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Reversed-phase high-performance liquid chromatography purification of methyl esters of C_{16}–C_{28} polyunsaturated fatty acids in microalgae, including octacosaoctaenoic acid [28:8(n-3)]

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Abstract

A preparative reversed-phase (RP; C_{18}) high-performance liquid chromatography (HPLC) method with gradient elution using acetonitrile (MeCN)–chloroform ($CHCl_3$) (or dichloromethane (DCM)) and evaporative light-scattering detection (ELSD) with automatic multiple injection and fraction collection was used to purify milligram quantities of microalgal polyunsaturated fatty acids (PUFA), separated as methyl esters (ME). PUFA-ME purified included methyl esters of docosahexaenoic acid (DHA; 22:6(n-3)), eicosapentaenoic acid (EPA; 20:5(n-3)) and the unusual very long-chain (C_{28}) highly unsaturated fatty acid (VLC-HUFA), octacosaoctaenoic acid [28:8(n-3)(4, 7, 10, 13, 16, 19, 22, 25)] from the marine dinoflagellate *Scrippsiella* sp. CS-295/c. Other PUFA purified from various microalgae using this RP-HPLC method to greater than 95% purity included 16:3(n-4), 16:4(n-3), 16:4(n-1) and 18:5(n-3). The number of injections required was variable and depended on the abundance of the desired PUFA-ME, and resolution from closely eluting PUFA-ME, which determined the maximum loading. The purity of these fatty acids was determined by electron impact (EI) GC–MS and the chain length and location of double bonds was determined by EI GC–MS of 4,4-dimethyl oxazoline (DMOX) derivatives formed using a low temperature method. Advantages over silver-ion HPLC for purifying PUFA-ME is that separation occurs according to chain length as well as degree of unsaturation enabling separation of PUFA-ME with the same degree of unsaturation but different chain length (i.e. between 18:5(n-3) and 20:5(n-3)). In addition, PUFA-ME are not strongly adsorbed, but elute earlier than their more saturated corresponding FAME of the same chain length. This method is robust, simple, and requires only a short re-equilibration time. It is a useful tool for preparing milligram quantities of pure PUFA-ME for bioactive screening (as free fatty acids), although many multiple injections may be required for minor PUFA-ME. It also enabled dose–response and structure–activity studies to be carried out. It can be used for the enrichment of low levels of VLC-HUFA-ME to facilitate elucidation of their chemical structure and so is a useful adjunct to EI GC–MS of DMOX derivatives and other techniques such as NMR, which requires milligram quantities of purified compounds.

Keywords: Dinophyceae; DMOX; Evaporative light-scattering detector (ELSD); Fatty acids; FAME; Lipids; Preparative HPLC; PUFA; *Scrippsiella* sp.; VLC-HUFA

1. Introduction

Long chain polyunsaturated fatty acids (LC-PUFA) such as eicosapentaenoic acid [EPA, 20:5(n-3)], arachidonic acid [AA, 20:4(n-6)] and particularly docosahexaenoic acid [DHA, 22:6(n-3)] have attracted the interest of many researchers in the areas of aquaculture nutrition and human nutraceuticals. Adequate dietary intake of LC-PUFA is considered to be beneficial in alleviating a variety of chronic diseases such as cardiovascular, hypertensive, inflammatory and autoimmune disorders, depression and certain disrupted neurological functions [1–6] suggested to be brought about by a relatively high ω6/ω3 [(n-6)/(n-3)] PUFA ratio in the "Western" diet.

Microalgae contain high contents of a variety of LC-PUFA and are major contributors to the marine food web as a renewable source of these LC-PUFA and other diverse bioactive compounds. They are amenable to mass culturing, biomass scale-up through photobioreactor and/or fermentation technology and the production of these compounds can be optimised by manipulating of culture conditions.

