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Novel anti-inflammatory ω-3 PUFAs from the New Zealand green-lipped mussel, *Perna canaliculus*

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Abstract

The present study has identified in the marine mollusc, *Perna canaliculus*, an homologous series of novel omega 3 polyunsaturated fatty acids (ω-3 PUFA) with significant anti-inflammatory (AI) activity. The free fatty acid (FFA) class was isolated from a supercritical-CO_2 lipid extract of the tartaric acid-stabilised freeze-dried mussel powder by normal phase chromatography, followed by reversed-phase high performance liquid chromatography (RP–HPLC). The RP–HPLC involved separation based on carbon numbers, followed by argentation–HPLC (Ag–HPLC) of the methyl esters based on degree of unsaturation. Identification of the FFA components was performed using gas chromatography (GC) with flame ionisation detection, and individual structures were assigned by GC-mass spectroscopy (GC-MS). Inhibition of leukotriene production by stimulated human neutrophils was used as an *in vitro* screening method to test the AI activity of the purified PUFAs. A structurally related family of ω-3 PUFAs was identified in the most bioactive fractions, which included C18:4, C19:4, C20:4, and C21:5 PUFA. The C20:4 was the predominant PUFA in the extract, and was a structural isomer of arachidonic acid (AA). The novel compounds may be biologically significant as AI agents, as a result of their *in vitro* inhibition of lipoxygenase products of the AA pathway.
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Keywords: Anti-inflammatory; Eicosanoid; Lipoxygenase; Lyprinol®; ω-3 PUFA; *Perna canaliculus*; Supercritical fluid extract

1. Introduction

Eicosanoids of the cyclooxygenase (COX) and lipoxygenase (LO) pathways of arachidonic acid (AA) metabolism are important modulators of inflammation (Bogatcheva et al., 2005). Often, excessive inflammatory responses progress to pathogenic states requiring pharmacological intervention. Elucidation of the AA pathways has led to understanding of modes of action of traditional anti-inflammatory (AI) drugs, and enhanced research into specific inhibitors of the AA pathways for further drug development (Morris et al., 2006). The reported side-effects and contra-indications of current AI drugs have lead to investigations into natural products for safer and more effective alternatives (Calder, 2006).

An area of recent investigation is the AI activity of *Perna canaliculus* (Bivalvia: Mytilidae), a marine mollusc commonly known as the green-lipped mussel of New Zealand. Lyprinol®, a commercially available preparation of *P. canaliculus*, is the mussel oil obtained by carbon dioxide-supercritical fluid extraction (CO_2–SFE) (Macrides and Kalafatis, 2000) of the tartaric acid-stabilised mussel powder (Kosuge and Sugiyama, 1989), formulated with olive oil and vitamin E as an antioxidant. Clinical trials and *in vivo* rat assays have established the AI effectiveness of Lyprinol®. The first randomised trial of Lyprinol® in the treatment of arthritis in humans was reported by Gibson and Gibson (1998). They showed that 76% of patients with rheumatoid arthritis and 70% of patients with osteoarthritis benefited from Lyprinol®. It has been found that increased consumption of ω-3 PUFAs alter the generation of AA-derived inflammatory metabolites, and reduce inflammatory responses (Stamp et al., 2005). Lyprinol® has been shown to have greater AI activity than the more commonly used ω-3 PUFA-containing therapeutic oils (Whitehouse et al., 1997). When administered therapeutically or prophylactically to Wistar and Dark Agouti rats with antigen-induced polyarthritis or collagen (II)-induced autoallergic

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Table 1
Solvent program for the FFA group purification by preparative NP–HPLC

Segment	Time (min)	Hexane (%)	Tetrahydrofuran (%)
0	0	100	0
1	20	96	4
2	25	90	10
3	45	0	100
4	48.8	90	10
5	50	100	0

arthritis, Lyprinol® was a more effective AI agent (Effective Dose, ED_{50} ≤ 15 mg/kg) than flaxseed, evening primrose, and fish oil (ED_{50} ≥ 1800 mg/kg) (Whitehouse et al., 1997). Lyprinol® has been shown to inhibit leukotriene B_4 (LTB_4) production in calcium and ionophore-stimulated human neutrophils (Whitehouse et al., 1997), and in interleukin-4-induced human monocytes (Dugas, 2000). Inhibition of prostaglandin-E_2 production in activated human macrophages has also been observed (Whitehouse et al., 1997). It has been shown that the AI activity of the CO_2–SFE mussel oil resides predominantly in the FFA fraction of the oil, with the greatest activity being exhibited by the PUFA class (McPhee et al., 2001; McPhee et al., 2007). The identities of the active PUFAs are however unknown.

