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High-performance liquid chromatographic analysis of fatty acid compositions of platelet phospholipids as their 2-nitrophenylhydrazides

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ABSTRACT

A novel high-performance liquid chromatographic method for biologically important fatty acids incorporated into platelet phospholipids in esterified form has been developed. 2-Nitrophenylhydrazine hydrochloride was used as a pre-column labelling agent to convert the saponified platelet phospholipids directly into corresponding fatty acid hydrazides, without a complicated isolation procedure. Isocratic separation was achieved within only 36 min for twenty-five saturated and mono- and polyunsaturated fatty acids ($C_{8:0}$–$C_{22:6}$), including *cis* and *trans* isomers, on a YMC-FA column. The analytical results showed good quantitative accuracy. Fatty acid compositions were determined in platelet phospholipids obtained from normal subjects and patients with diabetes mellitus. The method is simple, rapid and adequate for labelling esterified fatty acids in biological materials, and has several advantages with regard to resolution, analysis time and sensitivity over previously published methods.

INTRODUCTION

It is well known that alterations of the tissue fatty acid (FA) composition are found in macro- and microvascular diseases, reflecting a disturbed FA metabolism. For example, the decrease in the ratio of *cis*-5,8,11,14,17-eicosapentaenoic acid (EPA) to *cis*-5,8,11,14-eicosatetraenoic acid (arachidonic acid, AA) in blood lipids is one of the risk factors in coronary heart disease [1]. The aim of this work was to develop a suitable method for the analysis of FA incorporated into biological materials in esterified form, such as platelet phospholipids.

The introduction of gas chromatography (GC) of FA methyl esters in 1952 [2] developed the analysis of FAs extensively in various fields. However, the limited sensitivity and selectivity of GC can restrict the detection of FAs, such as EPA and AA, incorporated into platelet phospholipids, because a large sample volume

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of blood is required. More recently, a high-performance liquid chromatographic (HPLC) method has been developed for the analysis of FAs, in conjunction with pre-column derivatization. The HPLC method has certain advantages over the GC method in sensitivity and selectivity, but it does not show the degree of separation achieved by the use of capillary GC columns. We have used 2-nitrophenylhydrazine hydrochloride (2-NPH · HCl) as a labelling agent for FAs in HPLC analysis, and the method has been developed for the assay of carboxylic acids in biological materials [3–6].

This paper describes the satisfactory separation of saturated and mono- and polyunsaturated FAs, ranging from C_8 to C_{22} chain length, and *cis* and *trans* isomers of C_{18} FAs, and the application of the method to the analysis of the FA compositions of human platelet phospholipids.

EXPERIMENTAL

Reagent solutions

All FA solutions in ethanol were obtained from Yamamura Chemical Labs. (Kyoto, Japan). A 2-nitrophenylhydrazine hydrochloride (Tokyo Kasei Kogyo, Tokyo, Japan) solution (0.02 *M*) was prepared by dissolving the reagent in 0.3 *M* hydrochloric acid–ethanol (1:1, v/v). A 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (1-EDC · HCl) (Sigma, St. Louis, MO, U.S.A.) solution (0.25 *M*) was prepared by dissolving the reagent in a solution of pyridine (3%, v/v) in ethanol. A potassium hydroxide solution (10%, w/v) in methanol–water (1:1, v/v) and a 0.4 *M* potassium hydroxide–ethanol (1:1, v/v) solution were prepared. All the reagent solutions were stable for at least three months when kept below 5°C and were commercially available from Yamamura Chemical Labs. All reagents and chemicals were used without further purification.

Preparation of platelet phospholipids

Blood (*ca.* 7 ml) was drawn from normal and diabetic subjects by use of a vacuumed venous aspirator containing Na_2EDTA anticoagulant (Terumo, Tokyo, Japan). The blood was centrifuged at 225 *g* for 6 min to obtain platelet-rich plasma (PRP). The PRP was washed twice with 0.9% sodium chloride and centrifuged at 1000 *g* for 20 min after each washing, to obtain a platelet pellet. The lipid fraction was extracted by the method of Folch *et al.* [7], using a chloroform–methanol solution (2:1, v/v). A total phospholipid was separated from the other lipids by thin-layer chromatography (TLC) in one dimension on 5 cm × 20 cm × 0.25 mm silica plate (Silica-Gel 60 F_{254}, Merck, Darmstadt, Germany), which was developed with *n*-hexane–diethyl ether–acetic acid (65:35:1, v/v). The phospholipid fraction was scraped off from the TLC plate and extracted with 5 ml of methanol. The solvent was removed under a stream of nitrogen at room temperature, and the FAs of the phospholipid fraction were analysed as their hydrazides.

