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METHOD FOR EVALUATING THE BIOCONVERSION OF RADIOACTIVE POLYUNSATURATED FATTY ACIDS BY USE OF REVERSED-PHASE LIQUID CHROMATOGRAPHY

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SUMMARY

Reversed-phase high-performance liquid chromatography on a thermostatted octadecylsilyl column was used to separate mixtures of labelled polyunsaturated fatty acids (as their methyl esters) formed by successive desaturations and elongations of labelled linoleic (18:2 $n-6$) or linolenic (18:3 $n-3$) acid by rat liver microsomes. Acetonitrile–water mixtures were used for elution of the esters. Unsaturated and saturated esters were detected by their refractive indices. The order of elution of fatty acid methyl esters in complex mixtures varies as a function of the chain length and unsaturation, analysis temperature, water concentration and solvent flow-rate. The peak areas vary as a function of the unsaturation. Specific radioactivities of ^{14}C-labelled fatty acids and the percentage distribution of radioactivity among fatty acids from complex mixtures can be efficiently determined by collection and direct measurement of the radioactivity in the solvent by liquid scintillation counting. The method can be applied to complete compositional analysis, but is especially useful for determination of specific radioactivities during studies on the metabolic conversion of labelled polyunsaturated fatty acids.

INTRODUCTION

Polyunsaturated fatty acids (PUFAs) are significant constituents of biological membrane phospholipids[1]. They are also substrates for oxygen-containing compounds such as prostaglandins, leucotrienes, hydroxylated fatty acids and related substances[2].

In animal cells such as hepatocytes, the most important PUFAs are synthesized from linoleic acid (18:2 $n-6$)* and linolenic acid (18:3 $n-3$) of dietary origin according to Scheme 1.

* This symbolism means that the fatty acid comprises eighteen carbon atoms and two double bonds, the first one being at the sixth carbon atom when counted from the methyl end, the second one being three carbon atoms further on.

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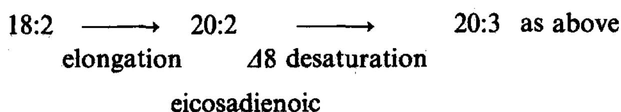
n-6 fatty acids	reaction	n-3 fatty acids
18:2 linoleic		18:3 α-linolenic
↓	Δ6 desaturation	↓
18:3 γ-linolenic		18:4 stearidonic
↓	elongation	↓
20:3 dihomo-γ-linolenic		20:4 eicosatetraenoic
↓	Δ5 desaturation	↓
20:4 arachidonic		20:5 eicosapentaenoic
↓	elongation	↓
22:4 docosatetraenoic		22:5 docosapentaenoic
↓	Δ4 desaturation	↓
22:5 docosapentaenoic		22:6 docosahexaenoic

Scheme 1. The symbol Δ6, etc. means that the double bond is formed at the sixth carbon atom when counted from the carboxyl end.

In the $n-6$ series, another pathway might start as follows:



The biosynthesis of PUFAs can be studied in animals by using ^{14}C-labelled fatty acids as precursors and by determining the radioactivity of the newly formed fatty acids after they have been separated.

Up to now this separation has usually been achieved by gas chromatography (GC) and the radioactivity measured either automatically at the column outlet for relatively high radioactivities[3] or after trapping on glass wool and counting by liquid scintillation for low radioactivities[4]. In both cases packed columns were used which

gave good separations. However, with this type of chromatography, only small quantities of fatty acids can be separated in one step. This represented a drawback when low specific radioactivities were encountered. Furthermore, the analysis was time-consuming since in the presence of polar phases permitting the best separations the most unsaturated fatty acids exhibited the longest retention times. Besides, they were eluted after the precursor, when biosynthesis experiments were performed. These newly formed fatty acids, generally of low radioactivity, may be affected by peak tailing of the precursor, usually highly radioactive. Finally, the method is still more time-consuming when the radioactive fatty acid has to be trapped before being counted.

It is now possible to separate fatty acids as esters by reversed-phase liquid chromatography (RPLC)[5–42], especially PUFAs, in less time than by GC[7,16,21,26,27,33,36,39] and in higher amounts when needed[11,41]. This prompted us to experiment with this type of chromatography to solve our analytical problems. The good results obtained with labelled trioleoylglycerol fractionated by RPLC[43] suggested that the same might be true with labelled polyunsaturated fatty acid esters, as demonstrated by others[21,27].

Accordingly we propose a method for separating and fractionating methyl esters of radioactive polyunsaturated fatty acids by RPLC and for measuring their radioactivity directly in the eluting solvent by liquid scintillation counting.