Isolation of active components from whole organisms requires several purification steps, with each step requiring a screening process to target the active components. Lipid extracts can be obtained by several methods, and SFE has been found to be effective in removing lipid fractions from solid or semi-solid material (Huang et al., 1994). Lipids obtained from whole organisms contain a large number of different lipid classes, each of which is itself heterogeneous. Therefore a sequential purification process is needed to purify and identify bioactive compounds from the lipid extracts. Gas chromatography offers a sensitive identification system for any novel or unusual compounds in the fractions. A sensitive and reliable assay of AI activity is the 5-LO inhibition assay (Cleland et al., 1990), which monitors the production by stimulated human neutrophils of pro-inflammatory leukotriene and hydroxy acid metabolites of the 5-LO pathway of AA metabolism.

In the present study, a simple purification procedure based on normal and reversed phase chromatography was carried out to isolate potential bioactive lipids from the FFA class of the CO_2–SFE oil from *P. canaliculus*. Novel fatty acids were detected by GC and structurally identified by GC-MS. Bioactivity was confirmed in an *in vitro* lipoxygenase-inhibition assay system.

2. Materials and methods

2.1. Chemicals

Solvents for all chromatographic procedures were of analytical grade quality and obtained from E. Merck (Darmstadt, Germany). Silica gel for column chromatography (Kieselgel 60, 230–400 mesh) and thin layer chromatography (TLC) plates (Kieselgel 60F254 nano DC) were sourced from Merck (Darmstadt, Germany). Lipid standards for thin layer chromatography (TLC) and HPLC analysis were obtained from Nu-Chek-Prep Inc., (Elysian, MO, USA).

2.2. Mussel extract

Tartaric acid stabilised green-lipped mussel (*P. canaliculus*) powder (McFarlane Marketing (Aust) Pty Ltd, Melbourne, Australia) was extracted for lipids by the procedure of SFE, utilising CO_2 as the extracting medium (Macrides and Kalafatis, 2000). Essentially, mussel powder (300 g) was charged to the pilot scale Super Critical Fluid Extraction Unit (Distillers MG Ltd. England, UK). Supercritical-CO_2 was delivered at a flow rate of 3.0 kg/h for two hours. The extractor temperature was set at 70 °C and the extractor pressure at 345 bar. The evaporator parameters were set at 40 °C and 35 bar. The extract presented as a concentrated oil, and was stored under nitrogen at −8 °C in amber vials to minimise autoxidation.

2.3. Open column flash chromatography

Initial fractionation to remove the highly polar mussel lipids was performed by open column flash chromatography using silica gel (Still et al., 1978). The mussel extract (4.5 g) dissolved in dichloromethane (DCM, 6.0 mL) was applied to 100 g of silica gel (230–400 mesh) in an open column (100 × 8 cm ID) fitted with a 1 L solvent reservoir (flow rate 175 mL/min). Separation of the lipid classes was achieved using a polarity gradient utilising two bed volumes of the following solvents; 100% DCM, 100% hexane, 10%, 50%, methyl-*tert*-butyl ether (MTBE) in hexane, 100% MTBE, and finally 100% methanol (MeOH) to remove the highly polar material. A total amount of 90 g of mussel oil was processed.