Derivatization of FAs

The residue was dissolved in 200 μl of ethanol containing 50 nmol of heptadecanoic acid as the internal standard and was saponified with 100 μl of 0.4 *M* potassium hydroxide–ethanol (1:1, v/v) at 80°C for 20 min. To the saponified sample, 200 μl of 2-NPH · HCl solution and 200 μl of 1-EDC · HCl solution were added, and the mixture was heated at 80°C for 5 min. After the addition of 200 μl of potassium hydroxide solution, the mixture was further heated at 80°C for 5 min and then cooled. To the resultant hydrazide mixture, 4 ml of 1/30 *M* phosphate buffer (pH 6.4)–0.5 *M* hydrochloric acid (7:1, v/v) were added. The FA hydrazides were extracted with 5 ml of *n*-hexane, and the solvent was evaporated under a stream of nitrogen at room temperature. The residue was dissolved in 100 μl of methanol, and an aliquot (5–20 μl) was injected into the chromatograph.

HPLC analysis

Chromatographic analyses were carried out using a Shimadzu LC-6A liquid chromatograph (Shimadzu Seisakusho, Kyoto, Japan) equipped with an on-line degasser ERC-3310 (Erma, Tokyo, Japan) and a Shimadzu SPD-6AV variable-wavelength UV–visible detector. The detector was set at 400 nm, and the detector signals were recorded on a Rikadenki multi-pen recorder (Tokyo, Japan). The column temperature was kept constant at 35°C using a Shimadzu GTO-6A column oven.

The separation of FA hydrazides was achieved isocratically using acetonitrile–methanol–water (75:11:14, v/v) as the eluent at a flow-rate of 1.2 ml/min on a YMC-FA (C_8) column (250 mm × 6 mm I.D.) packed with octyl-bonded silica gel (particle size 5 μm, Yamamura Chemical Labs.). The pH of the eluent was maintained at 4–5 by adding 0.1 *M* hydrochloric acid. The eluent was filtered through a Fluoropore filter (pore sized 0.45 μm) (Sumitomo Electric, Osaka, Japan).

RESULTS AND DISCUSSION

Derivatization conditions

In order to analyse a small amount of FAs, such as EPA and other polyenoic FAs in platelet phospholipids by HPLC, a labelling process is required to achieve high accuracy. The commonly used labelling procedure for esterified FAs is based on saponification of the sample, followed by extraction and derivatization. In this process, the extraction step is often tedious and can cause problems concerning the recovery and reliability of the FA analysis.

In our HPLC method of analysis of FAs in platelet phospholipids, an acidic solution of 2-NPH · HCl, as the labelling agent, can be successfully employed for the direct derivatization of the saponified sample without the extraction step.

The temperature is a very important factor in optimizing the derivatization rate. Investigation of the effect of the temperature on the formation of FA hydra-

zides showed that the derivatization rate gradually increased with increasing temperature, but the detector response decreased with reproducible quantitative yields. The detector response becomes constant at 3 min and thereafter at 80°C, which suggested that the derivatization was maximal in this period. Using an optimum reaction time of 5 min, the FAs studied were converted into their hydrazides without any deterioration.

Chromatographic conditions

In the field of FA analysis by HPLC, 9-anthrylmethyl esters [8–10] and phenacyl esters [11,12], including substituted phenacyl esters [13–15], are the most commonly used compounds. The separations are carried out on reversed-phase columns using isocratic or gradient elution with acetonitrile, methanol and water. However, much of the work [9–14] has not included certain biologically important FAs, such as EPA, AA and *cis*-4,7,10,13,16,19-docosahexaenoic acid (DHA). Therefore, we attempted the complete separation of naturally occurring polyenoic FAs, which has a significant value in clinical and biological chemistry, by a simple elution mode.

