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Analysis of Fatty Acids in Lipids by High Performance Liquid Chromatography (HPLC)

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ABSTRACT

Lipids were characterised by HPLC after hydrolysis and esterification (in a one-pot reaction) to prepare UV-absorbing esters of their fatty acids. The derivatives were separated by gradient elution on a reversed phase column and detected by dual-wavelength monitoring. This approach enabled identities to be verified before quantification was undertaken. Identity confirmation is an alternative to the conventional group fractionation of fats, prior to fatty acid profiling, when handling difficult or complex samples.

INTRODUCTION

Animal and vegetable fats are frequently characterised by the acid profiles of their triglycerides. Often, there are only a few long-chain acids present in a lipid and the identities and relative amounts characterise that fat, i.e. its source, processing history, adulteration, etc.

Such profiles are normally determined by gas chromatography. Usually, the methyl esters of the acids are prepared and then separated (Yazicioglu & Karaali, 1983; Kochar & Matsui, 1983) on packed columns. However, considerably improved separations are attainable on capillary columns (Gaydou & Bouchet, 1984; Cadden & Kennelly, 1984) and consequently their use is increasing. Gas chromatographic techniques for fatty acid (FA) profiling of lipids are now so numerous that most aspects of lipid characterisation have been investigated (Gaydou *et al.*, 1984).

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More recently, acids in lipids have been successfully determined by high performance liquid chromatography (HPLC). The free acids can be analysed on ODS columns with acidified eluants (Bailie *et al.*, 1982) or UV absorbing esters may be prepared and separated on reversed phase columns with aqueous–organic solvents (Kihara *et al.*, 1984). Fluorescent derivatives have also been examined (Sato, 1984). Several workers have compared gas and liquid chromatographic results for reproducibility, resolution, etc. (Tweeten & Wetzel, 1979; Jaselskis *et al.*, 1982; Manku, 1983) on a variety of samples. The expanding volume of literature on fatty acid analysis by HPLC has been recently reviewed (Smith, 1983).

Derivatisation enables the analyst to determine both macro and trace levels of acids in lipids as aromatic esters can conveniently be monitored with simple detectors, i.e. fixed-wavelength photometers (254 nm). Lipid hydrolysis and ester formation may be combined so that isolation of the released acids is not necessary. When multiple wavelength monitoring is used then confirmation of identity can also be achieved. The latter is attractive for examining trace constituents in a fatty acid profile as retention time and response ratio must match for positive confirmation. This is particularly useful when looking for evidence of adulteration or microbial attack in a foodstuff.

Lipids that contain a high level of non-saponifiable material often require fractionation prior to the fatty acid determination. This step is no longer necessary when the HPLC detector confirms identities as well as quantifying the FA levels. Appreciable savings in time and cost may be achieved by simplifying practical protocols in this manner.

EXPERIMENTAL

Apparatus and reagents

A Beckman gradient liquid chromatograph (model 334) fitted with an Ultrasphere Octyl (25 × 0·46 cm) column, coupled to a Beckman UV/visible (model 165) scanning detector.

A dual-pen chart recorder or dual-channel integrator.

A Reacti-Therm heating block (Pierce and Warriner) capable of taking 1-ml Reacti-Vials. All solvents (acetonitrile, methanol, dichloromethane and water) were HPLC grade. The derivatising agent was made up as follows: *p*-bromophenacyl bromide (25 mM) and 18-crown-6 (1·25 mM) were dissolved in acetonitrile. The reagent was stored in a refrigerator.

Standards were prepared as follows: fatty acids (each approximately 10 mg) were dissolved in methanol (50 ml). The solution was stored in a refrigerator.

Standardisation

An aliquot (200 microlitres) of the standard solution was mixed with a few crystals of potassium bicarbonate in a 1-ml Reacti-Vial and the solvent removed in a stream of air. The phenacyl bromide reagent (100 microlitres) and acetonitrile (100 microlitres) were added and the mixture heated for 30 min at 80°C. An aliquot (5 microlitres) was injected into the HPLC.

Response factors were calculated for unit weights of each compound injected and the FA profiles of hydrolysates computed by comparison with these standard runs. An internal standard (such as margaric acid) would simplify calculations in routine analyses.

