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# IMPROVED METHOD OF DETERMINATION OF BIOLOGICALLY IMPORTANT $C_{10:0}$–$C_{22:6}$ FATTY ACIDS AS THEIR 2-NITROPHENYLHYDRAZIDES BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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## SUMMARY

Fatty acids were separated by reversed-phase high-performance liquid chromatography after derivatization with 2-nitrophenylhydrazine hydrochloride in the presence of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride. The separation of a mixture of fourteen kinds of biologically important fatty acid hydrazides ($C_{10:0}$–$C_{22:6}$) was achieved within 15 min. Using margaric acid ($C_{17:0}$) as internal standard, each fatty acid could be quantitated over the range of 2.5–5000 pmol per injection. Analytical recoveries ranged from 98.1 to 102.6%. The intra- and inter-assay coefficients of variation were less than 2.5 and 3.2%, respectively. For the determination of esterified fatty acids in fats and oils, the saponified mixture was directly derivatized without extraction. This method was compared gave similar fatty acid profiles to those obtained with the conventional liquid–liquid extraction method. It is simple, rapid and accurate for routine analyses of esterified fatty acids in biological materials.

## INTRODUCTION

The development of an analytical method for the routine simultaneous identification and quantification of a variety of fatty acids is desirable for use in various fields. Recently, the combination of pre-column labelling and reversed-phase high-performance liquid chromatography (HPLC) has been extensively employed for the analysis of saturated and unsaturated fatty acids[1–23]. However, these studies did not consider quantitative aspects[1–9], were not very successful in the simultaneous separation of series of fatty acid derivatives and/or needed a fairly long analysis time or a complicated technique. In addition, for the analysis of esterified fatty acids in fats and oils, an extraction step was needed following saponification in order to obtain the free fatty acids prior to derivatization.

We previously demonstrated[24] that both short- and long-chain fatty acids can be converted into their 2-nitrophenylhydrazides and separated by reversed-phase HPLC with methanol–water as the eluent. This paper describes a method for the

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direct derivatization of saponified samples of fats and oils without an extraction step, and the simultaneous microanalysis of fourteen kinds of $C_{10:0}$–$C_{22:6}$ fatty acid hydrazides in a reversed-phase HPLC system.

## EXPERIMENTAL

### *Reagents and chemicals*

Oleic, linoleic, linolenic, arachidonic acids, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (1-EDC · HCl) and L-α-phosphatidylcholine were purchased from Sigma (St. Louis, MO, U.S.A.). Lauric, myristic, palmitic, palmitoleic and stearic acids were obtained from Japan Chromato Kogyo (Tokyo, Japan). Capric, margaric acids, pyridine, 2-nitrophenylhydrazine hydrochloride (2-NPH · HCl) and butylated hydroxytoluene (BHT) were purchased from Tokyo Kasei Kogyo (Tokyo, Japan). Eicosatrienoic, eicosapentenoic and docosahexenoic acids were purchased from Funakoshi (Tokyo, Japan). Analytical reagent grade methanol and acetonitrile were obtained from Wako (Osaka, Japan). Methanol and water were distilled before use and the other reagents and chemicals were used without further purification.

Aqueous 2-NPH · HCl solution (0.02 *M*) and acidic 2-NPH · HCl solution (0.02 *M*) were prepared by dissolving the reagent in water and 0.25 *M* hydrochloric acid, respectively. Solutions of pyridine (3%, v/v) in ethanol and 1-EDC · HCl (0.25 *M*) in ethanol were prepared, then mixed in equal volumes. A solution of potassium hydroxide (15%, w/v) in methanol–water (80:20, v/v) was prepared.

### *Derivatization procedure*

This procedure has been described previously[24]. Briefly, to 0.1 ml of an ethanolic mixture of fatty acids, 0.2 ml of 2-NPH · HCl solution and 0.4 ml of 1-EDC · HCl solution in pyridine were added, and the resulting mixture was heated at 60°C for 20 min. After the addition of 0.1 ml of potassium hydroxide solution, the mixture was heated at 60°C for 15 min, then cooled. An aliquot (1–5 μl) of the mixture was injected directly into the liquid chromatograph.