Our previous work [3] demonstrated that the fifteen FA hydrazides ($C_{8:0}$–$C_{22:6}$) were separated by isocratic elution, in which acetonitrile provided better retention control for the compounds than did methanol. Thus, we first tried to separate 25 FA hydrazides on a YMC-FA column (250 mm × 4.6 mm I.D.) with acetonitrile–water, but could not obtain satisfactory separation. By increasing the column I.D. to 6 mm, however, we achieved improved separation, with the exception of the two pairs of hydrazides: *trans*-9,12-octadecadienoic ($C_{18:2,trans,trans}$) and *cis*-11,14,17-eicosatrienoic ($C_{20:3}$), and *trans*-9-octadecenoic ($C_{18:1,trans}$) and *cis*-11,14-eicosadienoic ($C_{20:2}$). In order to resolve these adjacent peaks, we tried the use of a different elution system, comprising acetonitrile, methanol and water in the various proportions. Acetonitrile and methanol have different effects on the two parameters for the elution volumes of FA hydrazides, namely the number of carbon atoms and the number of unsaturated bonds in the FA chain.

Fig. 1 shows a typical separation of the 25 FA hydrazides by HPLC analysis with acetonitrile–methanol–water (75:11:14, v/v) as the eluent and with detection in the visible region. It can be seen that the retention time increases as the chain length increases and as the number of double bonds decreases. The *trans* isomers are eluted after the corresponding *cis* isomers. The satisfactory separation of the compounds could be accomplished by elution in the isocratic mode, which is a distinct advantage over gradient elution techniques [8,15].

Quantitative analysis

To construct calibration curves for quantitation, increasing amounts of the mixture of FAs were derivatized in the presence of heptadecanoic acid as the internal standard and analysed. The relationships between the relative peak heights and the amounts of FAs were linear, at least in the range from 1 pmol to 5