EXPERIMENTAL

Samples

[1-^{14}C]18:2 [Centre d'Etudes Nucléaires (CEA), Gif sur Yvette, France] was used in all the experiments carried out with a radioactive fatty acid. It was diluted with cold 18:2 (Sigma, St. Louis, MO, U.S.A.). Pure methyl linoleate was prepared by collecting the methyl ester separated by RPLC[43]. To study the separation of PUFAs by RPLC, two GC mixtures of methyl esters from Nu-Chek-Prep (Elysian, MN, U.S.A.) were used: the 1A mixture (16:0, 18:0, 18:1 $n-9$, 18:2 $n-6$, 18:3 $n-3$) and the 8A mixture (20:0, 20:1 $n-9$, 20:2 $n-6$, 18:3 $n-6$). The following fatty acid methyl esters were obtained from Sigma: 18:2 $n-6$, 20:4 $n-6$, 22:4 $n-6$, 20:5 $n-3$, 22:6 $n-3$. A distillate from blackcurrent seed oil (Nestlé, Vevey, Switzerland) provided 18:4 $n-3$. The methyl ester of 22:5 $n-6$ was isolated by RPLC from adipose tissue of trout deficient in 18:3 $n-3$ (ref. 44).

Liquid chromatography

A Model 6000 A solvent-delivery system and a R 401 differential refractometer (Waters Assoc., Milford, MA, U.S.A.) were used. The 250 mm × 4.0 mm I.D. Hibar Lichrocart, Superspher RP 18 (4 μm particles) column was obtained from Merck (Darmstadt, F.R.G.). It was maintained at a constant temperature in a jacket with circulating water. The water temperature was regulated by a thermostat (Model 33194 Polystat I, Bioblock, Strasbourg, France). The device, shown in Fig. 1, allows very reproducible analyses to be performed. The analyses were carried out isocratically at 30 or 40°C, using acetonitrile–water (95:5 or 93:7, v/v) as the mobile phase at a flow-rate of 1.2 or 1.0 ml min^{-1}. Acetonitrile was of analytical grade (SDS, Peypin, France) and water was twice-distilled. The mixture was filtered through a Millipore

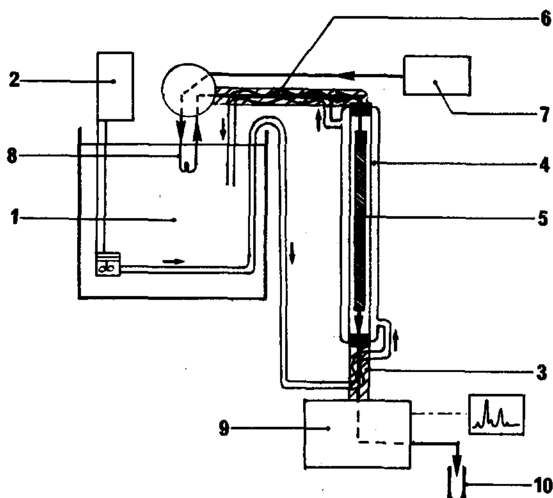


Fig. 1. Device for column thermostatting. Several litres of distilled water contained in a bath (1) are heated by a Polystat (2) which regulates the temperature and forces the water to circulate first close to the outlet column capillary into a glass wool, isolated muff (3), then through a jacket (4) containing the RPLC column (5) and lastly close to the column-inlet capillary into the same type of device as at the outlet (6), before returning to the water-bath. The solvent is delivered via a pump (7) from a reservoir at room temperature and is heated by circulating through a stainless-steel capillary loop (8) placed in the water-bath. Temperature decrease is avoided by the thermostatted devices (3, 6) before the solvent enters the column and at the outlet before it enters the refractive index detector (9). Samples are injected in the heated loop (8), avoiding possible precipitation of saturated long-chain compounds. A thermometer inside the water jacket (4) controls the column temperature. Solvent from the detector and containing an eluted labelled fatty acid methyl ester is collected in a 20-ml polyethylene vial (10) for radioactivity counting.

membrane (pore size 0.5 μm) and vacuum degassed for 1 min before use. The fatty acid methyl ester mixtures were dissolved in pure acetonitrile before injection. Peak areas were measured by means of an Enica 21 integrator-calculator (Delsi Instruments, Suresnes, France). Under the conditions used the detector response was found to be linearly related to the amount of the individual methyl ester detected.

To collect the radioactive methyl ester fractions, the solvent issuing from the detector through a 15 cm $\times$ 0.1 mm I.D. stainless-steel tube was collected into a polyethylene vial, routinely thirteen drops after the beginning of the peak registered on the chromatogram. This delay represents the void volume of the detector plus that of the collection tube. It was first determined after chromatography of [^{14}C]tri-oleoylglycerol as previously described[43] and verified with methyl-[1-^{14}C]linoleate in this work.

Gas chromatography

The fatty acid methyl esters were prepared from methanol–boron trifluoride[45]. The analyses were performed on a Becker-Packard Model 417 gas chromatograph, equipped with a 30 m $\times$ 0.4 mm I.D. glass capillary column coated in the laboratory with Carbowax 20M (Applied Science Labs., State College, PA, U.S.A.) at a constant temperature of 195°C and a nitrogen flow-rate of 3 ml min^{-1}. The column was equipped with a ROS injector[46] (Spiral, Dijon, France). Flame ionization detection

was employed. Peak areas were measured by means of an Enica 21 integrator-calculator (Delsi Instruments). Calibration factors for quantitations were calculated using standard mixtures of fatty acid methyl esters (Nu-Chek-Prep).