As part of a larger study screening microalgal PUFA (as free acids) for bioactivity, we needed a reliable method for purifying

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them in milligram amounts. A survey of the literature revealed a diverse range of methods for enrichment and purification of n-3 LC-PUFA. These included urea inclusion complexation, molecular distillation, iodolactonization, low temperature fractional crystallisation, salt solubility methods and liquid–liquid extraction-fractionation using aqueous silver nitrate solution [7]. Chromatographic techniques such as counter-current chromatography (CCC) [8], RP (C_{18}) HPLC [9,10], silver-ion HPLC (Ag^+-HPLC) [11,12], silver-ion solid-phase extraction (Ag^+-SPE) [13,14], Ag^+-silica gel TLC (Ag^+-TLC) [15,16] and Ag^+-silica gel open column chromatography (Ag^+-CC) [17] have also been used for separation and purification of individual LC-PUFA-ME.

After previously using Ag^+-TLC and Ag^+-SPE to purify LC-PUFA-ME, a method capable of purifying a variety of microalgal PUFA with a range of chain lengths ($C_{16–28}$) and unsaturation (up to 28:8(n-3)) at the milligram scale was required. To our knowledge, there was no published method so a RP-HPLC method was optimized for this purpose using evaporative light-scattering detection (ELSD) and automatic fraction collection.

2. Experimental

2.1. Culturing and harvesting

The marine dinoflagellate *Scrippsiella* sp. CS-295/c was obtained from the CSIRO Collection of Living Microalgae. This strain was originally isolated from ship ballast water. Cultures were grown in two 2 L Erlenmeyer flasks, each containing 100 mL of inoculum diluted to 1 L with GSe medium [18], a modification of the GP medium of Loeblich [19] with selenium added as selenite at 10^{-8} M and salinity adjusted to 28. The culture was maintained at 18.5 °C under 80 μmol photon PAR $m^{-2}\,s^{-1}$ of cool white fluorescent light (measured with a Biospherical Optics light meter) on a 12:12 h L:D cycle. The cultures were harvested after 7 days at late-logarithmic growth phase by centrifugation.

2.2. Extraction and transmethylation

The alga was extracted with $CHCl_3$–MeOH–H_2O (1:2:0.8) by a modified version of the method of Bligh and Dyer [20]. FAME were formed by heating in MeOH:HCl (10:1) at 80 °C for 2 h and extracted into hexane. FAME were purified by elution through a short silica–alumina (0.2 and 0.1 g, respectively) column with 10 mL diethyl ether:hexane (3:97, v/v). The purified FAME were reconstituted in DCM or $CHCl_3$ and stored at −20 °C until required for HPLC purification and GC/GC–MS analysis.

2.3. Analytical HPLC

The HPLC method was based on an industrial HPLC system for the purification of DHA from the thraustochytrid, *Schizochytrium* sp. SR21, which used isocratic elution [10]. We separated the fatty acids as methyl esters. HPLC was performed using a preparative pump with a low pressure quaternary gradient controller (Waters), an autosampler (Gilson) and a reversed-phase column (Alltima C_{18}, Alltech, 250 mm × 4.6 mm i.d.) packed with 5 μm spherical particles of 100 Å pore size coated with a polymerically bonded C_{18} stationary phase with a 16% carbon load and endcapped to remove free silanol groups. The elution was isocratic using acetonitrile (MeCN)/H_20 (97.5:2.5, v/v) with a flow rate of 1.5 mL/min. Samples (100 μL) of a 0.8 μg/μL solution of FAME were loaded onto the column.

2.4. Preparative HPLC

HPLC was performed using a RP column (300 mm × 22 mm i.d.) packed with the same type of particles as for the analytical column except they were 10 μm in size. A binary stepwise solvent gradient was used (Table 1) since the isocratic solvent system used for analytical separations did not elute any FAME (see Section 3). Samples (200 μL) of 50 μg/μL of total FAME from *Scrippsiella* sp. CS-295/c (10 mg) were loaded onto the column.

