Packard 824 chromatograph using a 6-ft. glass column packed with 20% of DEGS coated on Chromosorb W HMDS (60–80 mesh) (Varian, Fife, Scotland); nitrogen was the carrier gas at a flow-rate of 25 ml/min. The area under each fatty acid peak was estimated by triangulation.

Mass spectrometry

The mass spectra were obtained on an LKB 9000 S instrument with gas chromatograph and direct inlets. Recording conditions were as follows: ionising energy, 70 eV; trap current, 60 μA; and ion-source temperature, 270°. The temperature of the direct inlet to the mass spectrometer was set at approximately 100°. The column (2 m × 3 mm I.D.) used for GLC was 1% of OV-1 on Gas-Chrom P AW DMCS (100–120 mesh) prepared according to Horning *et al.*[6]. The flash heater, column and separator temperatures were, respectively, 260, 220 and 265°, and the carrier gas (helium) flow-rate was set at 30 ml/min.

Products

The following commercially available fatty acids were used: palmitic and stearic acids (BDH, Poole, Great Britain), arachidic and oleic acid (Fluka, Buchs, Switzerland), linoleic acid (Merck, Darmstadt, G.F.R.), arachidonic acid (Sigma, St. Louis, Mo., U.S.A.) and docosa-4,7,10,13,16,19-hexaenoic acid (Applied Science Labs., State College, Pa., U.S.A.). [1-^{14}C]Palmitic acid (57.5 mCi/mmole) certified to be 99% pure was purchased from the Radiochemical Centre (Amersham, Great Britain). All the organic solvents used were of analytical grade.

RESULTS AND DISCUSSION

Reaction of the fatty acids with bromine in chloroform solution

Sample of various fatty acids dissolved in chloroform (2 mg/ml) were allowed to react with bromine (40 μl/ml) in a PTFE screw-capped phial at room temperature for 16 h. Such treatment results in quantitative conversion of the fatty acids into less polar compounds, as was shown by TLC of radioactive palmitic acid that had been treated with bromine (see Fig. 1); no change in the R_F values of the different fatty acids was observed after brief treatment with bromine.

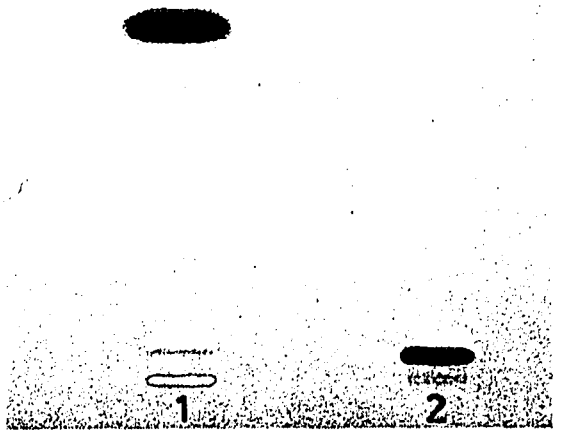

Fig. 1. Production of a non-polar derivative from palmitic acid treated with bromine in chloroform. 1, 1.08 nmole of [1-^{14}C]palmitic acid (0.062 μCi) treated with bromine; 2, the same amount of untreated labelled palmitic acid. Detection by autoradiography (Ferrania No-Screen film; exposure time, 5 days).

The polarities of these unknown derivatives were proportional to the number of double bonds of the parent fatty acids (see Fig. 2); in the solvent system light petroleum–diethyl ether (9:1), R_F values of 0.73, 0.61, 0.53, 0.37 and 0.31 were found for the derivatives of palmitic, oleic, linoleic, arachidonic and docosa-4,7,10,13,16,19-hexaenoic acid, respectively. However, the non-polar derivatives of several long-chain saturated fatty acids (*e.g.*, palmitic, stearic and arachidic acids) could not be resolved in this system.

From the experiments with [1-^{14}C]palmitate (see Fig. 1), it can be inferred that no decarboxylation occurs during the reaction, as there was no loss of radioactivity when this acid was converted into a non-polar substance.