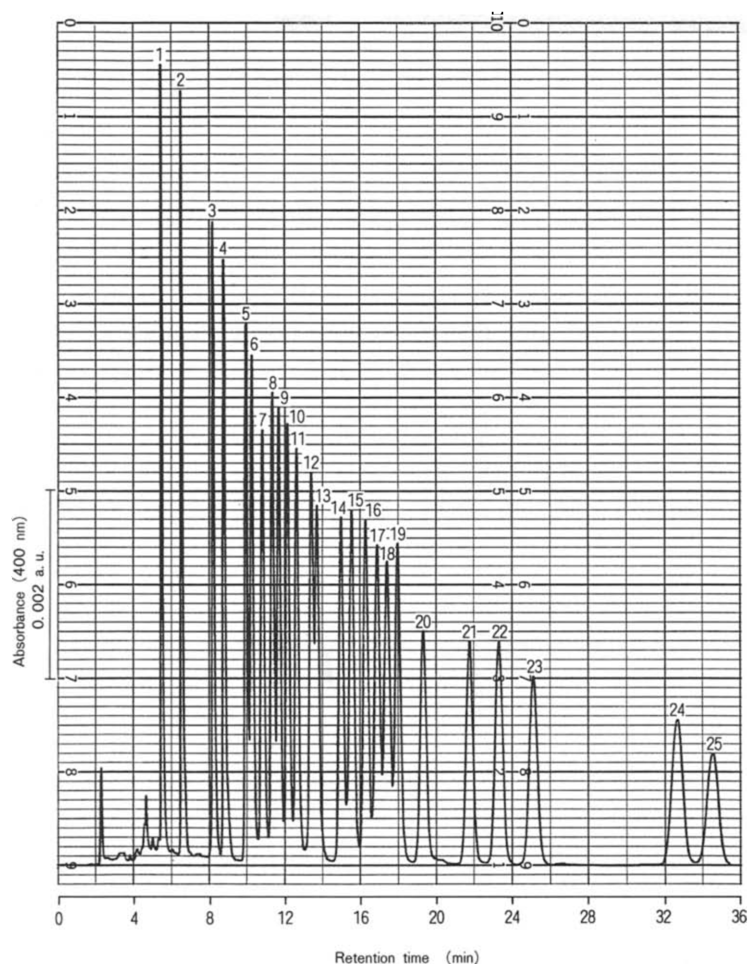

Fig. 1. Chromatogram of the 2-nitrophenylhydrazides of a standard mixture of 25 fatty acids obtained with visible detection. Peaks: 1 = octanoic ($C_{8:0}$); 2 = decanoic ($C_{10:0}$); 3 = dodecanoic ($C_{12:0}$); 4 = *cis*-9-tetradecenoic ($C_{14:1}$); 5 = *cis*-5,8,11,14,17-eicosapentaenoic ($C_{20:5}$); 6 = *cis*-9,12,15-octadecatrienoic ($C_{18:3}$); 7 = tetradecanoic ($C_{14:0}$); 8 = *cis*-4,7,10,13,16,19-docosahexaenoic ($C_{22:6}$); 9 = *cis*-9-hexadecenoic ($C_{16:1}$); 10 = *cis*-5,8,11,14-eicosatetraenoic ($C_{20:4}$); 11 = *cis*-9,12-octadecadienoic ($C_{18:2,cis,cis}$); 12 = *trans*-9,12-octadecadienoic ($C_{18:2,trans,trans}$); 13 = *cis*-8,11,14-eicosatrienoic ($C_{20:3}$); 14 = hexadecanoic ($C_{16:0}$); 15 = *cis*-7,10,13,16-docosatetraenoic ($C_{22:4}$); 16 = *cis*-9-octadecenoic ($C_{18:1,cis}$); 17 = *trans*-9-octadecenoic ($C_{18:1,trans}$); 18 = *cis*-11,14-eicosadienoic ($C_{20:2}$); 19 = heptadecanoic ($C_{17:0}$) (I.S.); 20 = *cis*-13,16,19-docosatrienoic ($C_{22:3}$); 21 = octadecanoic ($C_{18:0}$); 22 = *cis*-11-eicosaenoic ($C_{20:1}$); 23 = *cis*-13,16-docosadienoic ($C_{22:2}$); 24 = eicosanoic ($C_{20:0}$); 25 = *cis*-13-docosaenoic ($C_{22:1}$) acid hydrazide. Each peak corresponds to 150 pmol.

nmol per injection, with correlation coefficients of 0.999–1.000. The limits of detection, based on a signal-to-noise ratio of 2, were from 500 fmol to 1 pmol per injection.

Evaluation of the assay procedures

Platelet lipids extracted by Folch method [7] were isolated by developing on the TLC plate to obtain platelet phospholipids. In order to evaluate the isolation procedure, a standard solution containing known amounts of L-α-phosphatidylcholine (Sigma) (5 mg/ml in chloroform solution) was made as the model of platelet phospholipids. From this standard solution, six aliquots of 100 μl were developed on the TLC plates and analysed as described in the assay procedures. Another six aliquots of 100 μl were analysed directly. Table I lists the FA compositions obtained by the two methods. The relative recovery from TLC ranged from 98.4 to 101.0%. It can be seen that the values are not affected by the isolation procedure. The within-run precision of the proposed method, calculated from six repeated analyses of identical platelet lipid samples, ranged from 0.3 to 4.7%. The present method has a satisfactory precision in analysing the FA compositions of platelet phospholipids, while reducing the overall analysis time and cutting the requirement for skilled operation that applies in GC analysis [16].

Applicability

A number of studies have stimulated the belief that n-3 FAs, such as EPA and DHA, may have an essential function in certain animal cells [17–21]. In membrane lipids, however, these FAs are present as minor components and are contaminated with a variety of polyenoic FAs, which have similar chromatographic behaviour. Therefore, a complete separation of naturally occurring FAs, which is governed by the length of the carbon chain and the number of double bonds, is necessary.

To confirm the practical utility of our method in the assay of biological materi-

TABLE I

FATTY ACID COMPOSITIONS OF L-α-PHOSPHATIDYLCHOLINE ANALYSED WITH AND WITHOUT ISOLATION BY TLC

Fatty acid	Fatty acid composition (mean ± S.D., n = 6) (mol %)	
	With isolation	Without isolation
$C_{14:0}$	0.21 ± 0.01	0.21 ± 0.01
$C_{16:0}$	36.75 ± 0.25	36.67 ± 0.20
$C_{16:1}$	0.84 ± 0.03	0.83 ± 0.03
$C_{18:0}$	13.38 ± 0.15	13.25 ± 0.13
$C_{18:1,cis}$	29.55 ± 0.24	29.71 ± 0.08
$C_{18:2,cis,cis}$	14.16 ± 0.08	14.21 ± 0.13
$C_{20:4}$	3.08 ± 0.05	3.11 ± 0.10
$C_{22:5}$	1.39 ± 0.04	1.38 ± 0.05
$C_{22:6}$	0.64 ± 0.02	0.63 ± 0.02