### *Sample analysis*

*Method A (direct method).* Fat and oil samples were dissolved (0.5–1 mg/ml) in chloroform containing 0.005% (w/v) BHT as antioxidant. To 0.1 ml of the sample, 40 nmol of margaric acid were added as internal standard. After the solvent had been removed with a stream of nitrogen at room temperature, saponification was performed with 0.1 ml of 2.5 *M* potassium hydroxide–ethanol (2:8, v/v) at 90°C for 10 min. The mixture was cooled to room temperature and derivatized with acidic 2-NPH · HCl solution.

*Method B (extraction method).* To the saponified sample was added 1 ml of water. After acidification with 6 *M* hydrochloric acid, the free fatty acids were extracted twice with 2 ml of diethyl ether. The combined extracts were washed with 2 ml of water and then 3 ml of the ether layer was separated and evaporated. The residual fatty acids were dissolved in 0.1 ml of ethanol and derivatized with aqueous 2-NPH · HCl solution.

*HPLC analysis*

Analyses were carried out using a Model LC-5A liquid chromatograph (Shimadzu Seisakusho, Kyoto, Japan) equipped with an ODS guard columjn (30 × 4.6 mm I.D.) and a reversed-phase column of YMC-C8 (250 × 4.6 mm I.D., particle size 5 μm) obtained from Yamamura Chemical Institute (Kyoto, Japan). The column temperature was maintained at 30°C. A variable-wavelength UV detector (Shimadzu Model SPD-2A) was used to monitor the absorbance at 230 nm and the detector signals were recorded on a multi-pen recorder (Rikadenki Kogyo, Tokyo, Japan).

All analyses were performed isocratically using acetonitrile–water as the solvent system at a flow-rate of 1.2 ml/min. The pH of the solvents was maintained at 4.5 by adding acetonitrile–0.1 *M* hydrochloric acid. The solvents were filtered through Fluoropore filters (pore size 0.45 μm) (Sumitomo Electric Ind., Osaka, Japan) and degassed with a Sonifer B-12 (Branson Sonic, CT, U.S.A.) before use.

## RESULTS AND DISCUSSION

*Chromatographic conditions*

The conditions for HPLC separation of the fatty acid hydrazides were investigated using YMC-C8 reversed-phase column and different eluents comprising methanol and/or acetonitrile as the major solvent with varying amounts of water. The simultaneous separation of a mixture of fifteen kinds of fatty acid hydrazides with mobile phases comprising various mixtures of methanol and water was not possible because of the long retention times. Fig. 1a shows that with methanol–water (86:14, v/v) the separations of linolenic and eicosapentenoic acid hydrazides, and of linoleic, arachidonic and docosahexenoic acid hydrazides, were difficult.

The elution volumes of the fatty acid derivatives are affected principally by the number of carbon atoms and the number of unsaturated bonds in the fatty acid chains. In acetonitrile–water, unlike methanol–water, the number of unsaturated bonds seemed to be of greater importance. Also, the effect of the column temperature was important. A value of 30°C was used here to shorten the analysis time, with good resolution. Thus, satisfactory resolution and favourable retention times (within 15 min) of the $C_{10:0}$–$C_{22:6}$ fatty acid hydrazides were obtained in reversed-phase HPLC with isocratic elution using acetonitrile–water (85:15, v/v), as shown in Fig. 1b.

*Quantitative analysis*

Calibration curves were constructed by derivatizing increasing amounts of fatty acids in the presence of 40 nmol of margaric acid as internal standard and analysing as described above. The calibration test was replicated three times. From the chromatograms obtained, the relationships between the ratio of the peak heights of the acid hydrazides to that of the internal standard and the concentrations of the acids were calculated by the least-squares method. Table I lists the parameters and correlation coefficients of the calibration curves. Each plot was linear in the range from 2.5 to 5000 pmol per injection, and passed through the origin.

*Recovery and precision*

Recovery tests were performed by adding known amounts (10 and 40 nmol) of mixtures of fatty acids to L-α-phosphatidylcholine (0.5 mg/ml) and analysing by