The mass-spectrometric fragmentation of the substance derived from palmitic acid (see Fig. 3) shows a molecular ion at *m/e* 284, and the ion intensities relative to the base peak were similar to those quoted for the ethyl esters of palmitic acid[7]; moreover, the fragmentation pattern obtained with the palmitoyl ethyl ester was identical with that shown in Fig. 3. Further evidence for the identity of the unknown non-polar derivative with a palmitoyl ethyl ester was gained by GLC and TLC, which showed that both substances exhibited identical retention times and were characterized by the same R_F values.

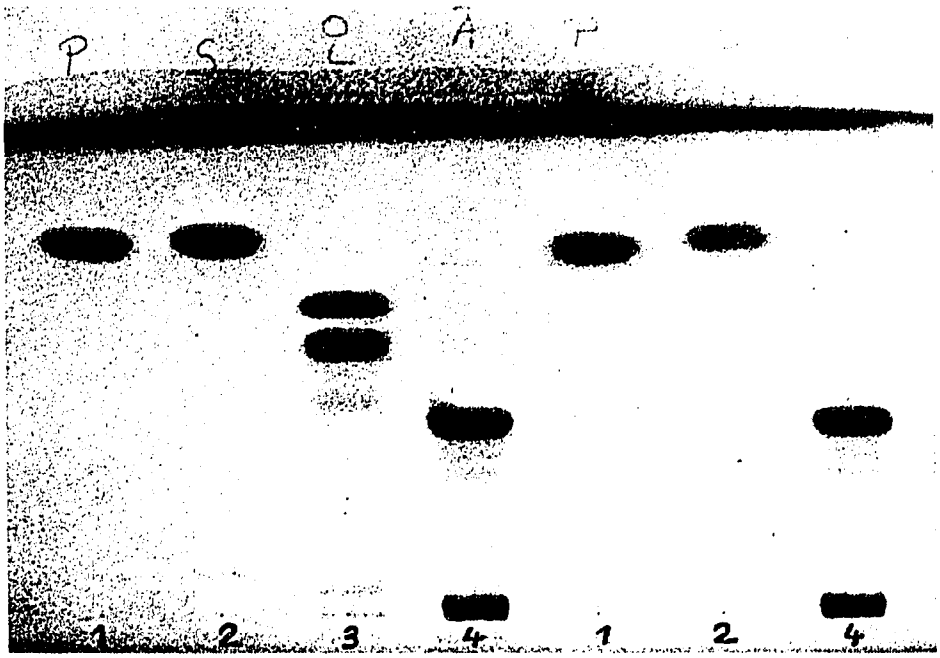

Fig. 2. TLC of the non-polar substances derived from various fatty acids treated with bromine. 1, Palmitic acid; 2, stearic acid; 3, mixture of oleic and linoleic acids; 4, arachidonic acid. Detection by the copper acetate reagent.

