Short communication

Counter-current chromatographic separation of polyunsaturated fatty acids

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Abstract

Hexadecatrienoic acid (C16:3), octadecatetraenoic acid (C18:4), eicosapentaenoic acid (C20:5) and docosahexaenoic acid (C22:6) were purified in two steps by counter-current chromatography (CCC). The selected liquid phases were heptane–acetonitrile–water for the first step and heptane–methanol–water for the second. The upper non-polar phase was kept stationary in the coil of the instrument by a rotational force field. The separation was followed by UV detection at 210 nm and by analysing the eluted fractions by gas chromatography after esterification. The purity of the fatty acids therefore purified was checked by capillary gas chromatography. CCC may be an alternative to HPLC for the preparative-scale purification of such compounds.

1. Introduction

The ω-3 fatty acids possess a number of properties that make them valuable molecules in the biomedical and nutrition fields [1,2]. Eicosapentaenoic acid (C20:5, EPA) and docosahexaenoic acid (C22:6, DHA) have in particular been studied in oils containing large amounts of the acids or as ethyl esters in the presence of large amounts of other fatty acids methyl esters. According to Holman [3], these essential fatty acids are active in the growth and maintenance of human skin.

Their full purification is very difficult owing in particular to their easy peroxydation. It has been more or less achieved on a preparative scale, however, by the combination of different techniques such as solvent extraction, molecular distillation [4], urea inclusion [5], counter-current distribution (CCD) [6,7], counter-current chromatography (CCC) [8–10], thin-layer chromatography on a silver nitrate-coated plate [11], high-performance liquid chromatography (HPLC) [12,13], supercritical fluid extraction [14], supercritical fluid chromatography [15], organic synthesis [16] and enzymology [17].

We present here a two-step method for the purification of these fatty acids on a semi-preparative scale. The results can be extended to the preparative scale provided that a suitable instrument is available.

2. Experimental

2.1. Materials

A mixture of fatty acids was prepared by

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saponification of an oil extract from microalgae (*Skeletonema costatum*) which was cultured under industrial fermentation conditions [18–20]. It was stabilized with butylated hydroxytoluene (BHT).

2.2. CCC system

The chromatographic system consisted of a Waters (Milford, MA, USA) M45 isocratic constant solvent-delivery pump, a six-way injection valve with a sample loop, a Waters Model 486 UV–visible spectrophotometer with a semi-preparative cell and an ISCO 328 fraction collector. The CCC apparatus was a multi-layer coil separator extractor (P.C. Potomac, MD, USA). The column was a 61.4 m × 1.6 mm I.D. PTFE tube wound on a 10-cm diameter holder [21]. The rotational speed was constant during elution and was controlled with a stroboscope. Detection was carried out at 210 nm and the fractions were analysed by GC and GC–MS after esterification of the fatty acids to methyl esters [22,23].

The fatty acid fraction was injected into the RP-CCC system with heptane as the stationary phase and acetonitrile–water as the mobile phase. Elution was performed at room temperature at a flow-rate of 2 ml/min. Fractions were collected every 5 min (10 ml). After analysis, the different fractions were pooled and the solvent was evaporated; an aliquot was esterified to methyl esters and analysed by GC. The separation of these essential fatty acids, in the third- and fourth-eluted fractions, was then studied with the biphasic system heptane–methanol–water.

In order to obtain a better separation, the resolution was increased by optimization of the CCC parameters, viz., the composition of the mobile phase, the flow-rate, the rotational speed and the injection loop volume.

2.3. Semi-preparative scale CCC

In order to improve this separation method for further purification, we injected into the column gram levels of material with a 9-ml sample loop.

On-line detection was not possible and analysis of the fractions by GC was applied.