Radioactivity

Radioactivity was measured by liquid scintillation counting with Permafluor III or Picofluor 15 (Packard Instrument, Rungis, France) using a Packard Model A 300 CD spectrometer. The polyethylene vials (7 or 20 ml) were obtained from Kartell (Noviglio, Italy). Each sample was counted four times for 5 min. Counting efficiencies were estimated by external standardization (Packard).

RESULTS AND DISCUSSION

Separation of polyunsaturated fatty acid methyl esters

In experiments on PUFAs biosynthesis, the mixture to be analyzed generally comprises different labelled fatty acids, *i.e.*, the precursor and the derived fatty acids. It also contains different unlabelled fatty acids originating from the biological material used. The first problem encountered with such a mixture is to separate these different fatty acids with sufficient resolution in order to provide accurate radioactivity measurements. Also, accurate mass determinations from peak areas must be made in order to determine the fatty acid composition.

The best separation of PUFAs in preliminary experiments, and from data in the literature, can be achieved by using RPLC[21,26,27,33,36,39], with a mixture of acetonitrile and water as the mobile phase[14,18,19,25,28,36,40], applied to methyl esters[10,12,16,21,22,27,29,41,42], especially when a differential refractometer was used as the detector[10,12,22,42]. Several chromatographic conditions were tested for different mixtures of fatty acid methyl esters.

Simple mixtures

Two chromatograms (Fig. 2) were obtained by RPLC of two synthetic mixtures under the conditions reported. The first mixture (A) comprised six PUFAs of the $n-6$ series, expected to be found after the bioconversion of 18:2 $n-6$ (see Introduction). The fatty acid methyl esters were well separated within a relatively short time (20 min) under the experimental conditions. The low peak tailing does not lead to cross-contamination between adjacent radioactive peaks. As previously stated by others[8,9,13,14,18,20,23,28–30,37], the retention time is related to chain length and to degree of unsaturation of the fatty acids. The higher the chain length, the longer is the retention time (compare 18:3 and 20:3 or 18:2 and 20:2), and the greater the unsaturation, the lower is the retention time (compare 20:4, 20:3 and 20:2). This illustrates the characteristic of RPLC; compounds are eluted in order of decreasing polarity[9,13,16,18]. Moreover, when a new double bond is added to a given fatty acid, the more unsaturated this fatty acid, the smaller is the influence of the additional double bond on the retention (compare 20:4 to 20:3 and 20:3 to 20:2)[18]. The reason may be that the new double bond is nearer to the polar (carboxyl) end of the molecule. Consequently, the notion of partition number[47], the number of carbon atoms $-2 \times$ number of double bonds, cannot apply to the separation of these fatty acid methyl esters by RPLC, as with triacylglycerols[48] and as is apparent from the effect of the position of the double bond on the retention time[13,18,23,42].

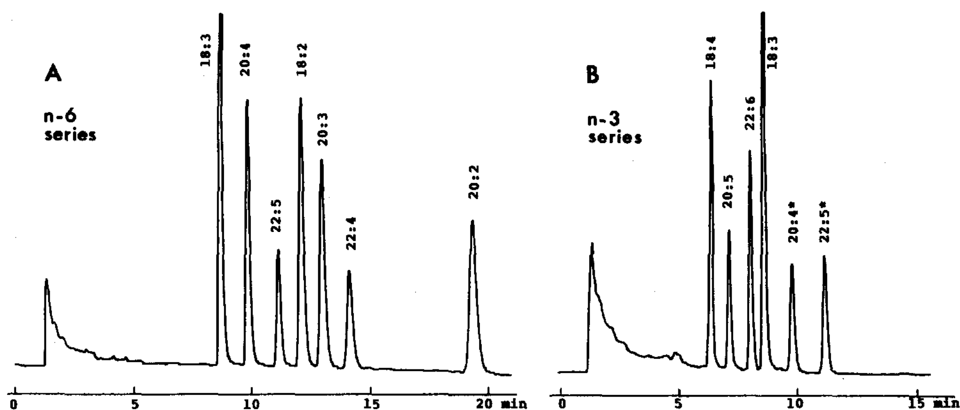


Fig. 2. Analysis by RPLC of a mixture of polyunsaturated fatty acid methyl esters of the $n-6$ family (A) and of the $n-3$ family (B). In the $n-3$ family the last two fatty acids 20:4 $n-6$ and 22.5 $n-6$ replaced their unavailable $n-3$ isomers. Stainless-steel column: 250 mm × 4 mm I.D. packed with 4 μm octadecylsilyl (C_{18}) reversed-phase materials. Eluent: acetonitrile–water (93:7, v/v) at 1 ml min^{-1}. Temperature: 30°C. Refractive index detector. Isocratic analysis.