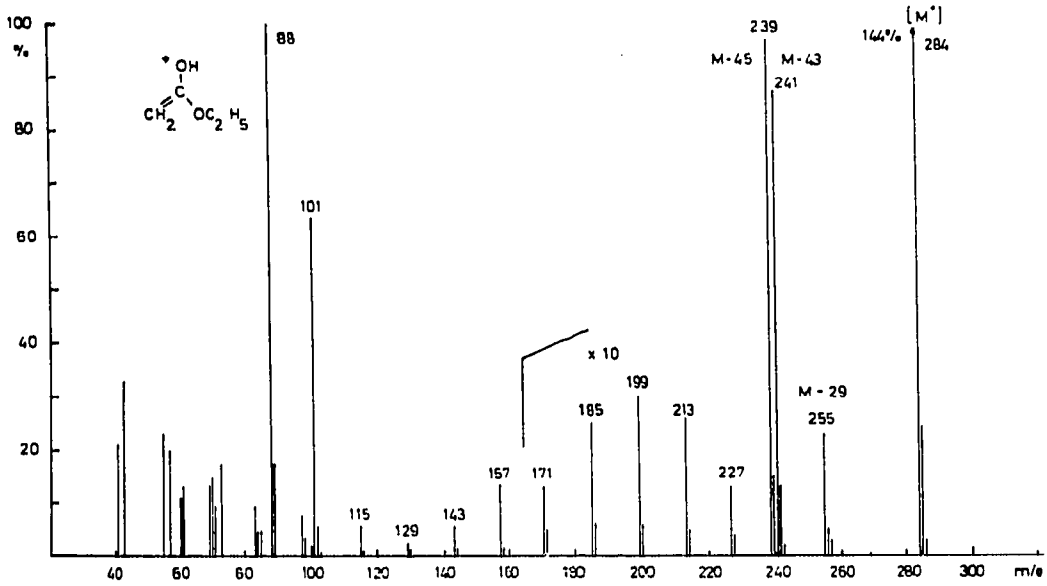

Fig. 3. Mass spectrum of the unknown substance derived from palmitic acid treated with bromine; the spectrum was recorded in the gas chromatograph inlet. An identical pattern was obtained by using the direct inlet.

The mass spectrum of the substance derived from linoleic acid treated with bromine (as recorded by direct inlet mass spectrometry) is shown in Fig. 4A and compared with that of the linoleyl ethyl ester (Fig. 4B); the isotopic-abundance ratio indicates the presence of four bromine atoms in the unknown derivative. It should be noted that the fragmentation pattern recorded via the gas chromatograph inlet differed from that in Fig. 4A and was similar to that of the linoleyl ester (Fig. 4B), probably owing to loss of halogen during GLC.

On TLC, the linoleyl ester proved to be less polar than the substance derived from linoleic acid after bromine treatment; however, within a few seconds of the reaction with bromine, the linoleyl ethyl ester could be converted into a more polar compound having a R_F value identical with that of the derivative of linoleic acid