2.4. Silica gel NP–HPLC purification of FFA class of mussel extract

Group purification of the FFA class of the phospholipid-free mussel extract was obtained by silica gel preparative HPLC using normal phase (NP) chromatography. The phospholipid-free fractions collected from the low resolution column procedure were pooled, filtered through a 0.45 μm polyvinylidene hydrofluoride filter (Activon Scientific Products, Thornleigh, Australia) and evaporated to dryness. The sample (450 mg) re-dissolved in hexane diluent (900 μL) was applied to a Prep Nova-Pak® HR 60Å, 6 μm silica, 100 mm × 40 mm (ID) column (Waters Chromatography Division Milford, MA, USA). Preparative HPLC analysis was performed using a Waters Delta Prep 3000

Table 2
Solvent program for the separation of FFA class by semi-preparative RP–HPLC

Segment	Time (min)	Water (%)	Acetonitrile (%)	Methanol (%)
0	0	13	87	0
1	35	13	87	0
2	40	0	100	0
3	42	0	0	100
4	57	0	50	50
5	59	13	87	0

Table 3
Gradient program for the separation of FAME by semi-preparative Ag–HPLC

Segment	Time (min)	A (%)	B (%)
0	0	70	30
1	31	8	92
2	35	8	92
3	43	0	100
4	53	0	100
5	55	70	30

A = DCM–DCE, 1:1 (v/v).
B = DCM–DCE–AcCN–MeOH, 40:40:10:10: (v/v/v/v).

solvent delivery system (flow rate 25 mL/min) with a 600E system controller (Waters Chromatography Division). The compounds were detected by an ACS Model 750/14 light scattering detector (Applied Chromatography Systems, Macclesfield, UK). The light scattering detector was set at an evaporator value (ESV) of + 80 °C and a nitrogen delivery pressure of 20 psi. The data was collected on a PE Nelson Model 1020 Personal Integrator (The Perkin Elmer Corporation, Norwalk, CT, USA). Sample collection was achieved by inserting a stream splitter between the column outlet and detector inlet, with 5% of the effluent going to the detector. The solvent system (Table 1) was developed by using commercial standards of tristearin (Nutritional Biochemicals Corporation, Cleveland, OH, USA), cholesteryl palmitate, stearic acid, and cholesterol (Sigma). The retention times of these standards were used to identify the lipid classes of the mussel extract applied. The identity of each peak was also confirmed by TLC analysis.

2.5. Separation of FFA by reversed phase semi-preparative HPLC

The FFA material obtained from the NP–HPLC procedure was evaporated to dryness and re-dissolved in acetonitrile (AcCN)-tetrahydrofuran (THF) (1:2 v/v) before being passed through a nylon 0.45 μm filter (Activon). The multi-solvent system (Table 2) developed for the FFA separation utilised AcCN as the polar carrying solvent with water and MeOH introduced to alter the solvent strength and selectivity of the mobile phase. The RP–HPLC utilised an Ultrasphere™ C18, 80 Å, 5 μm, 25 cm × 10 mm (ID) column obtained from Beckmann (Palo Alta, CA, USA) with a C18 pre-column, 1 cm × 4.3 mm (ID) (Activon). Semi-preparative HPLC analysis was performed on an Applied Biosystems Chromatograph, Model 150A with a 1400 A ternary high pressure pump system (flow rate 1.5 mL/min) equipped with a model 1783 A detector controller unit (Applied Biosystems, Foster City, CA). The light scattering detector was set at an ESV of + 45 °C and a gas delivery pressure of 14 psi with the data collected and stored on a 1020 Personal Integrator (Perkin Elmer). The sample was delivered through an automated sampling unit, WISP 712 (Waters Chromatography). Sample collection was achieved using a Gilson Model 202 programmable collector (Gilson Medical Electronics Inc., Middleton, WI, USA). The samples were collected in a preset time windows mode of collection with the column effluent diverted directly to the fraction collector.

2.6. Lipoxygenase inhibition assay

The fractions obtained from the RP–HPLC were evaporated to dryness and a known weight of each fraction was dissolved in 1 mL of MeOH. The *in vitro* assay was performed according to the method of Cleland et al. (1990). Briefly, the assay involved stimulation of human neutrophils with AA and calcium ionophore A23187, resulting in the production of the 5-LO