In desaturation experiments, the elution order of PUFAs in RPLC is favourable for radioactivity measurement, since the newly formed fatty acids are clearly eluted before their less unsaturated precursors. This is the case for the pairs 18:3–18:2 ($\Delta 6$ desaturation) and 20:4–20:3 ($\Delta 5$ desaturation).

The second mixture (chromatogram B in Fig. 2) comprised four PUFAs of the $n-3$ series. The other two fatty acids 20:4 $n-3$ and 22:5 $n-3$ necessary to complete the mixture (see Introduction) were not available. They were replaced by their respective isomers of the $n-6$ series, whose retention volumes must not differ under our experimental conditions, since the retention volumes of 18:3 $n-3$ and 18:3 $n-6$ do not differ (see below). Similarly, the six fatty acid methyl esters were clearly separated, particularly the derivative–precursor pairs, 18:4–18:3 ($\Delta 6$ desaturation) and 20:5–20:4 ($\Delta 5$ desaturation) for which accurate radioactivity determinations can be predicted. The same remarks as made above apply to the influence of the chain length and the degree of unsaturation of fatty acids on their retention volumes in this type of chromatography.

Complex mixtures

When an *in vitro* experiment is undertaken to study the biosynthesis of polyunsaturated fatty acids, labelled 18:2 $n-6$ or 18:3 $n-3$ is incubated in an appropriate medium containing rat liver microsomes. At the end of the experiment, an analysis of the radioactive fatty acids is performed on the lipid extract of the incubation medium containing the labelled precursors, the labelled newly synthesized fatty acid and the unlabelled microsomal fatty acids. The last are comprised mainly of palmitic acid (16:0), stearic acid (18:0), oleic acid (18:1 $n-9$), linoleic acid (18:2 $n-6$), arachidonic acid (20:4 $n-6$) and docosahexaenoic acid (22:6 $n-3$). Such a mixture was prepared qualitatively and analyzed by RPLC under the same conditions as above. The chromatogram thus obtained is reported in Fig. 3A. It shows that the different polyunsaturated fatty acids expected to be formed from the precursors can

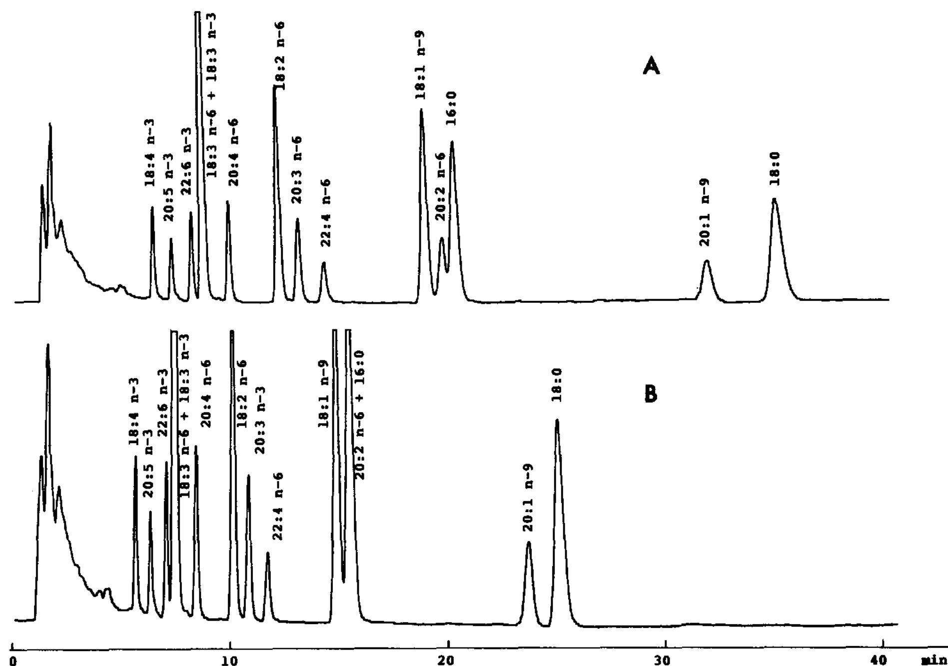


Fig. 3. Analysis by RPLC of a complex mixture of fatty acid methyl esters at 30 (A) or at 40°C (B). Other conditions as in Fig. 2.

easily be identified and thus their radioactivity measured after fractionation. Moreover, all the expected labelled and unlabelled fatty acids were sufficiently well separated to permit the fatty acid composition of the mixture to be determined precisely from the peak areas (see below). Under the conditions used, the analysis time was relatively short since 18:0 was eluted about 35 min after the injection. However, it can be shortened without decreasing the resolution too much, by modifying different chromatographic parameters, as will be shown below.

