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Preparative-scale liquid chromatographic separation of ω-3 fatty acids from fish oil sources

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Abstract

A preparative-scale chromatographic process for the enrichment and isolation of closely related compounds such as ω-3 fatty acids from fish oil supplements is described. An analytical-scale procedure was scaled up to a preparative level process to provide a simple, rapid, selective and sensitive method for the fractionation of these essential acids. The fish oil triglycerides were saponified into their free fatty acids. The polyunsaturated fatty acids were then enriched through urea crystallization. Subsequently, the individual ω-3 fatty acids were fractionated on a Waters PrepPak unit consisting of six cartridges (μBondapak phenyl silica) using an isocratic ternary mobile phase of acetonitrile–water–tetrahydrofuran (45:35:20, v/v/v). The separation was accomplished within 60 min without any recycling of the solutes. The fractions collected were further analysed by analytical liquid chromatography.

1. Introduction

The separation of biological compounds such as fatty acids from highly complex mixtures such as natural lipids is one of the key problems in current biochemical research. Further, isolation and purification on a preparative or process scale are also basic problems facing organizations attempting to translate the products of separation science from the laboratory to commercial viability. Therefore, for the commercial exploitation of high-value-added products such as ω-3 fatty acids, the acids must first be separated from their triglycerides and purified to a high degree for their effective end use.

ω-3 Fatty acids have many beneficial effects on human health from both therapeutic and nutritional points of view [1–5]. The main ω-3 fatty acids are eicosapentaenoic acid (EPA, C20:5 ω3) and docosahexaenoic acid (DHA, C22:6 ω3). These polyunsaturated fatty acids may be responsible for alleviating certain human health disorders related to heart and circulation problems, inflammation and cancer. They also play a dominant role in the visual, nervous and reproductive systems of the human body [6]. It was further reported recently that DHA afford some protection against Alzheimer's disease and stimulate mental growth in children [7]. The human body cannot produce large amounts of these acids to counter these problems, so external supplementation of these acids is necessary to achieve good health results.

The important natural sources of these acids are fish oils such as sardine, mackerel, cod and menhaden, which contain levels of about 20–30% [8]. Highly pure ω-3 fatty acids are very expensive and in demand in biochemical and biomedical research. Moreover, the separation of polyunsaturated fatty acids such as ω-3 acids

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from natural oils such as fish oils has been an important and basic research problem in fats and oil chemistry since 1945. Several traditional and modern separation methods have been developed since then to isolate and enrich these compounds. However, fish oils consists primarily of triacylglycerols, commonly called triglycerides, which are too complex and heterogeneous for individual components to be isolated efficiently with a single separation method. Therefore, most efforts have been directed towards isolating these acids or their derivatives such as methyl and ethyl esters at higher levels of purity without oxidation and isomerization through cost effective methodology. In the past two decades, several investigators have successfully accomplished modest separations of these acids at higher concentrations on analytical and preparative scales with the combination of one or two conventional and modern methods [9–15].

The main separation methods are crystallization, distillation, supercritical fluid extraction and chromatography. Some of these processes are recent and their application may not be feasible in industrial-scale processes [16]. Column liquid chromatography has become an important and often indispensable technique for analytical- and preparative-scale separations in a wide variety of fields, including fatty acid separations, during the last two decades owing to its speed of analysis, resolving power and relative ease of operation [17–21].

Reversed-phase high-performance liquid chromatography (RP-HPLC) is perhaps the most widely used technique in lipid analysis because of its versatility. The substantial literature dealing with its theory, instrumentation and application and also the ready availability of equipment prompted us to choose RP-HPLC as the main method in the separation of ω-3 fatty acids [22–25].