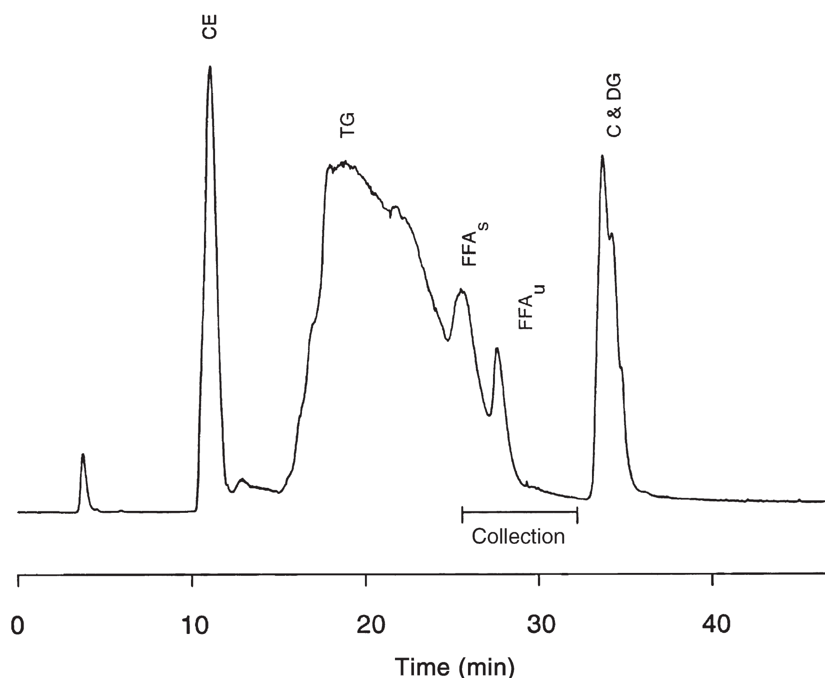

Fig. 1. Preparative NP–HPLC chromatogram of 450 mg of phospholipid-free mussel lipid extract. Chromatographic conditions: Nova Pak® silica preparative column, using the multi-solvent system shown in Table 1, and a flow rate of 25 mL/min. Light scattering detector settings: ESV + 80 °C gas pressure 20 psi. The peaks identified were: CE, cholesterol esters; TG, triglycerides; FFA_s, saturated free fatty acid region; FFA_u, unsaturated free fatty acid region; C, cholesterol; and DG, diglycerides.

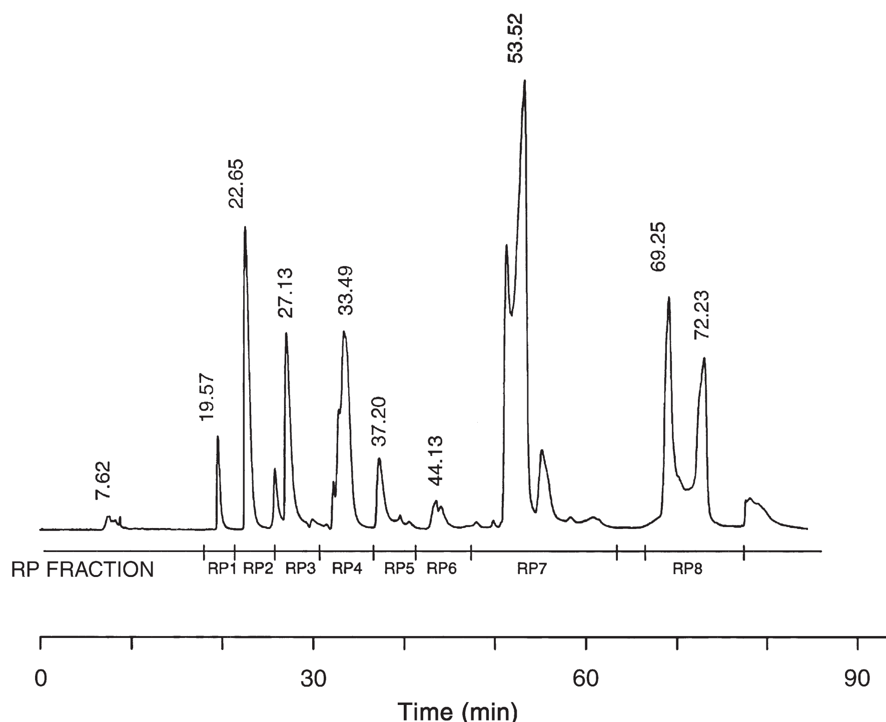

Fig. 2. Semi-preparative RP–HPLC chromatogram of mussel FFA class. Chromatographic conditions: Beckman™ C18 semi-preparative column, using the solvent system described in Table 2 and flow rate of 1.5 mL/min with 1 mg being applied. Light scattering detector settings: ESV, +45 °C gas pressure 14 psi. The retention times of the eluted FFA and the collection time windows are indicated.