Hydrolysis and derivatisation

The sample (50 mg lipid) was dissolved in dichloromethane (50 ml) and methanol (25 ml). An aliquot (100 microlitres) was transferred to a 1-ml Reacti-Vial, 0·25 M methanolic potassium hydroxide (10 microlitres) added and the mixture shaken. The sample was heated to 80°C for 20 min to hydrolyse the triglyceride. The solvent was removed in a stream of air, the phenacyl bromide reagent (200 microlitres) added and the sample heated for another 30 min, at 80°C, to prepare the esters. An aliquot (5 microlitres) was injected directly into the HPLC.

Chromatography

The bromophenacyl esters were separated by gradient elution on an Ultrasphere Octyl (25 × 0·46 cm) column.

The run commenced with a solvent of 80% acetonitrile in water at a flow of 1 ml/min. At 1 min the composition was changed to 100% acetonitrile over 15 min. At 20 min the flow rate was increased to 2 ml/min over 5 min. At 30 min flow rate and solvent composition reverted to starting values.

The column eluate was monitored at two wavelengths (214 nm and 254 nm). Both detector channels were set at an attenuation of 0·1 absorbance units, full scale deflection (AUFSD).

RESULTS AND DISCUSSION

Method development

Compounds are normally identified (in chromatography) on the basis of retention time. When response ratio is also measured then two criteria must

TABLE 1
Reproducibility of Calculated Fatty Acid Profiles[a]

Acid		*Repeat injections*						*Repeat analyses*					
						Av.	*CV*					*Av.*	*CV*
Palmitic	(C16)	30·0,	30·7,	29·3,	30·1	30·0	±1·66	30·0,	30·9,	30·3,	28·4	29·9	±3·09
Oleic	(C18:1)	28·7,	27·7,	29·1,	28·5	28·5	±1·79	28·7,	31·1,	29·5,	28·5	29·5	±3·47
Stearic	(C18)	21·1,	21·3,	21·2,	21·4	21·3	±0·57	21·1,	21·8,	20·6,	20·2	20·9	±2·86

[a] Expressed as % FA/total FA.
Av, average.
CV, coefficient of variation.

be satisfied before a result is judged positive and this can only improve the reliability of the acquired information. This is put to good use in examining materials with relatively high levels of non-saponifiable lipids, as the extra peaks on the chromatogram often respond at both wavelengths (i.e. 214 nm and 254 nm) but give incorrect ratios. They can then be eliminated from the calculations.

Repeatability is of utmost importance when developing any technique. Accordingly, reproducibility between repeat injections and between repeat analyses were both examined. The samples were hydrolysed, derivatised and relative fatty acid profiles calculated in the normal fashion. Results are given in Table 1. Re-examination shows a comparatively small variation. Effectively, re-injection produces no change in the calculated relative levels of constituent fatty acids in a triglyceride. Predictably, there is a greater variation after a complete re-analysis, but the change is still modest and would not alter conclusions based upon the FA profiles of the lipids. The results given in Table 1 were obtained from a butter fat sample (palmitic, oleic and stearic acids comprising the bulk of the fatty acids in this sample).

Response factors of individual esters vary considerably (Table 2) and are influenced greatly by the attached bromophenacyl molecule. Response ratios are more consistent, with an appreciable demarcation between saturated and unsaturated acids (as unsaturation increases relative response at 214 nm).

It was noticed during the course of this investigation that response ratios of the phenacyl esters varied between standard runs (to establish response factors and retention indices) and sample analyses (Tables 2, 3 and 4). The 214 nm traces (of samples) were noticeably more complex and it seems

TABLE 2
Response Factors and Retention Indices of Derivatives

Phenacyl ester of		*Relative response (254 nm)*[a]	*Response ratio (254 nm/214 nm)*	*Relative retention time*
Capric acid	(C10)	128	1·4	0·51
Lauric acid	(C12)	96	1·3	0·69
Linolenic acid	(C18:3)	68	0·9	0·81
Myristic acid	(C14)	88	1·3	0·85
Linoleic acid	(C18:2)	74	1·1	0·91
Palmitic acid	(C16)	83	1·3	1·00
Oleic acid	(C18:1)	79	1·3	1·04
Stearic acid	(C18)	63	1·3	1·11
Arachidic acid	(C20)	34	1·3	1·20

[a] Unit weights of derivative injected.