als, we applied it to the quantitation of FAs in platelet phospholipid samples from ten fasting normal subjects (age range 28–52 years) and eleven fasting patients with non-insulin-dependent diabetes mellitus (age range 18–76 years). The FA profiles of platelet phospholipid samples from a normal and a diabetic subject are shown in Figs. 2 and 3, respectively. There were no interfering peaks other than FA hydrazides on the chromatograms, which were easily identified by comparison of their retention times with those of standards. In Figs. 2 and 3 the peak X is unknown, but this FA is probably identical with *cis*-7,10,13,16,19-docosapentaenoic acid, on the basis of reference chromatograms of PUFA-1 and PUFA-2 obtained from Supelco (Bellefonte, PA, USA). In our analyses, fourteen FAs were detected in human platelet phospholipids.

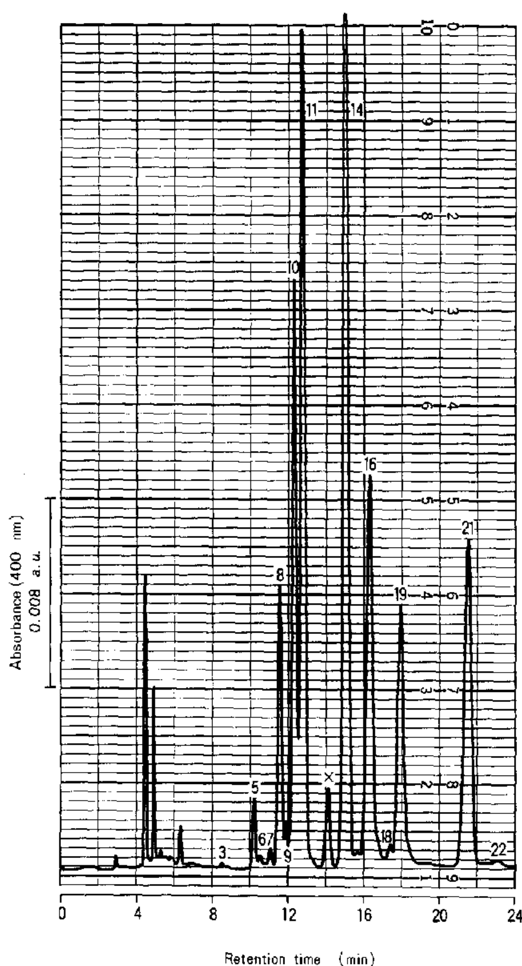

Fig. 2. Chromatogram of the derivatized fatty acids in platelet phospholipids obtained from a normal subject. Peak numbers as in Fig. 1.

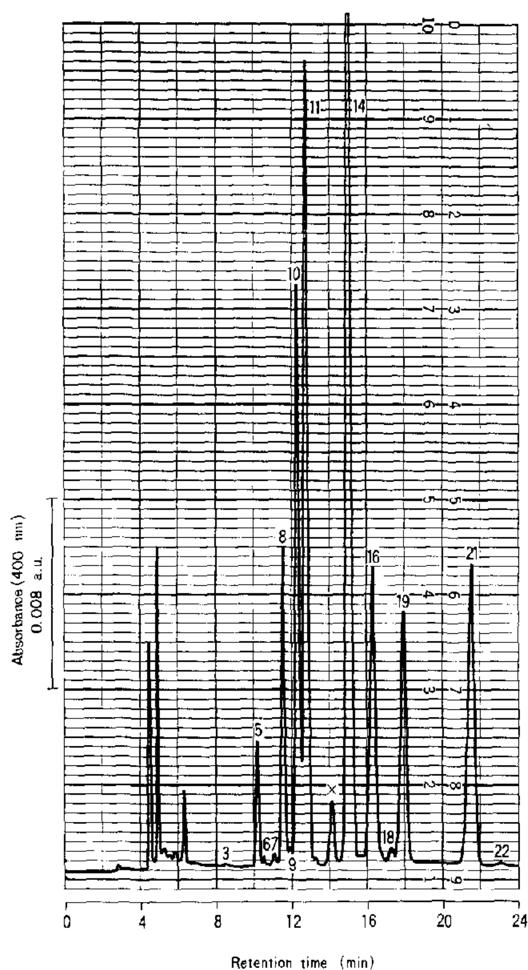

Fig. 3. Chromatogram of the derivatized fatty acids in platelet phospholipids obtained from a patient with diabetes mellitus. Peaks numbers as in Fig. 1.