2.5. Detection and collection

FAME were detected using an ELSD (Mass Detector Model 750/14, Applied Chromatography Systems, ICI). The settings were as follows: attenuation range, 16; time constant, 5 s; photomultiplier sensitivity, 2; evaporator set, 40 °C using nitrogen as the nebulizer gas. Fraction collection was achieved using a flow-splitter (Alltech) valve set at a split ratio of 20:1 (collector:detector) and an automatic fraction collector (Gilson model FC203B).

2.6. DMOX derivatization for determination of position of double bonds

A low temperature derivatization method [21] was used to avoid degradation of PUFA and to minimise the formation of by-products. HPLC-purified FAME mixtures or individual FAME were first hydrolysed to the free fatty acids by heating for 10 min at 80 °C with 10% KOH (w/v) in 80% MeOH (v/v) then acidified to pH 2 with concentrated HCl (supra pur). The free acids were extracted into hexane, dried using nitrogen gas then converted to the acid chloride by reaction with oxalyl chloride (0.5 mL) at ambient temperature overnight under nitrogen. Excess reagent was removed in a stream of nitrogen gas and the product reacted immediately with 0.5 mL of 2-amino-2-methyl-1-propanol in

Table 1
Stepwise solvent gradient program for preparative reversed-phase (C_{18}) HPLC separation of FAME

Time (min)	Flow (mL/min)	MeCN (%)	$CHCl_3$ (%)
0	10	100	0
1	10	90	10
8	10	80	20
12	10	40	60
14	10	0	100
26	10	100	0
35	10	100	0

DCM (10 mg/mL; stored over molecular sieve, type 5A (Alltech)) for 1 h at ambient temperature. The solvent was evaporated and trifluoroacetic anhydride (1 mL) was added and the mixture heated at 40 °C for 1 h. Excess reagent was then evaporated and the product taken up in hexane (5 mL) and washed with Milli-Q H_2O (2 mL × 2 mL). The solution was dried over anhydrous sodium sulphate, evaporated then redissolved in hexane for GC and GC–MS analysis.

2.7. Gas chromatography

GC was performed on a gas chromatograph (HP 5890, Hewlett-Packard) fitted with a BPX-70 bonded phase capillary column (50 m; 0.32 mm i.d.; 0.25 μm film thickness), an FID and an on-column injector. Samples were injected at 45 °C; after 2 min the oven temperature was raised at 30 °C min^{-1} to 120 °C and then at 3 °C min^{-1} to 240 °C, where it was held for 20 min. FAME and DMOX derivatives were also analysed on an HP-1 non-polar column (50 m; 0.32 mm i.d.; 0.17 μm film thickness) with the same conditions as above except the final temperature was 310 °C. Hydrogen was used as the carrier gas.

2.8. Mass spectrometry

EI GC–MS was performed on a quadrupole GC–MS (MD-800, Fisons) with an on-column injector set at 45 °C. Samples were injected into a retention gap attached to an HP-5 Ultra 2 bonded phase column (50 m; 0.32 mm i.d.; 0.17 μm film thickness). The initial temperature of 45 °C was held for 1 min, followed by temperature programming at 30 °C min^{-1} to 140 °C then at 3 °C min^{-1} to 310 °C, where it was held for 12 min. Helium was used as the carrier gas. Mass spectrometer operating conditions were: electron impact energy, 70 eV; transfer line, 310 °C; source temperature, 250 °C; scan rate, 0.8 scans s^{-1}; and mass range, 40–650 U. Mass spectra were analysed using Masslab software (Fisons).

3. Results and discussion

3.1. Analytical HPLC

The PUFA-ME of the marine dinoflagellate *Scrippsiella* sp. were well resolved within 12 min (Fig. 1) according to carbon length and degree of unsaturation. The elution order was similar to that on a non-polar capillary GC column [16], such that FAME with a greater degree of unsaturation eluted earlier than less unsaturated ones with the same carbon length and longer chain PUFA-ME eluted later than shorter chain analogs, though this relationship was non-linear (Table 2). The effect of increased retention time with increasing chain length is partly offset by the effect of decreased retention time with increasing degree of unsaturation. This effect is evident in the case of the very long-chain highly unsaturated fatty acid (VLC-HUFA), 28:8(n-3) which eluted at 10.6 min (Fig. 1).