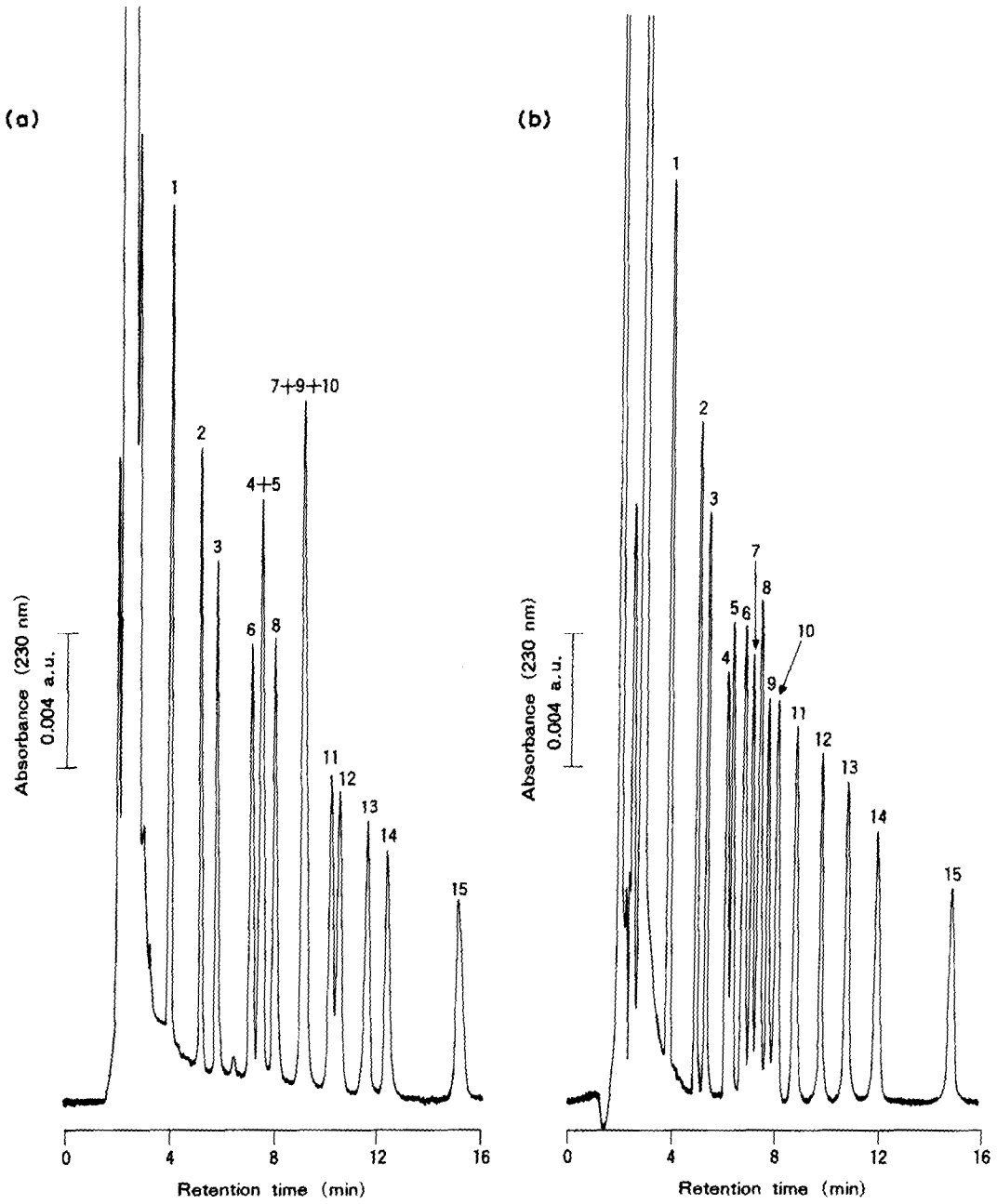


Fig. 1. Chromatograms of the 2-nitrophenylhydrazides of a mixture of saturated and unsaturated long-chain fatty acids obtained with UV detection. Flow-rate: 1.2 ml/min. Eluents and column temperatures: (a) methanol–water (86:14, v/v) and 50°C; (b) acetonitrile–water (85:15, v/v) and 30°C. Peaks: 1 = capric; 2 = lauric; 3 = myristoleic; 4 = eicosapentenoic; 5 = linolenic; 6 = myristic; 7 = docosahexenoic; 8 = palmitoleic; 9 = arachidonic; 10 = linoleic; 11 = eicosatrienoic; 12 = palmitic; 13 = oleic; 14 = margaric (internal standard); 15 = stearic acid hydrazide. Each peak corresponds to 150 pmol.