An analytical-scale chromatographic methodology in combination with a urea crystallization procedure was successfully developed in this laboratory for the separation and determination of ω-3 fatty acids [26,27]. The method consists of three main steps: (1) saponification of fish oil triglycerides into their free fatty acids, (2) enrichment of the polyunsaturated fatty acid content from the mixed acid concentrate by a urea crystallization procedure and (3) separation of the individual ω-3 fatty acids by RP-HPLC. A Waters μBondapak free fatty acid column with a ternary mobile phase of acetonitrile–tetrahydrofuran–water (45:20:35, v/v/v) was used. The fatty acids were isolated on the basis of carbon chain length and degree of unsaturation. The salient feature in this procedure was the achievement of rapid separations of these compounds through π–π interactions between fatty acids and the packing material. However, complete baseline separations of the individual acids were not accomplished owing to the short hydrocarbon chain length of the packing material.

This paper describes the development of a preparative-scale production method based on the above analytical procedure for the isolation of high-purity EPA and DHA concentrates. Six RCM PrepPak cartridges packed with the same μBondapak phenyl packing material were employed. The cartridges were connected in series with a cartridge holder. The preparative column was 85 times larger than the analytical column previously used. The other conditions were similar to those in the analytical-scale method. The oxidation of these acids was prevented by adding a small amount (0.005% w/v) of the antioxidant, butylated hydroxyanisole (BHA).

2. Experimental

2.1. Chemicals, reagents and fish oils

Fatty acid standards were obtained from Sigma (St. Louis, MO, USA). The mobile phase solvents acetonitrile and tetrahydrofuran were purchased from Fisher (Fair Lawn, NJ, USA). Water was deionized with an SA-105 Elgastat Spectrum unit (Elga) and filtered using a 0.45-μm filter. The mobile phase was prepared by mixing appropriate volumes of acetonitrile (ACN), tetrahydrofuran (THF) and water and then degassed in a B-1200 E3 ultrasonic bath (Brandon, Danbury, CT, USA).

Chemicals and the solvents such as potassium hydroxide, urea, anhydrous magnesium sul-

phate, hexane and ethanol, used in saponification and urea crystallization, were obtained from Merck and Fisher. The solvents were nitrogen degassed prior to use. Butylated hydroxyanisole was procured from Sigma.

The fish oils sardine oil (refined but not deodorized) and sanomega oil were provided by NOF (Tokyo, Japan) and the cod liver oil was supplied by Pronova Biocare (Sandefjord, Norway).

2.2. Equipment

A schematic diagram of experimental set up is shown in Fig. 1. An M-45 isocratic pump (Waters, Milford, MA, USA) was connected to a manually operated injection valve (Valco, Houston, TX, USA) which was fitted with a 2-ml sample loop. By turning the valve, the eluent pushed the sample in the loop into the column. The column effluent was monitored with a Waters R-401 differential refractive index detector. The detector signal was amplified using a laboratory-made voltage amplifier. It was then converted into digital form by a personal computer equipped with an analog-to-digital interface card (Flytech Technology, Taiwan) with a 12-bit operation. The digitized data were stored and processed by a program written in GWBASIC. The column temperature was controlled by circulating water from a thermostated bath (Braun) through a water-jacket. An RCM PrePak 25 mm × 10 cm cartridge (Waters) packed with 10-μm μBondapak phenyl packing material (pore size 125 Å) was employed. The mobile phase flow-rate was ca. 10 ml min^{-1}. Chromatograms for the injection of 2-ml samples of fish oil concentrates after urea adduction were recorded on chart paper with an R02 electronic chart recorder (Rikadenki, Tokyo, Japan) and digitally on a Phillips PC-3200. The chromatograms were subsequently analysed on a μBondapak free fatty acid analysis column (Waters).