Monounsaturated and saturated FAME were not eluted with this solvent composition (Fig. 1) indicating that they were selectively retained. However, this did not adversely affect or interfere with the chromatography of samples subsequently injected nor did it increase the backpressure of the column after 10 injections. These FAME were stripped off the column with $CHCl_3$ or DCM. This characteristic is useful for rapid preparation of various microalgal PUFA-ME mixtures for bioactive screening (as free fatty acids) and for potential use in nutraceuticals (see Section 3.2). Another advantage of this analytical system is that it allows rapid (<12 min) analysis of the major PUFA-ME up to 28:8(n-3) and so is suitable for high throughput screening of these components.

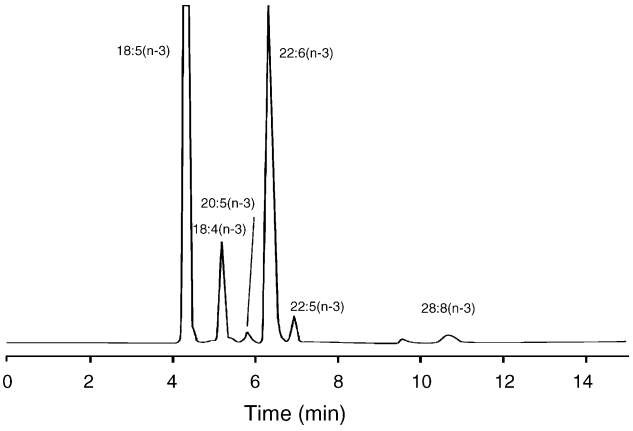

Fig. 1. RP (C_{18}) HPLC chromatogram of PUFA-ME from the dinoflagellate *Scrippsiella* sp. 295/c, separated on an analytical column (Alltima C_{18}, Alltech, 250 mm × 4.6 mm i.d., 5 μ particles) using an isocratic solvent system (MeCN: H_2O; 97.5:2.5, v/v) with a flow-rate of 1.5 mL/min and detected with an evaporative light-scattering detector (Mass Detector Model 750/14, Applied Chromatography Systems, ICI). Samples (100 μL) of a 0.8 μg/μL solution of FAME were loaded onto the column.

3.2. Preparative HPLC

When samples were injected onto the preparative HPLC column using the isocratic solvent system used for the analytical HPLC column, no FAME were eluted. This suggested that 10 μm packing in the preparative column was more selective than the 5 μm packing used in the analytical column and strongly retained all PUFA-ME, as well as the saturated and monounsaturated FAME. Hence, the eluting solvent system was modified by developing a simple stepwise gradient solvent system from 100% MeCN as the initial solvent with $CHCl_3$ or DCM as lipophilic solvent modifiers. This resulted in similar chromatography (Fig. 2) to that achieved using the isocratic solvent system on the analytical column (Fig. 1). Elution of the monounsaturated and saturated FAME also occurred with the

Table 2
Delta equivalent carbon lengths (ΔECL) between pairs of fatty acids differing in unsaturation or carbon length

Fatty acid pairs	ΔECL
18:5(n-3)/18:4(n-3)	1.2
22:6(n-3)/22:5(n-3)	0.8
18:5(n-3)/20:5(n-3)	2.0
20:5(n-3)/22:5(n-3)	0.9

preparative column. The resolution was such that this preparative system could be used for quantitative analysis of the main PUFA-ME within 26 min. It required no more than 10 min re-equilibration time with MeCN (Table 1).

The chromatography was reproducible and so this system is amenable to automation using an auto-sampler and auto-fraction collector. Separation of PUFA-ME allowed rapid analysis by capillary GC and GC–MS following fraction collection to determine purity and confirm identification of PUFA. This system can be adapted to LC–MS analyses of marine PUFA.

