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Semi-preparative supercritical chromatography scale plant for polyunsaturated fatty acids purification

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ABSTRACT

A one step supercritical fluid chromatography process has been developed using a semi-preparative supercritical fluid chromatography scale plant designed and built in-house, for isolation and purification of polyunsaturated fatty acid ethyl esters from different origins (fish and algae). A wide range of experimental conditions (pressure, temperature, sample load, co-solvent rate and CO_2 flow rate); columns (stationary phase and particle size); and UV/vis detectors have been investigated to optimise the isolation and purification of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) from an algae oil rich in EPA, and a fish oil rich in EPA and DHA. The particle size, followed by the stationary phase, sample loading, temperature/pressure and feed oil composition, were found to be the most important parameters for achieving separation. Under optimised conditions, EPA at greater than 95% purity from both oils and DHA at greater than 80% purity from fish oil was obtained in a single pass and without co-solvent.

1. Introduction

The long chain omega-3 fatty acids, EPA (eicosapentaenoic acid) and DHA (docosahexaenoic acid), are essential in the human diet for proper growth, development and good health. Unfortunately, these compounds cannot be chemically synthesised economically and it is necessary to recover them from natural sources in order to concentrate them for use in nutritional supplements. There is strong and growing evidence that increased consumption of EPA and DHA provides many important and even vital health benefits for humans, not least in reducing cardiovascular disease and deaths from heart problems [1]; autoimmune disorders [2,3], including type 2 diabetes [4]; improving children's brain development and maintaining good mental health [5]; cancer [6,7]; attention deficit/hyperactivity disorder (ADHD) [8]; dyslexia [9]; skin disorders [10]; and asthma [11]. The rising incidence of these conditions represents an imminent physical, mental and economical public health crisis. Health authorities worldwide advise adequate dietary consumption of EPA and DHA as being 1.1–1.6 g/day in adults and 0.5 g/day for children, depending upon age [12]. Both are entirely derived from the diet and are necessary for human health. An omega-6:omega-3 fatty acid ratio of 5:1 or less is desired, as suggested by nutrition experts [13]. However, nowadays food habits in Western countries result in this ratio reaching values of up to 100:1. Marine oils, especially fish oil, provide the major natural dietary source of EPA and DHA. The increasing market demand for omega-3 polyunsaturated fatty acids (PUFAs) has led to research and development into new raw materials, such as marine micro- and macro-algae and transgenic plants as potential sustainable sources of EPA and DHA [14,15] as alternatives to fish. Wild harvest fish stocks are in danger of over exploitation, whilst farmed fish can be contaminated with heavy metals such as copper or mercury and organic pollutants such as polychlorinated biphenyls (PCBs) or dioxins [16,17], and require feeding with fish oils to boost their levels of EPA and DHA. Interest has turned to algae (e.g. *Phaeoactylum tricornutum*, *Monodus subterraneus*, *Nannocloropsis*) as they are not seasonal products, and do not have either unpleasant odour or a high amount of cholesterol, and can contain squalene and phytosterols as additional beneficial compounds [18,19]. The main drawback to the use of these algae is both the cost of the extraction processes, because the desired fatty acids are mainly membrane-bound; and the cost of production by fermentation. Nevertheless, DHA in the form of triglycerides produced by microalgae is now widely used in infant formula [20–24]. Transgenic plant lipids containing EPA or DHA have not been commercialised yet, due to low consumer popularity around genetic engineering; and the levels of these fatty acids that can currently be produced.

To obtain a more desirable product in which EPA and DHA are highly concentrated, naturally derived oils have to be converted into fatty acid methyl or ethyl esters, or free fatty acids, since PUFAs in the triglyceride form are difficult to concentrate (separation of them has had limited success, because the fatty acids are more or less randomly distributed). There are many methods for the fractionation of fatty acid methyl or ethyl esters,

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including widely used chromatographic methods such as high performance liquid chromatography [25] and silver resin chromatography [26–29], molecular distillation [30,31], enzymatic splitting [32], urea complexation [33] and supercritical fluid extraction/fractionation techniques [5,34–38]. Some concentration of esters or fatty acids is possible by direct fractionation using supercritical CO_2 (SC-CO_2), but only on the basis of chain length and not on the degree of unsaturation. Supercritical fluid chromatography (SFC) makes this separation possible, because it combines the selectivity of a solid phase towards double bonds and the selectivity of supercritical CO_2 as a solvent towards the number of carbon atoms. Studies on the separation of PUFAs with SFC are limited, applying reversed phase chromatography [34] or using stationary phases like silica, silica gel or silica gel coated with a modifier [35]. There are some studies using fatty acids/ethyl esters from different fish oil origins (sardine, tuna, menhaden [5,35,38]). Perrut et al. [34] achieved EPA and DHA fractions with a purity of 92% and 85% respectively and yields of 99% starting from a feed material containing 50% EPA and 30% DHA. They coupled preparative supercritical chromatography and simulated countercurrent moving bed chromatography with several columns placed in series. Higashidate et al. [35] used a silver nitrate-loaded silica gel column at a laboratory scale to separate fatty acid extracts from the CO_2 extraction of esterified sardine oil (initial EPA and DHA contents of 12% and 13%, respectively). Using this method the authors obtained EPA and DHA-rich fractions with purities of 93% and 82%, respectively. Nilsson et al. [38] employed an increasing pressure programme in conjunction with a temperature gradient for fractionation of urea-crystallised fish oil ethyl esters (initial EPA, 48.6%; DHA, 22.2%). With this method they were able to recover more than 85% of the EPA and DHA from the feed with 90% purity. Snoey-Elich [37] was also able to concentrate fish oil EPA-EE from a purity of 50% to >95%. This was achieved using an industrial column of porous silicon dioxide impregnated with (3-aminopropyl)-triethoxysilane as the stationary phase (260–280 cm length, 50–60 cm I.D), and SC-CO_2 as mobile phase (100–150 bar, 313–333 K, 4000–7000 kg/h). The economic feasibility of producing industrial-scale quantities of EPA and DHA using SFC was investigated by Alkio et al. [5]. They reported that using SC-CO_2 at 65 °C and 145 bar and octadecyl silane coated silica as the stationary phase it was possible to obtain simultaneously DHA and EPA ethyl esters with purities of 90% and 50% respectively from a feed already highly enriched in EPA and DHA obtained by urea fractionation. Pettinello et al. [36] carried out pilot scale trials using quantities of feed materials on the order of hundreds of grams with CO_2 recycling. By optimising the feed loading, temperature and pressure for this process, a 93% pure EPA rich fraction at 25% yield was achieved. The purpose of this present study was to further establish a technology to obtain highly pure high value polyunsaturated fatty acids such as EPA and DHA using a one-step supercritical fluid chromatography process, for eventual potential use in the pharmaceutical, medical, functional food and cosmetic industries. Here we investigate the effect of particle size, temperature, pressure, co-solvent rate, and CO_2 flow rate on EPA and DHA ethyl ester recovery and purity for six semi-preparative scale column packings and two feed oils.