TABLE I

PARAMETERS AND CORRELATION COEFFICIENTS, $r$, OF CALIBRATION PLOTS FOR FATTY ACIDS

Calibration plots are expressed as regression lines of the form $y = ax + b$, where $Y$ is the peak height ratio and $x$, the amount of acid in pmol. Linear range: 2.5–5000 pmol.

| *Fatty acid* | *a* | *b* | *r* |
|---|---|---|---|
| Capric ($C_{10:0}$) | 0.030 | −0.043 | 0.9998 |
| Lauric ($C_{12:0}$) | 0.023 | −0.026 | 0.9999 |
| Myristoleic ($C_{14:1}$) | 0.020 | −0.013 | 0.9999 |
| Eicosapentenoic ($C_{20:5}$) | 0.015 | −0.025 | 1.0000 |
| Linolenic ($C_{18:3}$) | 0.017 | 0.028 | 0.9999 |
| Myristic ($C_{14:0}$) | 0.016 | 0.042 | 0.9999 |
| Docosahexenoic ($C_{22:6}$) | 0.016 | −0.019 | 0.9998 |
| Palmitoleic ($C_{16:1}$) | 0.018 | −0.032 | 1.0000 |
| Arachidonic ($C_{20:4}$) | 0.015 | −0.039 | 1.0000 |
| Linoleic ($C_{18:2}$) | 0.015 | −0.044 | 0.9999 |
| Eicosatrienoic ($C_{20:3}$) | 0.014 | −0.030 | 0.9998 |
| Palmitic ($C_{16:0}$) | 0.013 | 0.016 | 0.9999 |
| Oleic ($C_{18:1}$) | 0.012 | −0.026 | 0.9998 |
| Stearic ($C_{18:0}$) | 0.008 | 0.023 | 0.9998 |

the direct method. Table II shows the recoveries of each fatty acid; the range of 98.1–102.6% is sufficient for practical applications.

The intra-assay precision was evaluated by analysing the same sample of L-α-phosphatidylcholine (0.5 mg/ml) six times. The inter-assay precision was determined by analysing spiked L-α-phosphatidylcholine on different days over 1 week ($n$ = 6). Fig. 2 shows the fatty acid profile of L-α-phosphatidylcholine. The fatty acids in the sample were identified by comparison of the retention times of their

TABLE II

ANALYTICAL RECOVERY OF FATTY ACIDS ADDED TO L-α-PHOSPHATIDYLCHOLINE

| *Fatty acid* | *Added (nmol)* | *Found (nmol)** | *Recovery (%)** | *C.V. (%)* | *Added (nmol)* | *Found (nmol)** | *Recovery (%)** | *C.V. (%)* |
|---|---|---|---|---|---|---|---|---|
| $C_{10:0}$ | 10 | 9.84 ± 0.08 | 98.4 ± 0.8 | 0.8 | 40 | 39.47 ± 0.49 | 98.7 ± 1.2 | 1.2 |
| $C_{12:0}$ | 10 | 9.99 ± 0.14 | 99.9 ± 1.4 | 1.4 | 40 | 39.95 ± 0.35 | 99.9 ± 0.8 | 0.8 |
| $C_{14:0}$ | 10 | 9.98 ± 0.19 | 99.8 ± 1.9 | 1.9 | 40 | 39.65 ± 0.16 | 99.1 ± 0.4 | 0.4 |
| $C_{14:1}$ | 10 | 10.03 ± 0.14 | 100.3 ± 1.4 | 1.4 | 40 | 39.27 ± 0.40 | 98.2 ± 1.0 | 1.0 |
| $C_{16:0}$ | 10 | 10.00 ± 0.17 | 100.0 ± 1.7 | 1.7 | 40 | 39.88 ± 0.41 | 99.7 ± 1.0 | 1.0 |
| $C_{16:1}$ | 10 | 9.82 ± 0.17 | 98.2 ± 1.7 | 1.7 | 40 | 40.68 ± 0.85 | 101.7 ± 2.1 | 2.1 |
| $C_{18:0}$ | 10 | 10.04 ± 0.20 | 100.4 ± 2.0 | 2.0 | 40 | 39.52 ± 0.39 | 98.8 ± 1.0 | 1.0 |
| $C_{18:1}$ | 10 | 10.06 ± 0.11 | 100.6 ± 1.1 | 1.1 | 40 | 39.89 ± 0.16 | 99.7 ± 0.4 | 0.4 |
| $C_{18:2}$ | 10 | 10.02 ± 0.15 | 100.2 ± 1.5 | 1.5 | 40 | 40.24 ± 0.25 | 100.6 ± 0.6 | 0.6 |
| $C_{18:3}$ | 10 | 10.04 ± 0.15 | 100.4 ± 1.5 | 1.5 | 40 | 39.54 ± 0.20 | 98.9 ± 0.5 | 0.5 |
| $C_{20:3}$ | 10 | 9.83 ± 0.17 | 98.3 ± 1.7 | 1.7 | 40 | 41.05 ± 0.64 | 102.6 ± 1.6 | 1.6 |
| $C_{20:4}$ | 10 | 10.14 ± 0.10 | 101.4 ± 1.0 | 1.0 | 40 | 40.88 ± 0.77 | 102.2 ± 1.9 | 1.9 |
| $C_{20:5}$ | 10 | 9.87 ± 0.26 | 98.7 ± 2.6 | 2.6 | 40 | 40.27 ± 0.73 | 100.7 ± 1.8 | 1.8 |
| $C_{22:6}$ | 10 | 9.94 ± 0.12 | 99.4 ± 1.2 | 1.2 | 40 | 39.24 ± 0.89 | 98.1 ± 2.2 | 2.2 |