2.4. Identification methods

Esterification was performed with methanol in the presence of BF_3 (14%, w/v). The chromatograph (Shimadzu GC-14A) was equipped with a flame ionization detector and connected to an integrator (Shimadzu, Chrompack C-R5A). The column (Chrompack CP-Sil-5-CB) was a fused-silica megabore type (25 m × 0.25 mm I.D.). The conditions were as follows: column temperature, increased from 140 to 230°C at 3°C/min; injector and detector temperatures, 240°C; carrier gas, helium with a split of 40 ml/min. Before analysing the fatty acid methyl ester fractions by GC, it is possible to remove undesirable compounds from the esterification reaction and repurify the polyunsaturated fatty acids by described methods [9,10,24], i.e., a CCC run of the methyl esters with heptane–acetonitrile as a biphasic system.

For identification by GC–MS, experiments were performed on a Nermag R10-10C instrument combined with a gas chromatograph controlled by a Digital PDP11-23 Plus system (Delsi-Nermag, Argenteuil, France). The same column and temperature conditions as mentioned above were used. The retention times and mass spectra of the samples were compared with those obtained with standard C20:5ω3 and C22:6ω3 methyl esters (Sigma).

The free and methyl ester fatty acids were stored in solution in the dark with BHT as an antioxidant.

3. Results and discussion

3.1. Recovery of fatty acids from marine source

The analysis by GC of the total fatty acids of *Skeletonema costatum* shows a large number of fatty acids in the starting extracted oil. Dried algae could also be directly saponified with alcoholic KOH to recover the fatty acids quickly with the same ratios [25].

3.2. Purification of essential fatty acids

In this first CCC step, purification was carried out with RCOOH prepared directly from microalgae. All the saturated fatty acids were eliminated by a first CCC step with heptane–acetonitrile–3% water as a biphasic system. The main advantage of CCC is its efficiency, which allows one to remove all the non-essential fatty acids. In the microalgae we used, the recovered polyunsaturated fatty acids constituted about 20–40% (w/w) of the total fatty acids.

3.3. Chromatographic studies and separation

The equivalent chain length (ECL) of a fatty acid is defined as $ECL = N - 2n_{(C=C)}$, where N is the number of carbon atoms and $n_{(C=C)}$ is the number of double bonds in the fatty acid [26]. The ECL value for C16:3, C18:4, C20:5 and C22:6 is 10; the separation of these four compounds, with the same ECL, from marine oils is a challenging task.

In the reversed-phase mode, where heptane was the stationary phase and methanol–water was the mobile phase, the configuration was analogous to that in an octadecylsilyl (C_{18}) reversed-phase adsorption liquid chromatographic system. Polyunsaturated fatty acids have a COOH end, which could be ionized during elution, thus causing distortion of chromatograms. In order to suppress this ionization, a small amount of hydrochloric acid was added to the mobile phase. The elution mechanism depends on the alkyl chain length and the degree of unsaturation of the individual fatty acids.

In CCC we have $V_c = V_0 + V_s$ [27] with V_0 and V_s known (V_c = column volume; V_s = stationary phase volume); the general equation of chromatography, $V_R = V_0 + KV_s$, can also be written as $K = (V_R - V_0)/(V_c - V_0)$, so from the chromatograms, we can calculate directly the partition coefficients and the selectivity coefficients. As in HPLC, with an increasing proportion of water in the mobile phase to retain the compounds in the column better, the separation was increased as shown in Fig. 1. For each biphasic composition,
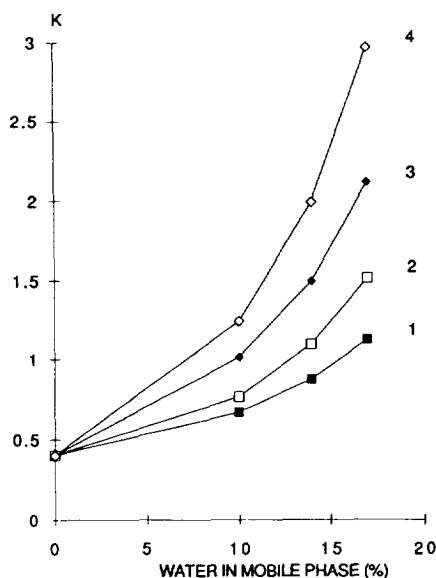

Fig. 1. Partition coefficients of four critical pairs of fatty acids versus the percentage of water in the mobile phase in RP-CCC. 1 = C16:3; 2 = C18:4; 3 = C20:5; 4 = C22:6.

the K values are a logarithmic function of the carbon chain length (Fig. 2).