Influence of temperature. The preceding complex mixture of fatty acid methyl esters was analyzed at 40 instead of 30°C, the other conditions remaining the same. The chromatogram thus obtained (Fig. 3B) shows that the retention times were markedly reduced when the analysis temperature was increased, confirming the results reported by Tsuchiya *et al.*[28] for a wide variety of fatty acid 4-bromomethyl-7-acetoxycoumarin derivatives and by Ichinose *et al.*[26] for the 9-anthryldiazomethane derivative of eicosapentaenoic acid. In our experiment, the retention time of the stearic acid methyl ester was reduced from *ca.* 35 to 25 min for a 10°C increase, that is a 30% reduction in the analysis time of the mixture.

However, the time reduction was not the same for the different fatty acids. In Table I (first line) are reported the ratios of the retention times measured from the beginning of the solvent peak, at 40 and 30°C respectively for the different fatty acids listed according to the elution sequence. The ratios decreased when the retention times increased, which means that the fatty acids eluted last at 30°C had the most shortened retention times at 40°C or, in other words, that the retention time reduction affected the saturated or monounsaturated fatty acids much more than the polyunsaturated

TABLE I

EFFECT OF TEMPERATURE, FLOW-RATE AND NATURE OF SOLVENT ON RETENTION TIME FOR FATTY ACIDS OF DIFFERENT CHAIN LENGTHS AND DEGREES OF UNSATURATION

	18:4 $n-3$	*20:5* $n-3$	*22:6* $n-3$	*18:3* $n-3$, $n-6$	*20:4* $n-6$	*18:2* $n-6$	*20:3* $n-6$	*22:4* $n-6$	*18:1* $n-9$	*20:2* $n-6$	*16:0*	*20:1* $n-9$	*18:0*
40/30°C*	0.87	0.87	0.86	0.85	0.84	0.82	0.82	0.81	0.78	0.78	0.76	0.74	0.71
1.2/1.0 ml**				0.81	0.80	0.80	0.80	0.80	0.80	0.80	0.80	0.79	0.79
95:5/93.7***				0.75	0.73	0.72	0.71	0.69	0.69	0.67	0.70	0.65	0.66

* Ratios of the retention times, at 40 and 30°C respectively, with acetonitrile–water (93:7, v/v) at 1 ml min^{-1}.

** Ratios of the retention times, at 30°C with acetonitrile–water (93:7, v/v) at flow-rates of 1.2 and 1.0 ml min^{-1} respectively.

*** Ratios of the retention times at 30°C with acetonitrile–water, in the proportions 95:5 and 97:3 (v/v) respectively, at 1 ml min^{-1}. All the retention times were measured from the beginning of the solvent peak.

fatty acids. Consequently, for the latter, the loss of resolution was low, not impairing the accuracy of radioactivity determinations. In view of the data reported in Table I, it appears that the fatty acid chain length has a greater contribution to the reduction than the degree of unsaturation, when the analysis temperature is increased. This discriminative effect has been previously underlined by Ichinose *et al.*[26] with different fatty acid derivatives. This is illustrated in Fig. 3B by the cochromatography of 20:2 and 16:0 at 40°C, whereas these two fatty acids were separated at 30°C.

In conclusion, from a practical point of view, in order to reduce the duration of analysis, the temperature can be slightly increased since the resolution of the principal PUFAs is not greatly affected.

Influence of solvent flow-rate. Another mixture of fatty acids was analyzed at 30°C with the same mixture of acetonitrile–water (93:7, v/v) as the eluting solvent but at two different flow-rates, 1.0 and 1.2 ml min^{-1}. The ratios of the two retention times observed for the different fatty acids are reported in Table I (second line). The ratios were the same, *ca.* 0.80. Thus, whatever the type of fatty acid the retention time was reduced in the same proportion, *i.e.*, 20% for a 20% increase in the solvent flow-rate, indicating that the retention volume was not affected by the change in flow-rate. This implies that there is practically no loss of resolution between two close peaks. An increase in the solvent flow-rate thus appears to be a good means of reducing the analysis duration without modifying the elution pattern.

Influence of solvent nature. The same mixture as above was analyzed with the same acetonitrile–water mixture but at two different proportions of water, namely 93:7