* Mean ± S.D. ($n$ = 3).

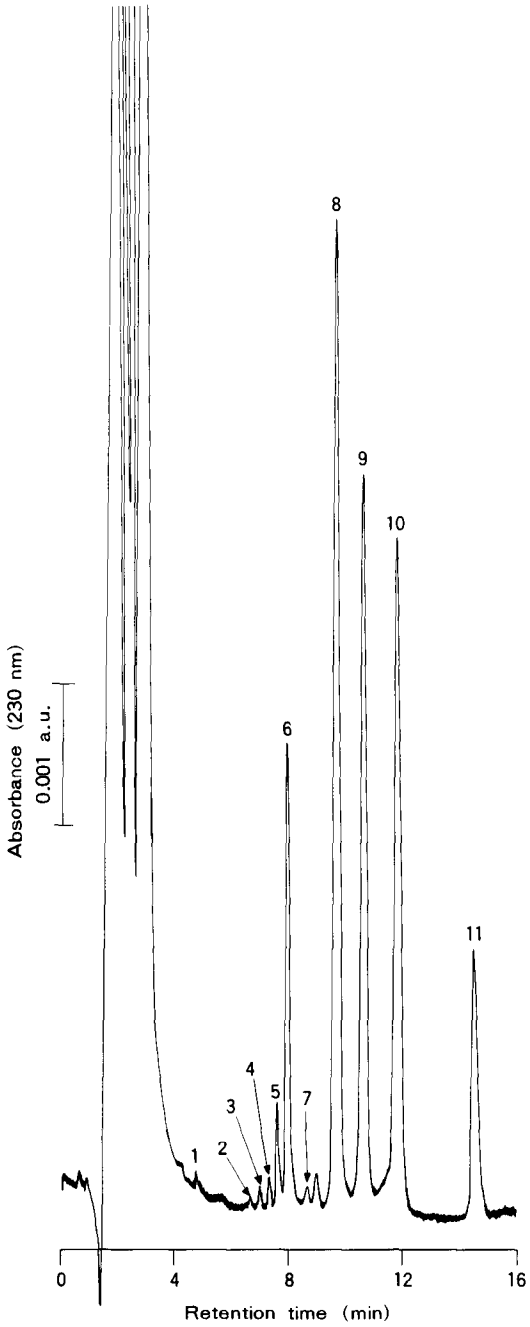


Fig. 2. Fatty acid profile of L-α-phosphatidylcholine. Eluent: acetonitrile–water (85:15, v/v); flow-rate 1.2 ml/min. Column temperature; 30°C. Peaks: 1 = lauric; 2 = myristic; 3 = docosahexenoic; 4 = palmitoleic; 5 = arachidonic; 6 = linoleic; 7 = eicosatrienoic; 8 = palmitic; 9 = oleic; 10 = margaric (internal standard); 11 = stearic acid hydrazide.