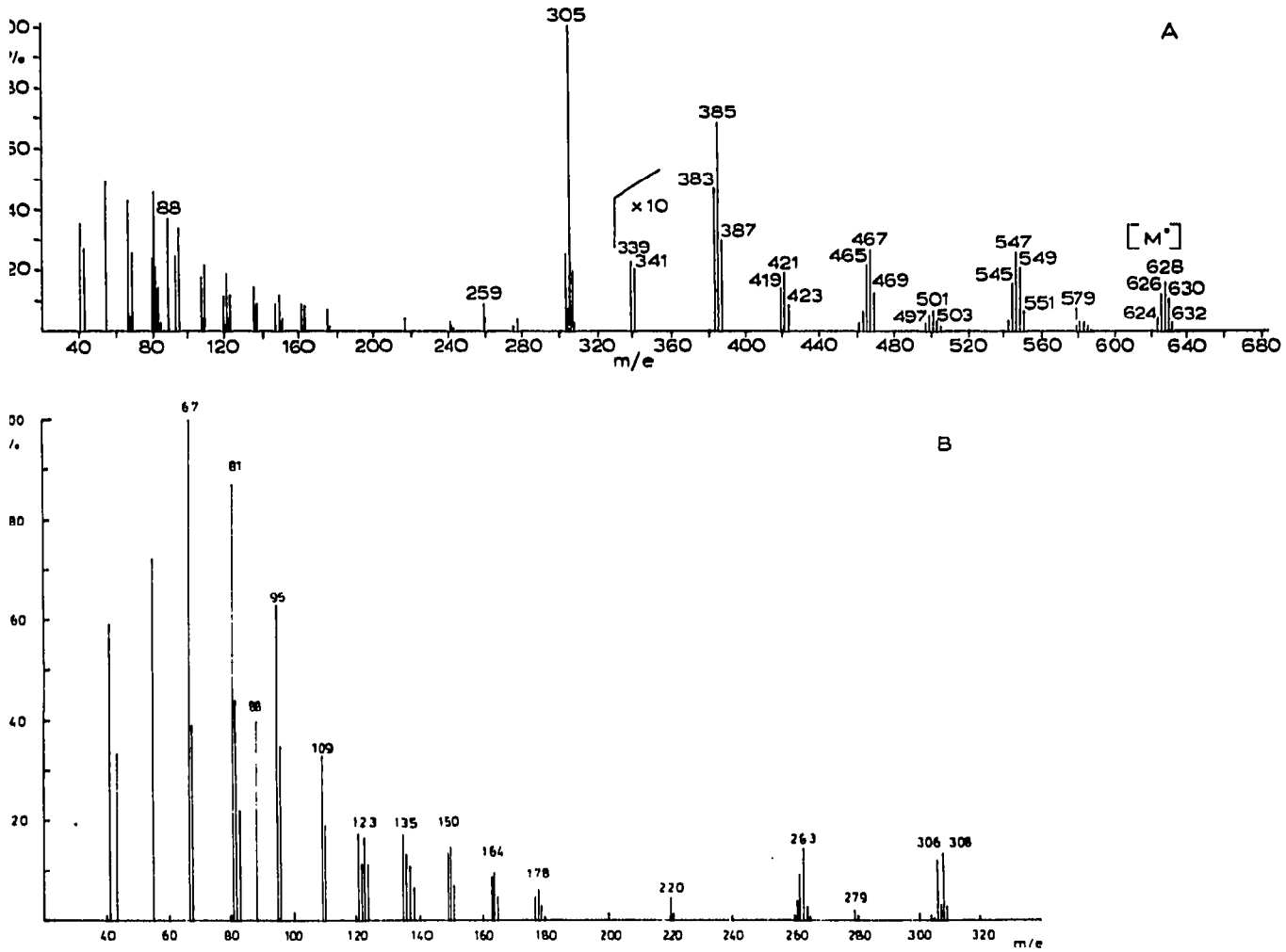

Fig. 4. Mass spectra of the unknown derivative of linoleic acid (A) and of linoleyl ethyl ester (B); the spectra were recorded either in the direct inlet (A) or through the gas chromatograph inlet (B).

(see Fig. 5). These observations suggest that the latter compound is a polybromo-derivative of linoleyl ethyl ester, which is in agreement with the mass-spectrometric fragmentation studies.

It thus appears that bromine treatment of fatty acids in chloroform medium results not only in addition of bromine to the double bonds, but also in esterification of the carboxyl group. Although the mechanism of the latter reaction is not yet known, esterification would depend on the presence of small amounts of ethanol in the chloroform: indeed, the formation of the non-polar derivatives did not occur when the reaction with bromine was carried out in other solvents, such as carbon tetrachloride or benzene, or in chloroform that had been freed of ethanol by passage through an alumina column. Formation of a reactive acid bromide intermediate may be a possible mechanism.

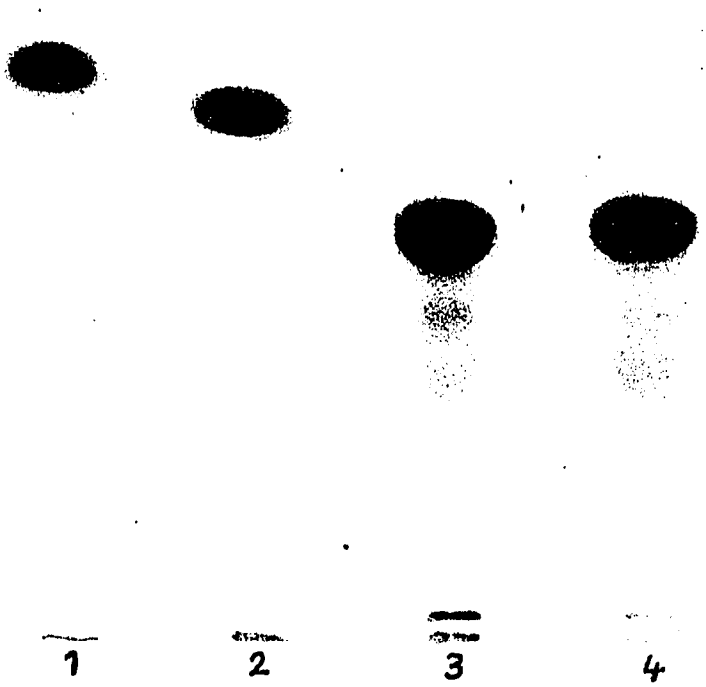

Fig. 5. Chromatographic evidence for identity of the unknown linoleyl derivative with the bromo-derivative of linoleyl ethyl ester. 1, Palmitoyl ethyl ester; 2, linoleyl ethyl ester; 3, linoleyl ethyl ester treated with bromine; 4, unknown substance derived from linoleic acid treated with bromine. Detection by the copper acetate reagent.