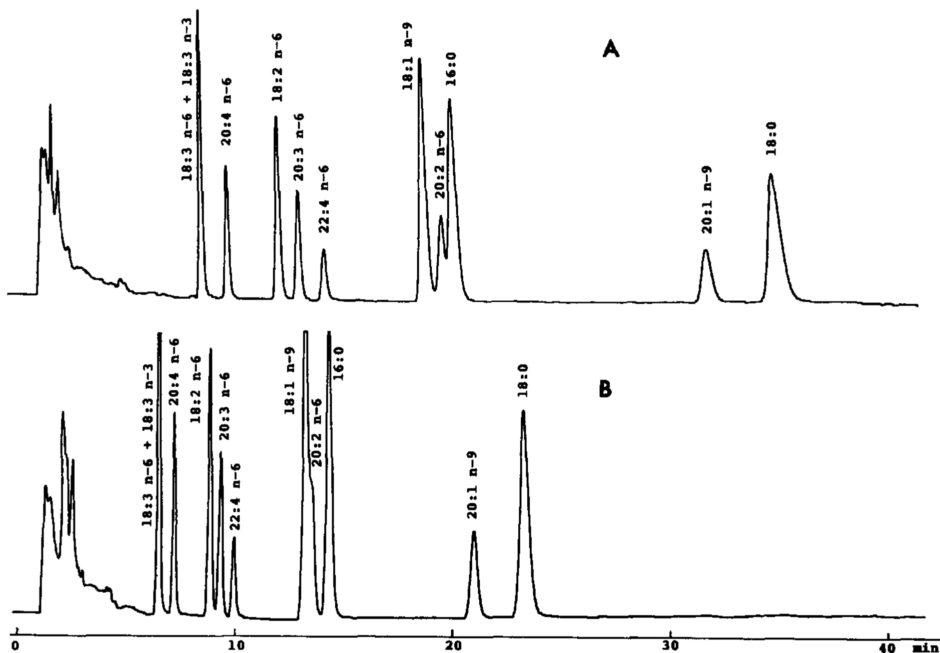


Fig. 4. Analysis by RPLC of a complex mixture of fatty acid methyl esters with acetonitrile–water at two different water concentrations: 93:7, v/v (A), 95:5, v/v (B). Other conditions as in Fig. 2.

TABLE II

RESPONSE OF THE REFRACTIVE INDEX DETECTOR TO FATTY ACID METHYL ESTERS ANALYZED BY RPLC

	18:4 n − 3	*20:5 n − 3*	*22:6 n − 3*	*18:3 n − 3, n − 6*	*20:4 n − 6*	*18:2 n − 6*	*20:3 n − 6*	*22:4 n − 6*	*18:1 n − 9*	*20:2 n − 6*	*16:0*	*20:1 n − 9*	*18:0*
GC/RPLC* (n = 5)	0.90 ±0.03	0.84 ±0.04	0.86 ±0.04	0.92 ±0.03	0.93 ±0.04	0.95 ±0.02	0.92 ±0.02	0.88 ±0.03	1.00 ±0.03	1.01 ±0.02	1.08 ±0.03	1.01 ±0.02	1.08 ±0.03

* Ratios of the percentages of the fatty acid methyl esters present in the complex mixture previously analyzed (Fig. 3A) as determined by GC after application of calibration factors, to the percentages of areas determined after analysis of the mixture by RPLC. Analytical conditions: temperature, 30°C; solvent, acetonitrile–water (93:7, v/v) at 1.0 ml min^{-1}. Values are means ± S.E. of five determinations.

TABLE III

RADIOACTIVITY MEASUREMENT BY LIQUID SCINTILLATION OF FATTY ACID METHYL ESTERS ACCORDING TO THE RELATIVE PROPORTION OF THE ELUTING SOLVENT AND SCINTILLATING LIQUID

Scintillating liquid*	*Permafluor III*		*Picofluor 15*		*Permafluor III*							
Quantity of elution solvent (ml)	0	1	0	1	0	1	2	3	0	1	2	3
Quantity of scintillating liquid (ml)	5	4	5	4	10	10	10	10	15	15	15	15
Counting efficiency (%)**	94.2	90.8	92.5	90.4	96.6	94.7	94.2	93.3	97.1	96.4	94.7	94.4

* Scintillating liquids from Packard (Rungis, France).
** Efficiencies (cpm/dpm · 100) were determined by external standardization (Packard).

and 95:5 (v/v). The two chromatograms are shown in Fig. 4 and the ratios of the two retention times determined from the solvent peak start are listed in Table I (third line). It is seen that for a low decrease in water proportion (from 7 to 5%), the retention times were considerably reduced (from 25 to 35% according to the fatty acids). The significant influence of water (or of the solvent strength) has been observed by several authors using different fatty acid derivatives and different reversed-phase columns[8,9,11,14,15,18,20,21,25–27,30,32,34,35,38]. The most extensive study on free fatty acids was done by Aveldano *et al.*[21] who made the same observations as can be made for the methyl esters from our results (Table I). That is, the higher the hydrophobicity (the longer the chain length or the lower the unsaturation) the larger is the decrease in retention time with decreasing water proportion. Thus, with regard to chain length, for 16:0 and 18:0 the decrease was 30 and 34%, for 18:1 and 20:1 31 and 35%, for 18:2 and 20:2 28 and 33% and for 18:3 and 20:3 25 and 29% respectively. With regard to unsaturation, for the series 20:4, 20:3, 20:2, 20:1 the decrease in retention time was 27, 29, 33 and 35% respectively.