TABLE 3
Butter Fat Hydrolysate

Peak No.	*Response ratio (254 nm/214 nm)*	*Relative retention time*	*Identification*	*% FA / total FA*	*Expected FA composition*[a]
1	1·2	0·67	C12	3·5	3·0–4·3
2	1·2	0·83	C14	12·6	11·7–13·8
3	1·2	0·85	*	—	—
4	1·0	0·91	C18:2	4·8	1·7–4·4
5	1·2	1·00	C16	30·0	28·6–35·3
6	1·2	1·03	C18:1	27·7	32·5–34·3
7	1·2	1·12	C18	21·3	11·2–19·5

* Possibly palmitoleic acid (C16:1).
[a] Harwood & Geyer (1968). Eyres (1979). Egan *et al.* (1981).

likely that the response was not entirely due to the prepared ester. For this reason, these traces were never used for quantification.

Fatty acid (FA) profiles in lipids

The butter fat hydrolysate gives a clean profile (Fig. 1). There is no trouble in identification or quantification and the results are shown in Table 3. All the major peaks have been assigned names and the percentage fatty acids/

TABLE 4
Marine Fat (Shrimp Oil) Hydrolysate

Peak No.	*Response ratio (254 nm/214 nm)*	*Relative retention time*	*Identification*	*%FA/total FA*
1	0·5	0·72	—	—
2	0·4	0·78	—	—
3	0·8	0·83	C18:3*	17·3
4	0·9	0·91	C18:2*	3·6
5	1·4	0·94	—	—
6	1·2	1·00	C16	33·9
7	1·2	1·03	C18:1	22·6
8	1·5	1·09	—	—
9	1·2	1·13	C18	22·2

* Probable identification.

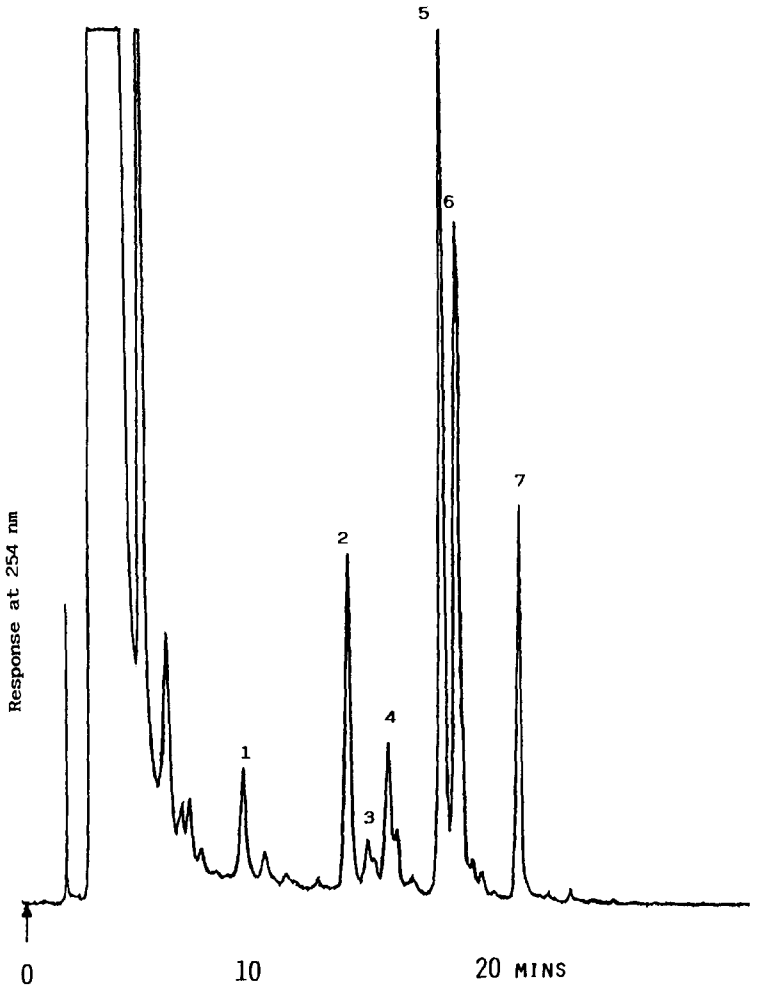

Fig. 1. Chromatogram of butter fat hydrolysate.