While the bromine-catalyzed formation of fatty acid ethyl esters is a rather slow reaction, addition of bromine to the double bonds of unsaturated fatty acid ethyl esters takes place immediately, so that a kinetic study of this latter reaction could not be undertaken.

Application to fatty acid analysis

The above-mentioned findings form the basis of a simple method for the rapid estimation of the distribution of various fatty acid classes in a mixture.

The bromo-derivatives of fatty acid ethyl esters can be prepared in two ways depending on the starting material. For unesterified fatty acids, the sample is simply dissolved in a known amount of chloroform containing 4% of bromine and the solution is set aside overnight at room temperature. When starting from phospholipids, the fatty acid ethyl esters are first prepared by trans-esterification as described in Experimental; an appropriate sample is then evaporated under nitrogen in a conical tube, and the residue is taken up in 4% bromine solution in chloroform and used for TLC in the system light petroleum–diethyl ether (9:1). The TLC can be carried out on a small amount of material, preferably 20–40 μg of total fatty acids (corresponding to 1–2 μg of lipid phosphorus for analyses of phospholipids).

The validity of our method was assessed by comparing the fatty acid distribution as measured by the optical scanning of the TLC plate with the percentage distribution determined by GLC of the methyl esters. Table I shows that the percentage distribution obtained by our method closely approximates to the value calculated from the GLC results.

The bromo-derivatives of fatty acid methyl esters were found to be slightly more polar than those of the corresponding ethyl esters; the former substances, how-

TABLE I

PERCENTAGE DISTRIBUTION OF THE VARIOUS FATTY ACID CLASSES. COMPARISON OF THE TLC TECHNIQUE WITH GLC ANALYSIS

Sample No.	*Acid structure*	*Distribution (%)*		*Sample No.*	*Acid structure*	*Distribution (%)*	
		*TLC**	*GLC***			*TLC**	*GLC***
Fatty acid mixtures				*Rat-liver phosphatidylcholine*			
1	16:0	27	28	1	16:0	—	28
	18:1	23	22		18:0	—	11
	18:2	26	24		Total saturated	42	39
	20:4	24	25		16:1	—	4
					18:1	—	12
2	16:0	45	48		Total monoenes	14	16
	18:1	10	8		18:2	26	26
	18:2	28	24		20:4	18	19
	20:4	17	20				
				Rat-liver phosphatidylethanolamine			
3	16:0	43	55	1	16:0	—	29
	18:1	12	10		18:0	—	24
	18:2	36	28		Total saturated	47	53
	20:4	9	7		16:1	—	—
					18:1	—	9
4	16:0	11	12		Total monoenes	9	9
	18:1	28	28		18:2	21	14
	18:2	42	39		20:4	23	24
	20:4	18	20				
5	16:0	27	23				
	18:1	31	27				
	18:2	12	10				
	20:4	30	39				

* Results obtained by TLC of bromo-derivatives of fatty acid ethyl esters.
** Results obtained by GLC of fatty acid methyl esters.

ever, may also be used for chromatographic separation according to the degree of unsaturation. Small additional spots have occasionally been detected after TLC analysis of the polybromo-derivatives of arachidonyl methyl esters, which suggests that these esters may exhibit some degree of instability. On the other hand, the bromine-addition compound of arachidonyl ethyl ester never gave more than one spot during TLC.

The heating period after spraying with the copper acetate reagent should be sufficiently long (around 2 h at 180°) to allow complete detection of the saturated fatty acid esters; reduction in the heating time could lead to erroneous estimation of the percentage distribution of each fatty acid class.

CONCLUSIONS

The analytical method described in this paper can be used to estimate the relative proportions of the various fatty acid classes in a mixture and therefore offers an alternative to argentation chromatography; its main advantage over the latter method lies in its lower cost and greater simplicity. In view of the increasing use of photodensitometric techniques, it may also be noted that our method can be used as an alternative to GLC, at least to some extent; however, no separation of the different saturated fatty acid ethyl esters could be achieved on layers of unmodified silica gel.

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