metabolites; LTB_4, the two non-enzymic isomers, 6-*trans* LTB_4 and 6-*trans*,12-*epi* LTB_4, and 5-hydroxyeicosatetraenoic acid (5-HETE), which were quantified by HPLC. Samples were typically assayed at three dilutions, i.e. 1:100, 1:1000, and 1:10,000, with an inhibition of the production of the LO metabolites indicating bioactivity. Percent inhibition of control production of LO metabolites was calculated by: 100×(concentration of LO metabolite of test sample/concentration of LO metabolite of control).

2.7. Analysis of fractions by gas chromatography

The FFA fractions from the chromatography procedures were dissolved in hexane (1 mL) and a C17:0 methyl ester internal standard (IS) (Nu-Chek-Prep) was added (fraction:IS, 5:1 w/w). The fatty acids were converted to their methyl esters (FAME) by reaction with BF_3–MeOH, as described in the Association of Official Analytical Chemists method 963.22 (AOAC, 1995). Briefly, 10 mL of 12% BF_3 in MeOH was added to 500 mg of FFA and refluxed for 60 min at 70 °C. A 20 mL aliquot of heptane was added and the reaction mixture refluxed for a further 10 min. The reaction was terminated by the addition of 15 mL of saturated sodium chloride. The FAME were extracted using 2 volumes of 50 mL MTBE. The organic layer was washed with 20 mL volumes of water until the solution was acid free. The extract was dried over anhydrous Na_2SO_4 and analysed by TLC. The unreacted material was removed by a clean-up procedure using 2 g of fine silica in a 10 mL syringe, and the flow rate (4 mL/min) was achieved by applying compressed air. The FAME were separated by a stepwise elution gradient utilising 8 mL of each of the following solvents; 100% hexane, 2%, 10%, 50% MTBE in hexane, and finally 100% MTBE.

GC analysis was performed using a Shimadzu model GC-17A with a flame ionisation detector set at 260 °C and linked to a Shimadzu Chromatopac integrator (Shimadzu Corporation, Tokyo, Japan), which was equipped with a split/splitless injector. The FAME (1 μL injections) were separated using a fused silica capillary column (50 m×0.22 mm (ID), film thickness 0.2 μm) coated with BPX70 (biscyanopropyl polysiphenenylene-siloxane, SGE, Ringwood, Victoria, Australia), with helium as the carrier gas. The oven temperature was held at 120 °C for 1 min, then increased to 170 °C (at a rate of 5 °C/min) and held for 4 min, then increased to 220 °C (at a rate of 10 °C/min) and held for 17 min. The injector temperature was set at 260 °C and the linear velocity of the helium gas was 20 cm/min. Compounds were identified by comparison of the retention time to known FAME standards (Nu-Chek-Prep) and were quantified by comparison to the C17 internal standard peak.

2.8. Analysis by thin layer chromatography

A commercial TLC standard lipid mixture, containing cholesterol ester (CE), triglyceride (TG), cholesterol (C), phospholipid (PL), diglyceride (DG) and free fatty acid (FFA), together with the crude mussel extract and semi-purified fractions were applied to a silica gel plate. The plates were developed in a mobile phase of hexane-diethyl ether-acetic acid (80:20:2 v/v/v) until the solvent reached approximately 2 cm from the top of the plate (Christie, 1982). Lipid compounds were visualised by spraying with 10% $CuSO_4$, 8% H_3PO_4 in water and heating at 110 °C for 20 min