Two parameters, the flow-rate of the mobile phase and the composition of the biphasic system, were important for optimizing the separation. Baseline resolution between EPA and DHA was obtained with 17% water in the mobile phase and after optimization of the chromatographic parameters, i.e., decreasing the flow-rate to 2.0 ml/min [21], increasing of the coil column rotational speed to 850 rpm and using a 0.5-ml injection loop [28]. For this separation (Fig. 3), the K and α values were as reported in Table 1.

The spectrophotometer response is a function of the degree of unsaturation in the carbon chain; for this reason, the mass ratio could not be obtained by comparison of peak areas without a calibration graph for each fatty acid.

The large range of flow-rate that can be used in CCC allows one to achieve the separation quickly at a high flow-rate and then to increase the resolution by decreasing the flow-rate.
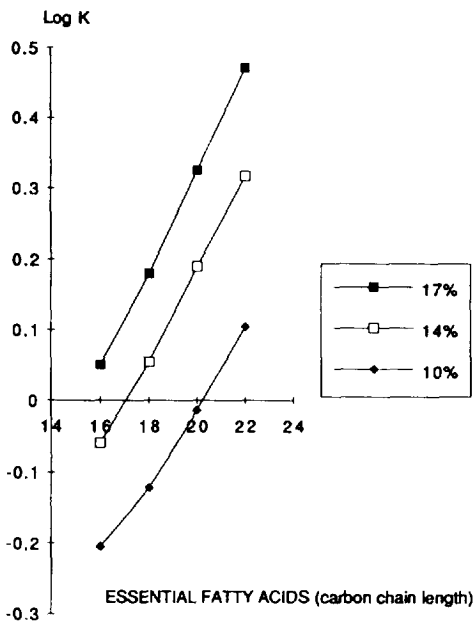
Fig. 2. Partition coefficients of four polyunsaturated fatty acids (critical pairs) from marine oil with different chain lengths with heptane–methanol containing different percentages of water as a biphasic system.

Fig. 3. RP-CCC of four essential fatty acids from *Skeletonema costatum*. 1 = C16:3; 2 = C18:4; 3 = C20:5; 4 = C22:6. $R_{s(EPA-DHA)} > 1$.

3.4. Semi-preparative scale

The high stationary volume increases the injection capacities in comparison with HPLC and decreases the saturation broading effects. The limiting factor is mainly due to the decrease in the stationary phase from variations of physical parameters such as viscosity and density difference when injection is being made. The crude sample is then eluted without separation occurring, but it can be totally recovered, e.g., through evaporation of solvents by a rotary evaporator under reduced pressure. Gram-level separations were achieved with a bench-top apparatus; we injected 2.4 g of total fatty acids into the 143-ml column and recovered 1 g of essential fatty acids (C16:3 43%, C18:4 7.5%, C20:5 45%, C22:6 4.5%).