The dinoflagellate VLC-HUFA, 28:8(n-3) was resolved from other major PUFA-ME (Fig. 2) and eluted prior to the 18:1 and 16:0. Capillary GC analysis showed the 28:8(n-3) fraction to be 94% pure. GC–MS of this peak identified it as a HUFA-ME due to the high abundance of the diagnostic ions *m/z* 91/79, but there was insufficient detail in the fragmentation pattern and no detectable M^+. EI GC–MS was performed on the DMOX derivative to confirm the carbon length, degree on unsaturation and the positions of the double bonds. An interval of 12 U between the most intense peaks of clusters of fragments containing n and $n-1$ carbon atoms indicates a double bond occurs between carbons n and $n+1$ in the molecule. Hence, this fatty acid was confirmed to be 28:8(n-3) (4, 7, 10, 13, 16, 19, 22, 25) according to the fragment ion pairs: *m/z* 126/138, 166/178, 206/218, 246/258, 286/298, 326/338, 366/378, 406/418 and $(M\text{-}1)^+$ of 460 [16].

For bioactive screening, milligram quantities of PUFA-ME from various microalgal strains were purified to greater than 95% purity by repeated injections using this RP-HPLC method. These included 18:5(n-3) (30.5 mg, 99% pure, 10 injections) and 28:8(n-3) (1 mg, 94% pure, 16 injections) from the dinoflagellate, *Scrippsiella* sp. CS-295/c, 16:3(n-4) (17.9 mg, 98% pure, five injections) from the diatom *Amphora* sp. CS-10, 16:4(n-3) (16.8 mg, 97% pure, seven injections) from the green alga *Dunaliella tertiolecta* CS-175 and 16:4(n-1) (5.5 mg, 95% pure, five injections) from the diatom *Skeletonema* sp., CS-252.

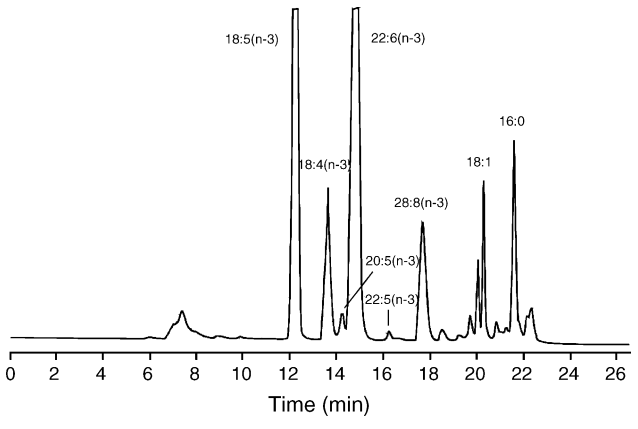

Fig. 2. Preparative RP (C_{18}) HPLC chromatogram of FAME from the dinoflagellate *Scrippsiella* sp. 295/c using a RP column (Alltima C_{18}, Alltech, 300 mm × 22 mm i.d., 10 μm particles) using gradient elution (100% MeCN to 100% $CHCl_3$) at a flow-rate of 10 mL/min and detected with an evaporative light-scattering detector (Mass Detector Model 750/14, Applied Chromatography Systems, ICI). Samples (200 μL) of 50 μg/μL of total FAME from *Scrippsiella* sp. CS-295/c (10 mg) were loaded onto the column.

28:8(n-3) and 28:7(n-6) were purified previously on an analytical scale from the dinoflagellate, *Prorocentrum micans* [16] using several Ag^+-impregnated TLC plates to obtain a sufficient amount for confirming the identification. This method did not, however, separate 28:8(n-3) and 28:7(n-6). It was laborious, time consuming, messy and was not able to prepare milligram quantities due to lower loading capacity, resolution and recoveries.