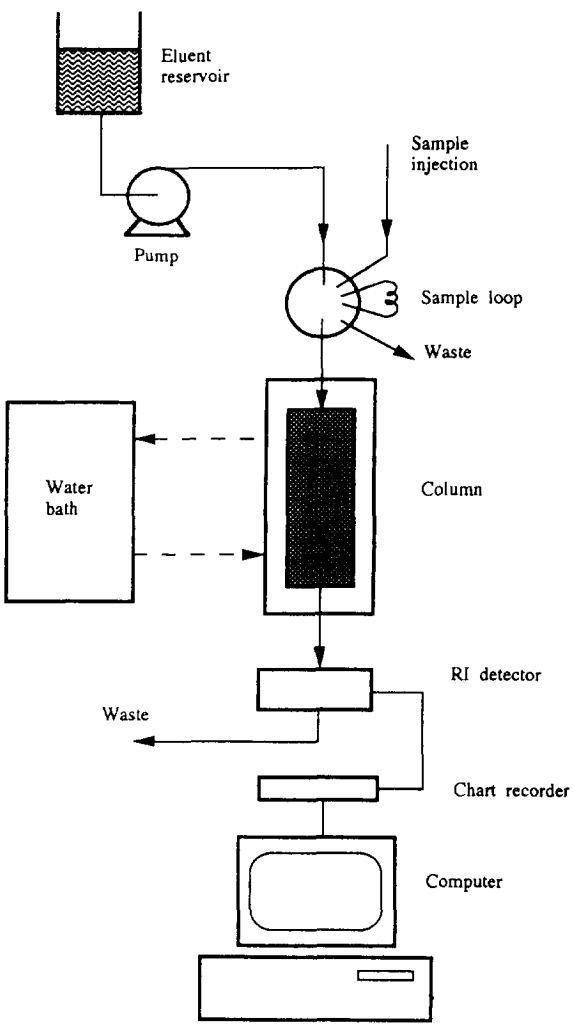

Fig. 1. Schematic diagram of chromatographic set-up.

2.3. Saponification

Saponification hydrolyses the triglycerides into their free fatty acids. The procedure reported by Ratnayake et al. [28] was employed with a few modifications. Fish oil (40 g) was saponified by refluxing with a mixture of KOH (9.2 g), water (17.6 ml) and absolute ethanol (52.8 ml) for 1 h in a nitrogen atmosphere. The saponified mixture was diluted with water (100 ml) and unsaponifiable matter was extracted thoroughly with 3 × 100 ml of hexane and discarded. The aqueous phase was then neutralized with 12.0–12.5 ml of dilute HCl (pH 4–5) and subsequently free fatty acids were extracted with 3 × 100 ml of hexane. The hexane extract was dried over anhydrous magnesium sulphate. The solids in the resulting slurry were removed with a Buchner funnel under suction, then the remaining solvent

was evaporated to dryness by using a rotary evaporator and the free fatty acids were recovered. The average yield of the mixed fatty acids for the three different fish oils in these experiments was 85%, which is comparable to that reported by Ratnayake et al. [28].

2.4. Urea crystallization

The free fatty acids (34 g) obtained from above saponification method and urea (102 g) were mixed with 180 ml of absolute ethanol. The mixture was heated with constant stirring until a clear, homogenweous solution formed. The solution was cooled to room temperature and subsequently crystallized at 4°C for 24 h. After the solution had thawed, the urea crystals (urea complex fraction) were separated from the liquid (non-urea complex fraction) with a Buchner funnel under suction. The non-urea complex fraction was diluted with an equal amount of water and acidified with dilute HCl to pH 4–5. The liberated fatty acids were extracted with 3×100 ml of hexane. The hexane layer was dried over anhydrous magnesium sulphate and subsequently hexane was evaporated to yield 8.0–8.5 g of polyunsaturated fatty acid concentrate. The resulting fatty acid concentrate was kept under a blanket of nitrogen and 0.005% (w/v) of BHA was added. The polyunsaturated fatty acid yields in these experiments were 20–21% of crude fish oils. These yields agree with those reported in the literature [25].