Instruments with a larger column volume are available: an injection of 130 g of fatty acid esters was made with a 6.8-l apparatus. The liquid stationary phase makes the filling of the
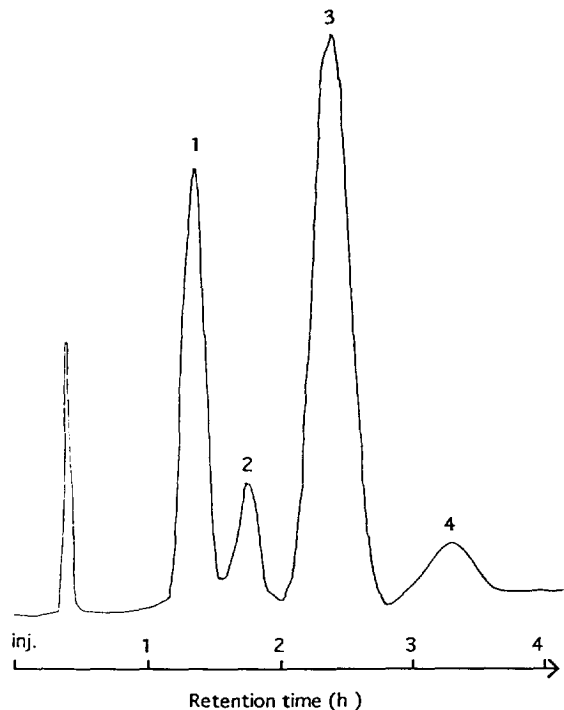
column faster and prevents irreversible adsorption phenomena with the support.

These results are encouraging for the separation of essential fatty acids on an industrial scale. Long-chain unsaturated fatty acids, which

Table 1
Partition coefficients of four essential fatty acids in the biphasic system heptane–methanol–water (500:415:85, v/v/v) used in counter-current chromatography

Fatty acid[a]	K	Selectivity factor
C16:3	1.1	$\alpha_{12} = 1.3$
C18:4	1.5	$\alpha_{23} = 1.4$
C20:5	2.1	$\alpha_{34} = 1.4$
C22:6	2.9	

[a] C20:5 = EPA and C22:6 = DHA.
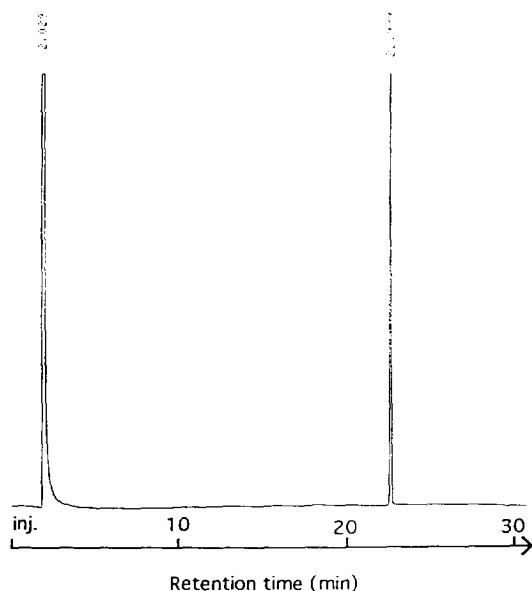
Fig. 4. GC of EPA methyl esters from microalgae purified by CCC. GC column, CP-Sil-5CD, 30 m × 0.25 mm I.D.; column temperature, increased from 140 to 230°C at 3°C/min; carrier gas, helium; flame ionization detection.

are generally susceptible to oxidation on a catalytic surface, are readily purified by CCC.

3.5. Peak identification

During elution when studying the chromatographic conditions for separation, the CCC fractions were systematically analysed, after esterification, by GC. The peaks of the fatty acid methyl esters were identified by comparison of retention times and mass spectra. Peak identification for the C20:5ω3 and C22:6ω3 esters was confirmed by comparison with the data for commercial standards. Fig. 4 shows the EPA and DHA purity, monitored by GC, after esterification to methyl esters.

4. Conclusions

CCC has been widely applied to the separation of various organic and inorganic materials, as an alternative and a complement to HPLC. CCC becomes especially attractive with increase in the column capacity and for the purification of labile compounds. Therefore, these results indicate that a separation process with a CCC instrument in order to obtain different compounds from microalgae is practicable and that this process will compete favourably with classical processes such as liquid and supercritical fluid extraction. Further experiments will be performed in order to increase the separations of different biological compounds from microalgae which are widely exploited and studied particularly for active molecule screening.

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