3.3. Advantages of C_{18} HPLC over other methods

Preparative RP-HPLC is a useful tool for purifying milligram quantities of PUFA-ME. It is simple, rapid, reproducible, robust and has desirable selectivity and resolution characteristics particularly suited to marine microalgal PUFA-ME. The elution order is similar to that seen in non-polar capillary GC. Most other techniques lack either the selectivity and/or resolution required for purifying individual PUFA-ME. This method can be automated and generally uses low volumes of solvent to elute PUFA-ME than argentation-based separations since highly unsaturated FAME are not as strongly retained. Variable amounts of total FAME from different microalgal strains could be loaded each cycle requiring 260 mL of total solvent each time. The total amount of solvent and number of injections needed to prepare a certain amount of PUFA-ME of a required purity, however, depended on the proportion of the PUFA-ME in the sample and loading on the column, which is determined by how well it is resolved from close eluting FAME. Since 18:5(n-3) composed 43% of the total FAME of *Scrippsiella* sp. CS-295/c (determined by GC) 10 injections were required to prepare 30.5 mg of 18:5(n-3), whereas 10 injections with a further 6 re-injections of the semi-purified 28:8(n-3) fraction were required to increase the to purity of 1 mg of 28:8(n-3) to a level of 94%, since 28:8(n-3) is only 2% of the total FAME of *Scrippsiella* sp. CS-295/c.

While this method was useful and allows rapid analysis, with short cycle time many multiple injections are required to purify milligram quantities particularly for minor PUFA-ME as is the case for 28:8(n-3). In the case of preparing a variety of PUFA-ME, careful selection of algal strains which had both higher proportions of these PUFA-ME and simple profiles with good separation allowed higher loadings to be applied to the column and hence reduced the number of cycles, amount of solvent and time required. For *Amphora* sp. CS-10, *Skeletonema* sp. CS-252, *Scrippsella* sp. CS-295/c, *Dunaliella tertiolecta* the amounts of total FAME loaded each cycle were 14, 9, 11 and 12 mg, respectively.

Other techniques such as Ag^+-TLC have been used to separate FAME according to degree of unsaturation and *trans*/*cis* geometry of the double bonds [12,13,17]. Unlike C_{18} HPLC, separations are not based on chain length of PUFA-ME and this is a limiting factor. Řesenka and Sokolov [22] found that using Ag^+-TLC resulted in unsatisfactory resolution of PUFA-ME homologues and partial degradation of FAME. They concluded that C_{18}-HPLC was the best method of enriching the PUFA fraction. Highly unsaturated PUFA-ME such as DHA-ME and 28:8(n-3)-ME are strongly adsorbed onto the Ag^+-silica stationary phase due to the formation of very stable coordination complexes and require strong polar solvents to elute them and

extensive cleaning between analyses. Leaching of the silver-ions occurs with use, especially when polar solvents such as methanol are used and this can affect chromatographic reproducibility and column durability [17]. New polar bonded phases such as diol phase [12] or cation-exchange resins (e.g. *p*-propylbenzene sulfonic acid and propyl sulfonate) [13] are physically and chemically stable alternatives to silica and can also be loaded with Ag^+ in SPE cartridges and HPLC columns to separate FAME according to unsaturation. These phases are more easily cleaned and re-equilibrated with the initial solvent after each analysis. This is also a characteristic of this RP preparative column, which allows a rapid analysis and short cycle time for multiple injections. It is also highly durable, very reproducible and unaffected by solvents with a wide range of polarities.

The HPLC method demonstrated here allows preparation of sufficient amounts of purified PUFA for in vitro bioactive screening and structural elucidation by EI GC–MS of DMOX derivatives. It was particularly useful for the enrichment and purification of the uncommon VLC-HUFA, 28:8(n-3) (4, 7, 10, 13, 16, 19, 22, 25) present in very low proportions (∼2% of total fatty acids). It also allowed us to carry out structure–activity and dose–response relationship studies of a variety of other PUFA (as free fatty acids) from several algal strains. In addition, there is scope for modifying this method for the purification of other bioactive fatty acids for bioactive screening and other applications including high throughput chemical screening and to augment other structural elucidative techniques such as NMR and LC–MS.

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