Table 4
Percent inhibition of the production of LO metabolites by RP fractions

Code	Dilution	Inhibition (%)[1]			
		6-*trans* LTB_4	6-*trans*,12-*epi* LTB_4	LTB_4	5-HETE
RPFA-1	1:100	13±5.2	13±4.7	2±3.8	0±23.2
	1:1000	0±5.7	0±5.1	3±4.0	0±15.9
	1:10,000	8±4.2	10±4.1	4±3.0	4±13.8
RPFA-2	1:100	100±0.7	100±0.0	100±0.8	100±3.1
	1:1000	24±1.1	40±1.1	47±1.1	3±7.1
	1:10,000	12±2.8	12±2.6	20±2.7	0±10.8
RPFA-3	1:100	94±1.6	100±2.4	79±3.0	100±7.2
	1:1000	29±4.5	43±3.9	13±3.0	12±16.3
	1:10,000	12±4.7	26±3.9	8±3.0	7±12.2
RPFA-4	1:100	100±2.7	100±2.4	100±2.2	100±7.2
	1:1000	43±4.5	58±4.0	25±3.1	23±13.4
	1:10,000	20±4.3	29±3.8	13±3.3	6±14.2
RPFA-5	1:100	97±3.7	100±2.4	89±3.3	83±11.3
	1:1000	25±4.8	43±4.3	13±3.0	0±19.7
	1:10,000	19±3.5	36±3.6	6±3.1	0±13.7
RPFA-6	1:100	22±2.3	33±2.1	16±2.0	10±9.3
	1:1000	0±4.6	0±4.0	0±2.4	0±14.6
	1:10,000	0±4.7	0±4.2	0±2.4	0±11.7
RPFA-7	1:100	44±3.5	59±3.3	22±3.3	22±11.6
	1:1000	0±4.4	6±4.1	0±3.5	0±13.6
	1:10,000	8±4.1	19±3.6	6±2.7	5±10.1
RPFA-8	1:100	26±1.1	43±1.0	14±1.4	3±7.0
	1:1000	11±2.2	12±1.6	14±1.3	0±7.2
	1:10,000	0±3.6	0±2.7	8±3.6	0±7.6
FFA[2]	1:100	89±2.1	100±0.6	100±0.8	89±10.1
	1:1000	0±3.3	0±2.6	0±2.1	0±13.6
	1:10,000	0±2.2	0±1.8	0±1.4	0±11.5

[1]Control values were, typically (ng/10^6 cells, mean±s.d, n=4): 6-*trans* LTB_4, 28.7±2.7; 6-*trans*,12-*epi* LTB_4, 21±2.4; LTB_4, 20.2±2.2; 5-HETE, 147±7.2. The activity of the test samples is expressed as the % inhibition of control production of LO metabolites (mean±s.d, n=4). [2]Total free fatty acids isolated by open column NP flash chromatography.

(Bitman and Wood, 1982). The analysis of the FAME was carried out by TLC in a solvent system of 4% MTBE in 96% hexane, with a visualising standard of palmitic methyl ester.

2.9. Argentation–HPLC (Ag–HPLC) separation of the FAME

The RP–HPLC fractions which were identified as bioactive and novel were individually purified by Ag–HPLC. These fractions (as their methyl esters) were evaporated to dryness, dissolved in 1, 2-dichloroethane (DCE) (1:10 w/v) and passed through a 0.45 μm nylon filter (Activon). The optimal separation system was developed by applying a commercial standard mixture (Nu-Chek-Prep 84) containing C16:0, C17:0, C18:0, C18:1, C18:2, C18:3, C20:0, C20:4 and C22:6 methyl esters. A gradient, multi-solvent system (Table 3) was developed with DCM–DCE (1:1 v/v) as the equilibrating solvent and a stepwise increase of AcCN–MeOH (1:1 v/v) to alter the solvent strength and selectivity of the mobile phase. The Ag–HPLC utilised a Chromsphere™ 5 μm, 25 cm × 10 mm (ID) C18 silver impregnated column (Chrompack International, Middelburg, Netherlands) with a 75 mm × 4.6 mm Valco Polar bonded phase (Chrompack) pre-column. The same instrumentation as the RP–HPLC was used with the detector setting ESV at +70 °C and a gas delivery pressure of 14 psi. The samples were collected in a preset time windows mode of collection with the column effluent diverted directly to the fraction collector.