2.5. Chromatographic analysis

A 2.5-g amount of polyunsaturated fatty acid concentrate was dissolved in 5 ml of mobile phase and injected into the column through a 2-ml sample loop. The resulting response curves for each acid were integrated numerically in order to obtain first moments by using the following equation:

$$\mu = \frac{\int_0^\infty ct\,dt}{\int_0^\infty c\,dt}$$

The mean retention times were calculated by subtracting the dead times (t_0) from the first moments. The capacity factors (k') of the fatty acids were obtained from the mean retention times by using the equation

$$k' = \frac{t_R - t_0}{t_0}$$

The capacity factors obtained at various flow-rates are given in Table 1. It can be seen that the capacity factors not only are independent of flow-rates but are within the range 2.0–5.0, indicating economic viability of large-scale separation [29].

3. Results and discussion

Perfect baseline separations of EPA and DHA derived from three different fish oils were accomplished within 60 min. The chromatograms are shown in Figs. 2–4. The other minor peaks have not been identified. The elution sequence of these two ω-3 fatty acids was similar to that in analytical-scale separations [26]. However, baseline separations of the acids were not achieved in analytical chromatography, which indicates that the column size parameters such as diameter and length also play a role in the

Table 1
Capacity factors (k') of ω-3 fatty acids at various mobile phase flow-rates

ω-3 Fatty acid	k'		
	5.0 ml/min	7.5 ml/min	9.9 ml/min
Eicosapentaenoic aicd (C20:5 ω3)	3.35	3.87	3.96
Docosahexaenoic acid (C22:6 ω3)	4.24	4.39	4.83

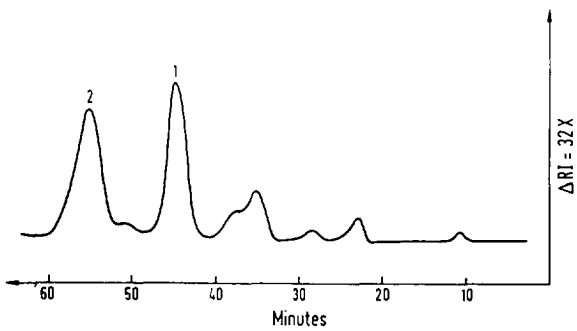

Fig. 2. Preparative chromatograms of ω-3 fatty acids from sardine oil. Stationary phase, RCM PrePak 25 × 10 cartridges packed with μBondapak phenyl-bonded silica; mobile phase, ACN–THF–H_2O (45:20:35, v/v/v) at a flow-rate of 9.9 ml/min; detection, refractive index at ambient temperature (24°C) and attenuation ×32; chart speed, 10 cm/h. Peaks: 1 = EPA; 2 = DHA.

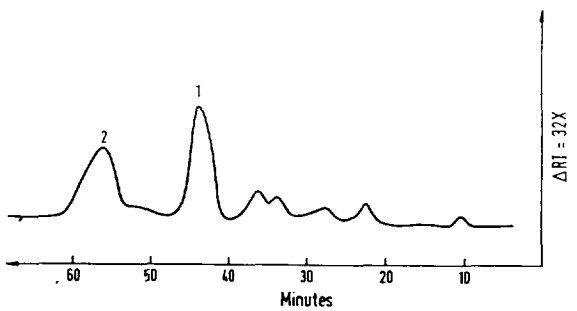

Fig. 3. Preparative chromatograms of ω-3 fatty acids derived from sanomega oil. Conditions and peak identification as in Fig. 2.

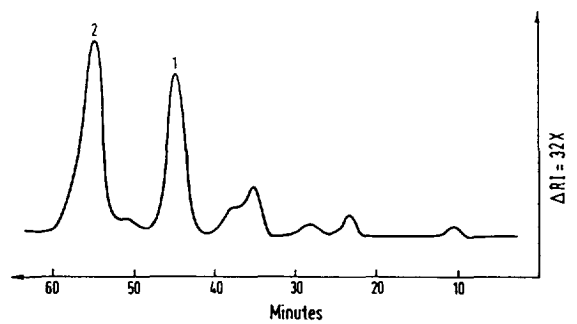

Fig. 4. Preparative chromatograms of ω-3 fatty acids derived from cod liver oil. Conditions and peak identification as in Fig. 2.

achievement of adequate separations of these components in addition to the chain length, saturation and position of the double bonds of the fatty acids [26,27,30,31].