2. Materials and methods

2.1. Materials

Algae oil (local supplier) and fish oil (Ocean Nutrition Canada Ltd., Darmouth, Canada) fatty acid ethyl ester compositions are shown in Table 1. Palmitic acid ethyl ester, palmitoleic acid ethyl ester, linoleic acid ethyl ester, linolenic acid ethyl ester, arachidonic acid ethyl ester, eicosapentaenoic acid ethyl ester, and docosahexaenoic acid ethyl ester standards with estimated purities of 99% were supplied by Cayman Chemical Company (Ann Arbor, MI, USA) to determine residence times. Ethanol (Merck, Darmstadt, Germany) was used as received. CO_2 liquefied at high pressure and used in supercritical chromatography was supplied by BOC New Zealand (Auckland, New Zealand). Silica gel MN Kieselgel 60, 0.063–0.2 mm, for column chromatography was supplied by Macherey–Nagel GmbH&Co (Düren, Germany). The algae oil ethyl esters were produced from algae oil in a two-step process. In step 1, the lipid was converted to free fatty acids using KOH in an ethanol/water mixture at 323 K for 2 h with subsequent neutralisation with H_2SO_4, phase separation, washing and removal of solvent by vacuum evaporation. In step 2 the free fatty acids were converted to ethyl esters using H_2SO_4 in absolute ethanol at 323 K for 3 h with subsequent neutralisation using concentrated KOH solution, phase separation and evaporation of solvent. Fish oil ethyl esters were produced from the supplied fish oil using KOH in absolute ethanol at 323 K for 1.5 h with subsequent neutralisation with HCl, phase separation, washing and removal of solvent by vacuum evaporation.

Table 1
Sample composition (%) according to origin.

	C14	C16	C18	C20	AA	EPA	C22	DHA
Algae oil	6.3	58.0	16.6	18.3	*2.5*	*15.8*	0.7	*0.7*
Fish oil	9.3	29.9	20.3	19.1	*0.9*	*17.6*	11.0	*9.1*

2.2. Experimental set-up

The semi-preparative supercritical chromatography scale plant shown schematically in Fig. 1 was designed in-house, built and commissioned to study highly purified polyunsaturated fatty acid ester fractionation and recovery. This plant consists of two syringe pumps (ISCO Teledyne 260 HL, Lincoln, NE, USA) that can either provide a continuous supply of high pressure CO_2, or CO_2 and a co-solvent. The supercritical CO_2 is then supplied to the temperature controlled water bath (GD120, Grant Instruments (Cambridge) Ltd., Shepreth, England) containing the remainder of the apparatus. Neat ethyl ester sample is injected via a fixed volume loop (121, 364, 750 and 1000 μL) and mixed with CO_2 in an electrically actuated 6 port valve (VICI Valco Instruments Co. Inc., Houston, TX, USA). The mixture then passes through a guard column and semi-preparative column. Pre-packed, commercially supplied columns employed for this study are summarised in Table 2. The sixth column was packed in-house with silica 63–200 μm particle size in a Phenomenex column. The eluent from the column passes through a UV/vis detector (three different UV/vis detectors were used for comparison: Gilson UV/VIS-155 detector, Middleton, WI, USA; USB4000 with integrated semi-preparative scale flow cell; and high-sensitivity Deep UV Maya2000 Pro detector (Ocean Optics, FL, USA) combined with a Milton Roy LDC high pressure flow cell). A low flow rate

Table 2
Columns used for this study.

Manufacturer	Stationary phase	Length × diameter (mm)	Particle size (μm)
GreenSep™	Silica	250 × 20	5
GreenSep™	Pentafluorophenyl (PFP)	250 × 20	5
GreenSep™	Nitro	250 × 20	5
GreenSep™	Amino Phenyl	250 × 20	5
Phenomenex Luna (2)	Silica	250 × 21.2	15
Phenomenex[a]	Silica	250 × 21.2	63–200

[a] Empty column from Phenomenex but stationary phase supplied by Macherey-Nagel GmbH&Co.