2.10. GC-MS analysis of active FAME fractions from Ag–HPLC separation

The analysis was carried out using a Hewlett-Packard 5890 GC (Palo Alta, CA, USA) fitted with an BPX5 capillary column (12 m × 0.22 mm (ID), film thickness 0.33 μm, (SGE) inlet pressure of 5 psi. The oven temperature was held at 75 °C for 2 min, and then heated at 30 °C/min to 300 °C, with a final holding time of 9 min. Splitless injections were used with an injector temperature of 250 °C.

The GC-MS was carried out in both electron impact (EI) and chemical ionisation (CI) modes with both data sets providing supportive data. The EI-MS was performed using the above GC conditions and utilising a quadruple Finnigan MAT INCOS-50 MS, with an interface temperature of 280 °C and the ion source temperature of 180 °C. The scan range was 50 to 500 Da at 0.6 s/scan. The positive ion CI-MS using methane as the ionisation gas was performed with the same conditions as described above on a Hewlett-Packard 5890 GC (Palo Alta, CA, USA) and a Finnigan MAT TSQ-70 MS detector.

3. Results

3.1. Supercritical fluid extraction

The maximum yield of CO_2–SFE extractable oil from *P. canaliculus* freeze dried mussel powder was 4.76% w/w. The mussel extract was orange-amber with a viscous waxy appearance at ambient temperature. Analysis of the oil by TLC afforded several lipid classes including cholesterol esters (CE), triglycerides (TG), free fatty acids (FFA), diglycerides (DG), cholesterol (C), phospholipids (PL) and monoglycerides (MG).

3.2. Group separation of the FFA class of the mussel extract

A representative chromatogram of the fractions obtained in the preparative NP–HPLC system is shown in Fig. 1, with the elution order of the lipid classes indicated (i.e. CE, TG, FFA, DG and C). The lipid classes were identified in the chromatogram by comparison of the relative retention times to commercial standards, as well as by TLC analysis.

The FFA class was eluted in a retention volume of 640–775 mL, and divided into two regions. The first region designated "FFA_s", which eluted close to the TG, was identified by GC as the saturated FFA component. The second region designated "FFA_u" had a longer retention time, and contained the unsaturated FFA component. The later region was determined to be the more biologically active fraction by the *in vitro* assays. The collection of the FFA fraction commenced from the middle of the first region in order to avoid excessive carry-over of the TG class. The overall yield of the collected FFA fraction was 10% of the total lipid material.