total fatty acids calculated from the 254 nm trace. The absence of extraneous material, together with the general response, indicates that this sample contained predominantly only triglycerides. The lower fatty acids were not specifically determined in this exercise but could easily be examined by altering the chromatographic conditions and reinjecting the sample. The high level of stearic (C18) acid suggests blending at some time in the past (possibly with fractionated butterfat).

The marine fat is a typical 'difficult' sample. The level of triglyceride is quite low and the level of non-saponifiable matter correspondingly higher. Whilst much of this material elutes in the solvent front (and is therefore not visible) some interferes with the FA trace. Under these circumstances dual-wavelength monitoring (to identify constituents before quantification) is

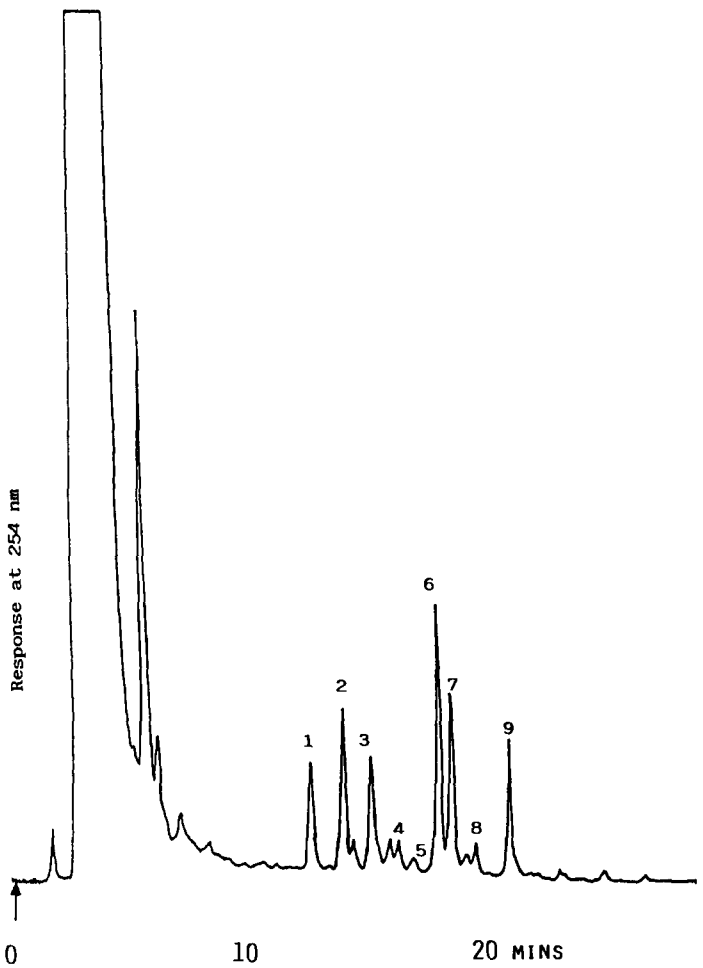

Fig. 2. Chromatogram of marine fat hydrolysate.

essential. In this example peaks 1 and 2 (Fig. 2) were not esters (incorrect response ratios) and most of the components were probably subject to baseline interference (especially on the 214 nm trace). Nevertheless, the fatty acids were identified and their relative levels calculated (Table 4). One is not likely to encounter many samples of this nature in practice; this sample was chosen to demonstrate the advantages of measuring two parameters for confirmation of identity (prior to calculation of results). Marine invertebrate oils are known to contain high levels of odd-carbon numbered and branched-chain fatty acids. Traditionally, such samples would be subjected to a group fractionation prior to the fatty acid profiling of the triglycerides.

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