TABLE III

PRECISION OF THE DIRECT METHOD FOR DETERMINATION OF FATTY ACIDS IN L-α-PHOSPHATIDYLCHOLINE

| *Fatty acid* | *Intra-assay (n = 6)* | | *Inter-assay (n = 6)* | |
|---|---|---|---|---|
| | *Mean ± S.D. (nmol/mg)* | *C.V. (%)* | *Mean ± S.D. (nmol/mg)* | *C.V. (%)* |
| $C_{12:0}$ | 10.51 ± 0.26 | 2.5 | 11.12 ± 0.23 | 2.1 |
| $C_{14:0}$ | 15.97 ± 0.22 | 1.4 | 14.82 ± 0.24 | 1.6 |
| $C_{16:0}$ | 931.73 ± 21.43 | 2.3 | 908.42 ± 16.35 | 1.8 |
| $C_{16:1}$ | 26.83 ± 0.40 | 1.5 | 27.51 ± 0.63 | 2.3 |
| $C_{18:0}$ | 403.24 ± 7.66 | 1.9 | 421.38 ± 5.90 | 1.4 |
| $C_{18:1}$ | 767.90 ± 7.68 | 1.0 | 785.10 ± 18.06 | 2.3 |
| $C_{18:2}$ | 372.62 ± 4.47 | 1.2 | 351.47 ± 7.38 | 2.1 |
| $C_{20:3}$ | 15.33 ± 0.37 | 2.4 | 16.43 ± 0.46 | 2.8 |
| $C_{20:4}$ | 94.94 ± 1.71 | 1.8 | 90.89 ± 2.45 | 2.7 |
| $C_{22:6}$ | 20.19 ± 0.42 | 2.1 | 21.95 ± 0.70 | 3.2 |

hydrazides with those of standards. Table III shows that the direct method has a satisfactory precision, the intra- and inter-assay coefficients of variation being $\leqslant 2.5$ and $\leqslant 3.2\%$, respectively. These results indicate that the direct method can be used for quantitative analyses of esterified and non-esterified fatty acids.

*Application*

The direct and extraction methods were applied to the determination of fatty acids in fat and oil samples. The amount of each fatty acid was calculated from the calibration curves. The fatty acid compositions as determined by the two methods are compared in Table IV, and are in excellent agreement.

TABLE IV

COMPARISON OF ANALYSES OF FATTY ACIDS IN FAT AND OIL SAMPLES BY METHODS A AND B

| *Sample* | *Method* | *Total (mmol/g)** | *Composition (mol %)*** | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | $C_{10:0}$ | $C_{12:0}$ | $C_{14:0}$ | $C_{16:0}$ | $C_{16:1}$ | $C_{18:0}$ | $C_{18:1}$ | $C_{18:2}$ | $C_{18:3}$ |
| Corn oil | A | 3.248 ± 0.045 | | | | 14.76 | | 2.56 | 26.28 | 54.64 | 1.76 |
| | B | 3.218 ± 0.074 | | | | 15.21 | | 2.65 | 26.21 | 54.36 | 1.57 |
| Olive oil | A | 2.375 ± 0.074 | | | | 12.17 | 0.66 | 4.14 | 75.47 | 6.80 | 0.75 |
| | B | 2.458 ± 0.096 | | | | 12.88 | 0.64 | 4.39 | 74.65 | 6.72 | 0.73 |
| Safflower oil | A | 2.952 ± 0.073 | | | | 10.05 | | 2.89 | 12.97 | 74.08 | |
| | B | 2.855 ± 0.104 | | | | 10.39 | | 2.99 | 12.94 | 73.68 | |
| Butter fat | A | 1.954 ± 0.077 | 5.08 | 3.87 | 13.68 | 42.51 | 1.40 | 12.25 | 21.21 | | |
| | B | 2.034 ± 0.079 | 4.86 | 3.54 | 14.23 | 40.65 | 1.60 | 12.24 | 22.89 | | |
| Margarine fat | A | 2.196 ± 0.072 | | 0.76 | 1.53 | 35.72 | | 7.52 | 39.92 | 13.12 | 1.43 |
| | B | 2.100 ± 0.083 | | 0.86 | 2.15 | 36.06 | | 7.56 | 39.21 | 13.03 | 1.20 |

* Mean ± S.D. ($n = 3$).
** Mean ($n = 3$).

## CONCLUSIONS

In the case of the conventional methods of analysis of esterified fatty acids in fats and oils an extraction step is needed following saponification in order to obtain the free fatty acids. This step might cause difficulties in the analysis of low levels and/or volatile fatty acids. In the desired method, the saponified sample can be derivatized directly without extraction. Furthermore, pmol amounts of fourteen biologically important fatty acid hydrazides were separated in a short retention time (15 min), with symmetrical peaks. This method may be very useful for the routine analysis of fatty acids in biological tissues and fluids.

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