Comparison of the present method with another published method, the 9-anthryldiazomethane (ADAM) method [22], was made for nine platelet phospholipid samples taken randomly from the two groups of subjects. The correlation coefficients of 0.972–0.991 were acceptable for eleven acids but not for the $C_{20:1}$, $C_{20:2}$ and $C_{22:5}$ acids, which could not be measured by the ADAM method.

The mean values for the relative FA compositions of platelet phospholipids obtained from normal subjects and patients with diabetes mellitus are listed in Table II. The EPA composition was significantly higher in the patients (2.61 $\pm$ 0.30 mol %) than in the normal subjects (1.59 $\pm$ 0.30 mol %). The proportion of DHA was considerably higher in the patients than in the normal subjects, but without reaching statistical significance. There was a tendency for the proportion

TABLE II

FATTY ACID COMPOSITIONS OF PLATELET PHOSPHOLIPIDS FROM NORMAL AND DIABETIC SUBJECTS

Fatty acid	Fatty acid composition (mean ± S.E.) (mol %)	
	Normal (n = 10)	Diabetic (n = 11)
$C_{12:0}$	0.06 ± 0.01	0.06 ± 0.01
$C_{14:0}$	0.67 ± 0.21	0.63 ± 0.12
$C_{16:0}$	27.26 ± 0.52	28.39 ± 1.03
$C_{16:1}$	0.22 ± 0.03	0.23 ± 0.03
$C_{18:0}$	16.13 ± 0.62	13.53 ± 1.31
$C_{18:1,cis}$	10.39 ± 0.54	9.00 ± 0.91
$C_{18:2,cis,cis}$	20.37 ± 1.43	20.40 ± 2.11
$C_{18:3}$	0.12 ± 0.02	0.08 ± 0.01
$C_{20:1}$	0.22 ± 0.03	0.21 ± 0.03
$C_{20:2}$	0.25 ± 0.04	0.10 ± 0.02
$C_{20:4}$	14.28 ± 1.02	14.16 ± 0.83
$C_{20:5}$	1.59 ± 0.30	2.61 ± 0.30[a]
$C_{22:5}$	2.25 ± 0.13	1.83 ± 0.15
$C_{22:6}$	6.19 ± 0.37	8.77 ± 0.92

[a] $P < 0.05$.

of octadecanoic acid and *cis*-9-octadecenoic acid in the patients to decrease. One possible explanation for our observations is that the diabetic patients have undergone treatment with an EPA-rich diet, which is thereafter obviously incorporated into the platelet membranes. These findings are in keeping with observations by Siess *et al.* [23], who found marked changes in platelet FAs caused by dietary supplements.

Increased platelet aggregation, which is probably mediated by AA metabolites formed *via* both the cyclooxygenase and lipoxygenase pathways, has been described in diabetes mellitus. In such patients, however, the AA composition has been found both to increase [24] and to decrease [25] compared with normal subjects. In our study, there was no significant difference in the AA composition between the two groups. A likely explanation for the discrepancy is related to patient selection, in particular the duration of diabetes and the existence of complications, as well as the dietary factors [25].

We are currently trying to obtain detailed information about the relationship between the FA composition of platelet phospholipids and platelet aggregation in diabetic patients suffering from vascular complications, such as retinopathy.

CONCLUSION

The use of non-volatile hydrazine derivatives with HPLC has an unquestionable advantage over GC in the analysis of lipid samples containing short- to medium-chain FAs. Another advantage of the proposed HPLC method is that the polyunsaturated FAs are eluted at the beginning with HPLC, not at the end as with GC. This increases the sensitivity for polyunsaturated FA. In addition, the HPLC method did not produce any artifacts in the procedure involving the sample preparation, and improved the column life-time (more than 500 analyses). Because of its simplicity and accuracy, the present method may become useful for the assay of FAs in biological materials that can be collected in only small amounts.

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