Quantitative analysis of fatty acid methyl esters

At the end of *in vitro* desaturation experiments, in addition to determining the radioactivity of certain fatty acids present in the medium lipids, it can also be useful to calculate the fatty acid composition of the total lipids, starting from the peak areas, or to calculate a specific activity, assuming that a measured radioactivity can be ascribed to a known quantity of the labelled fatty acid. To determine the detector (differential refractometer) response to the fatty acid methyl esters according to chain length and unsaturation, the most complex synthetic mixture (Fig. 3) was analyzed both by liquid chromatography and gas chromatography (GC) under the conditions reported in the Experimental section. Calibration factors determined by use of standard mixtures were applied to peak areas in GC analysis. The peak area percentage calculated from the values thus obtained represented the exact weight percentages of the fatty acid methyl esters present in the mixture. The ratios of these figures to those of the peak area percentages calculated after RPLC analysis are reported in Table II. It is seen that PUFA methyl esters exhibited a ratio less than one, and the more unsaturated the fatty acid, the lower was the ratio. The lowest value was observed with 20:5 $n-3$. Conversely, the saturated fatty acids showed the highest ratios. The question can be raised whether the ratios increase with retention time and not with decreasing unsaturation. Comparison of the ratios obtained with 22:4 (0.88) and 18:1 (1.00), which are not too distant on the chromatogram, or with 18:1 and 16:0 (1.08) which are very close to each other, shows that unsaturation is mostly responsible for the decrease in ratio.

The values reported in Table II indicate that for the same quantity of fatty acid methyl ester the area registered by using the differential refractometer in RPLC was higher with polyunsaturated than with saturated fatty acids. A similar result has been obtained previously with methyl esters[10,42] or with triacylglycerols[48].

Therefore, to calculate the composition of fatty acid mixtures analyzed by RPLC under the experimental conditions, it is necessary first to apply calibration factors to peak areas. These are the ratio values reported in Table II. Such factors can be determined directly using commercial standard mixtures. Analyses of fatty acid mixtures as methyl esters by RPLC under these conditions can replace GC analyses,

TABLE IV

RADIOACTIVITY RECOVERED AND AREA REGISTERED DURING PEAK COLLECTION

Results are expressed as means ± S.E. for n determinations.

							Mean
Injected radioactivity (dpm) $n = 4$	839 ±6	1626 ±16	3195 ±40	6317 ±12	12 635 ±88	24 715 ±132	
Recovered radioactivity (dpm) $n = 6$	820 ±10	1578 ±32	3110 ±44	6192 ±116	12 259 ±277	24 177 ±280	
Recovered / Injected	0.973 ±0.008	0.970 ±0.016	0.973 ±0.014	0.980 ±0.018	0.970 ±0.022	0.978 ±0.011	0.974* ±0.004
Peak area (arbitrary units) $n = 6$	3462 ±214	7109 ±347	13 420 ±337	26 840 ±338	53 173 ±1361	105 373 ±965	
Recovered radioactivity / peak area	0.233 ±0.008	0.222 ±0.002	0.232 ±0.004	0.231 ±0.002	0.231 ±0.001	0.229 ±0.001	0.229* ±0.004

* Mean ± S.E. of the six means.

when the amount of the available material is not too low. However, to separate positional isomers of PUFAs, capillary GC has to be used, in our case, whereas Pei *et al.*[13] succeeded in separating 18:3 $n - 3$ and 18:3 $n - 6$ *p*-bromophenacyl esters by RPLC. When labelled fatty acids are present in the mixture, mass determinations in addition to radioactivity measurement allow calculation of the specific radioactivity of these fatty acids.

Radioactivity measurements

Particular attention was paid to radioactivity measurements in the eluting solvent. In order to save time it was essential that radioactivity be measured directly after collection of a peak, in the presence of the eluting solvent. The latter contains water generally not miscible with scintillating liquids, which can impair radioactivity measurements. Accordingly, we have tested two scintillating liquids from Packard Instruments, namely Permafluor III in which water is insoluble and the more expensive Picofluor 15 in which water is soluble. When Permafluor III was added to the acetonitrile–water mixture used as the eluting solvent, water first appeared as an emulsion and then progressively precipitated leaving a clear supernatant. In contrast, when Picofluor 15 was added, water was maintained in solution and the mixture remained clear.

Tests of radioactivity counting were undertaken with both scintillating liquids. In a first set of experiments, vials of low capacity (7 ml) were chosen because of the high cost of the scintillating liquids. They contained 12.000 dpm of [1-^{14}C]18:2 and 1 ml of acetonitrile–water (93:7, v/v) corresponding to the elution of a peak at 20 min under the usual conditions of analysis. The vials were shaken, then placed in 20 ml polyethylene vials and the radioactivity was immediately counted (Table III). In the absence of eluting solvent (columns 1 and 3), the efficiency of counting was 94.2% with Permafluor III and less (92.5%) with Picofluor 15. In both cases, the presence of solvent decreased the efficiency to values too low for accurate counting. Apparently the presence of water droplets with Permafluor III did not hamper counting and this scintillating solution was preferred because of its lower cost, in the search for better conditions of counting. We therefore tested ethylene vials of higher capacity (20 ml) which can contain a higher amount of solvent, in the case of radioactive peaks eluted after 18:2 and a higher amount of scintillating liquid to improve the counting efficiency. Experiments were carried out in the presence of 0–3 ml of solvent added with a constant volume of 10 or 15 ml of scintillating liquid. Results reported in Table III (columns 5–12) show that the efficiency of counting was higher than before. It was also slightly higher with 15 ml than with 10 ml of scintillating liquid, but not sufficiently for 15 ml to be preferred.