Twenty fractions of preparative column effluents were collected at different points on the chromatograms of ω-3 fatty acids derived from Sanomega oil and analysed with the free fatty acid column. The integrated areas of the chromatograms thus obtained were used to calculate the percentages of these acids present in the fractions, as summarized in Table 2. The highest concentrations of EPA and DHA were 96.7% and 92.4%, respectively.

Interfering saturated and monounsaturated fatty acids such as palmitic acid (C16:0), stearic acid (C18:0) and oleic acid (C18:1 ω9) were successfully removed by the urea crystallization method. It was evident from Bengen's discovery that urea occludes straight-chain compounds such as long-chain saturated and monounsaturated fatty acids in a hexagonal structure and excludes methylene-interrupted polyunsaturated fatty acids due to the irregularities in their

Table 2
Compositions of ω-3 fatty acids in collected fractions determined by analytical chromatography

Fraction no.	EPA (C20:5 ω3) (%)	DHA (C22:6 ω3) (%)
1	62.4	
2	76.9	
3	88.2	
4	89.9	
5	96.7	
6	90.3	
7	84.8	
8	50.1	
9	20.4	
10	4.5	
11		34.9
12		53.5
13		78.2
14		86.6
15		92.4
16		90.1
17		81.7
18		40.1
19		12.4
20		1.4

Table 3
Composition of ω-3 fatty acids obtained at various ratios of urea to fatty acids and a crystallization temperature of 4°C

ω-3 Fatty acid	Urea: fatty acid ratio		
	2:1	3:1	4:1
EPA (C20:5 ω3) (%)	69	81	79
DNA (C22:6 ω3) (%)	52	74	71

molecules caused by the bends at each double bond [32]. It was observed that the extent of crystallization varies with the concentration ratio of urea and fatty acids and also with the temperature of crystallization, as shown in Tables 3 and 4. These tables also show that the higher concentration of ω-3 fatty acids was achieved when the urea concentration was three times higher than that of the fatty acids. The optimum temperature of crystallization was found to be 4°C. These results agree with previously reported results [33]. It was also found that saponification and urea crystallizations are simple chemical operations that can be performed under mild conditions in which no isomerization or degradation of the fatty acids occurs. Further, as oxidized products do not form urea adducts, the peroxidation of these highly unsaturated ω-3 fatty acids could be avoided during the extraction of free acids from fish oil triglycerides [34].

4. Conclusions

A simple, rapid and cost-effective preparative-scale chromatographic method was developed for the separation of gram-scale amounts of highly pure ω-3 fatty acids. These acids were eluted on the basis of their equivalent chain length (ECL), a parameter introduced by Miwa et al. [35] to determine the relative retention times of the fatty acids and their derivatives in gas and liquid chromatography. The ECL values for these acids are calculated by using the equation $ECL = N - 2n_{C=C}$, where N is the number of carbon atoms and $n_{C=C}$ is the number of double bonds present in the fatty acids. The ECL value for both EPA (C20:5 ω3) and DHA (C22:6 ω3) is 10. Such fatty acids having the same ECL values are termed as critical pairs and their separation was reported to be difficult in the past. However, the present methodology clearly demonstrates the enrichment and isolation of these high-value-added similar products within 60 min through urea crystallization folowed by RP-HPLC. Moreover, improvements are now under investigation such as converting the batch-scale operation to a continuous counter-current mode and total optimization of the process.

Table 4
Composition of ω-3 fatty acids obtained at various crystallization temperatures and a urea to fatty acid ratio of 3:1

ω-3 Fatty acid	0°C	4°C	10°C
EPA (C20:5 ω3) (%)	69	81	85
DNA (C22:6 ω3) (%)	80	74	64

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