Thus the use of 10 ml of Permafluor III in 20 ml polyethylene vials was adopted. The only problem was the formation of water droplets when the Permafluor solution was added to the elution solvent. Additional tests were carried out by counting for only 1 min the radioactivity 1, 5, 11, 16 and 60 min after the addition of Permafluor. At 60 min the water emulsion had completely disappeared, the supernatant over the precipitated water being quite clear. For each time tested, the efficiency of counting was exactly the same, showing that the presence of water in the elution solvent did not hamper radioactivity measurement.

Peak collection

Increasing amounts of pure methyl-[1-^{14}C]linoleate were injected onto the RPLC column. The peak was collected and its radioactivity was measured. Results in Table IV show that the radioactivity eluted with the peak increased in proportion to the radioactivity injected. The ratio was equal to 0.974 ($\pm$0.004). Thus 2.6% ($\pm$0.4%) of the injected radioactivity was not recovered. Experiments carried out under the same conditions but in the absence of the column showed that 1.6% ($\pm$0.1% for four determinations) was lost during injection. Peak tailing can most probably account for the rest (1.0%). Indeed, the radioactivity collected after elution of the labelled peak and during the same elution time amounted to 0.44% ($\pm$0.10% for six determinations). The radioactivity eluted just before was found to be 0.25% ($\pm$0.11%). It probably represented the beginning of the peak emergence.

The radioactivity following elution of the labelled peak is very low. However, it could cause relatively high contamination in biological experiments, when a newly synthesized PUFA of low radioactivity would be eluted just after the labelled precursor of high radioactivity. That is the reason why this method in which the more unsaturated fatty acids formed from the precursors are eluted first is preferred.

Table IV also shows that area of the labelled peak collected increased proportionally to the radioactivity collected. The ratio (radioactivity/peak area) was found to be 0.225 ($\pm$0.004). It is equivalent to a specific radioactivity. The lowest value was observed for the lowest amount of labelled material injected. The relative lack of precision is ascribable to the peak area determination (precision: $\pm$6%), not to the radioactivity measurement (precision: $\pm$1.2%). In consequence, the radioactivity distribution among fatty acids can be determined more precisely, even for low radioactivities, than the specific radioactivities of fatty acids.

CONCLUSIONS

In *in vivo* or *in vitro* experiments on the biosynthesis of polyunsaturated fatty acids, using radioactive precursors, one or several mixtures of fatty acids have eventually to be analyzed. The mixtures contain both labelled and unlabelled fatty acids. We have shown that it is possible by using RPLC both to measure the radioactivity of the newly formed fatty acids and to determine the fatty acid composition from the peak areas. From these two determinations, it is also possible to calculate the specific radioactivity of the labelled fatty acids. In RPLC, under the conditions used, the elution sequence of fatty acids is quite different from that observed in GC with polar phases. Polyunsaturated fatty acids are eluted very early, as narrow peaks, so that the solvent volume containing such a radioactive fatty acid is low. The subsequent measurement of radioactivity by liquid scintillation counting is thus accurate without high quenching which might be due to water in the elution solvent. Morever, the fatty acids synthesized by desaturation from the labelled precursors are more unsaturated than the precursors and thus are eluted earlier. This prevents them from being highly contaminated by peak tailing of the precursor which is generally highly labelled. The opposite is true in GC and in addition the method is much more time-consuming.

The RPLC separations of the different fatty acids in a mixture are generally of good quality. When the amount of material to be analyzed is not too low, the

sensitivity of the refractometer being relatively low, RPLC can replace GC advantageously. Different conditions can be used according to the nature of the fatty acids present in the mixture. If the mixture comprises long-chain monounsaturated and saturated fatty acids, the temperature can be increased and/or the water content of the solvent in acetonitrile can be decreased to shorten the analysis duration without loss of resolution. Conversely, if the mixture is rich in polyunsaturated fatty acids, as for example when PUFA biosynthesis is studied, it seems appropriate to work at a relatively low temperature (30°C) at a relatively high water content in acetonitrile (7%) and to increase the solvent flow-rate to decrease the duration. However, to achieve separation of isomers, capillary GC remains the method of choice.

To solve our problems linked to PUFA metabolism, RPLC presents the same accuracy as GC with, as additional qualities, simplicity and reduction in analysis time.

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