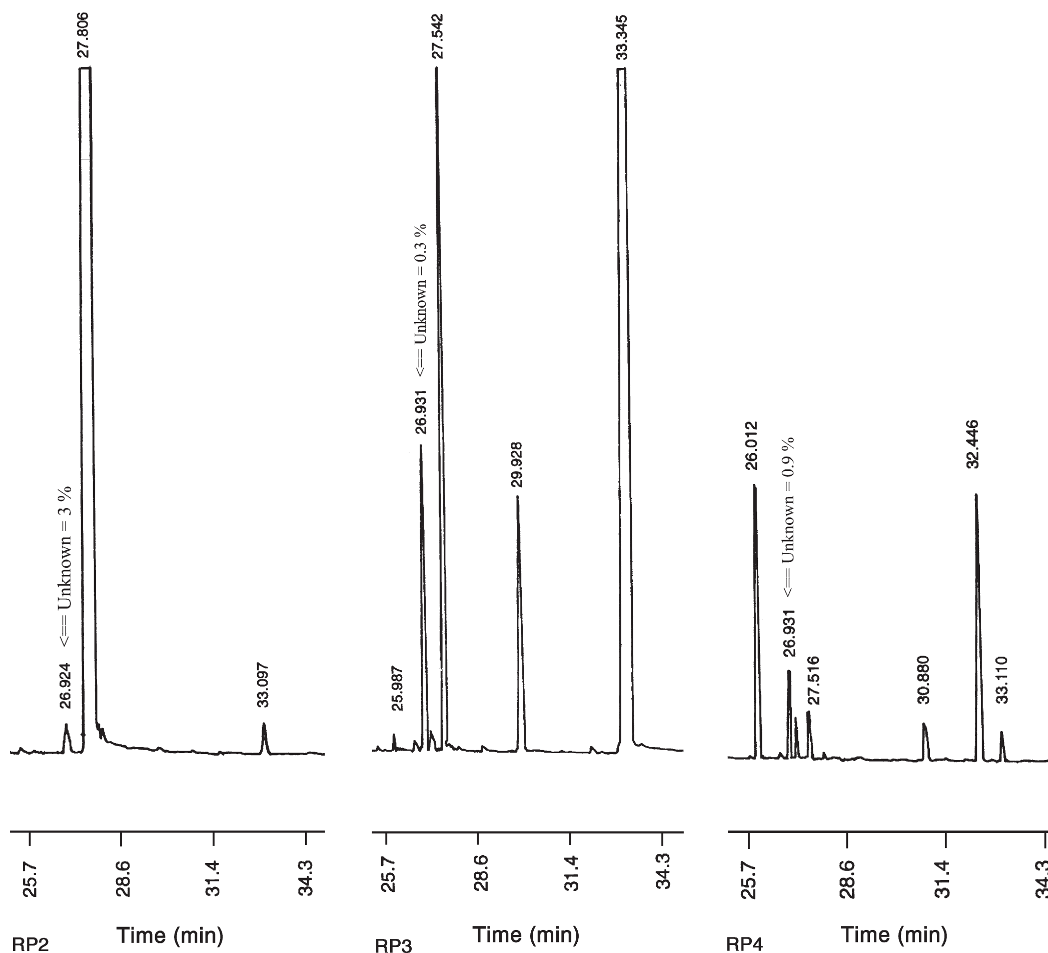

Fig. 3. The significant regions of GC chromatograms of the bioactive fractions RP2, RP3, and RP4, which correspond to time windows shown in Fig. 2. Chromatographic conditions: BPX70 capillary column (50 m × 0.22 mm (ID), 0.2 μm), injector temperature 260 °C, using the temperature program in methods Section 2.7. The common unknown compound is expressed as a percentage of the total FAME in each of the fractions.

3.3. Semi-preparative reversed-phase–HPLC

A preliminary separation of the FFA fraction obtained from NP-chromatography was carried out by RP–HPLC. Fig. 2 indicates the separation achieved by the RP–HPLC with the relative elution times shown above each peak. The fractions were collected according to the time windows shown in Fig. 2 and coded “RP1” through to “RP8”, and screened for AI activity in the *in vitro* leukotriene inhibition assay.

3.4. Leukotriene in vitro assay of RP1 to RP8

The RP–HPLC fractions RP1-RP8 and the FFA class were screened by the *in vitro* assay and the results of this assay are summarised in Table 4. The FFA class contained activity at the 1:100 dilutions only. Fractions RP2–RP5 exhibited the most inhibition of the LO products formed (at all dilutions), thus indicating that bioactive compounds were located in these fractions.

3.5. Analysis of bioactive fractions by GC

Fractions RP2–RP5 were analysed by GC as their methyl esters. Fraction RP5 contained known PUFA but no novel compounds when compared to the retention times of commercial standards, therefore it was not further purified. The fractions RP2–RP4 all contained unusual compounds, and are shown in Fig. 3 which illustrates the significant region of each of the GC chromatograms. The chromatogram of the profiles of RP2, RP3, and RP4 shared a common unknown compound at the retention time of 26.93 min under the experimental conditions. This unusual FAME had separation characteristics which indicted it was 20 carbons in length with 4 unsaturated double bonds (C20:4). Fractions RP2, RP3 and RP4 were further purified by Ag–HPLC.

3.6. Semi-preparative Ag–HPLC separation of RP-FAME

The bioactive fractions were converted into their methyl esters, with a 93% conversion. Using the 12% BF_3 in MeOH method, RP2 (365.6 mg), RP3 (519.5 mg), and RP4 (757.2 mg) were individually converted to obtain 340.0 mg, 405.6 mg and 673.9 mg, respectively.

The Ag–HPLC silver impregnated C18 column was able to separate the FAME by their degree of unsaturation, with the most saturated compound being eluted first. The separation of the fractions RP2, RP3 and RP4 are indicated by a

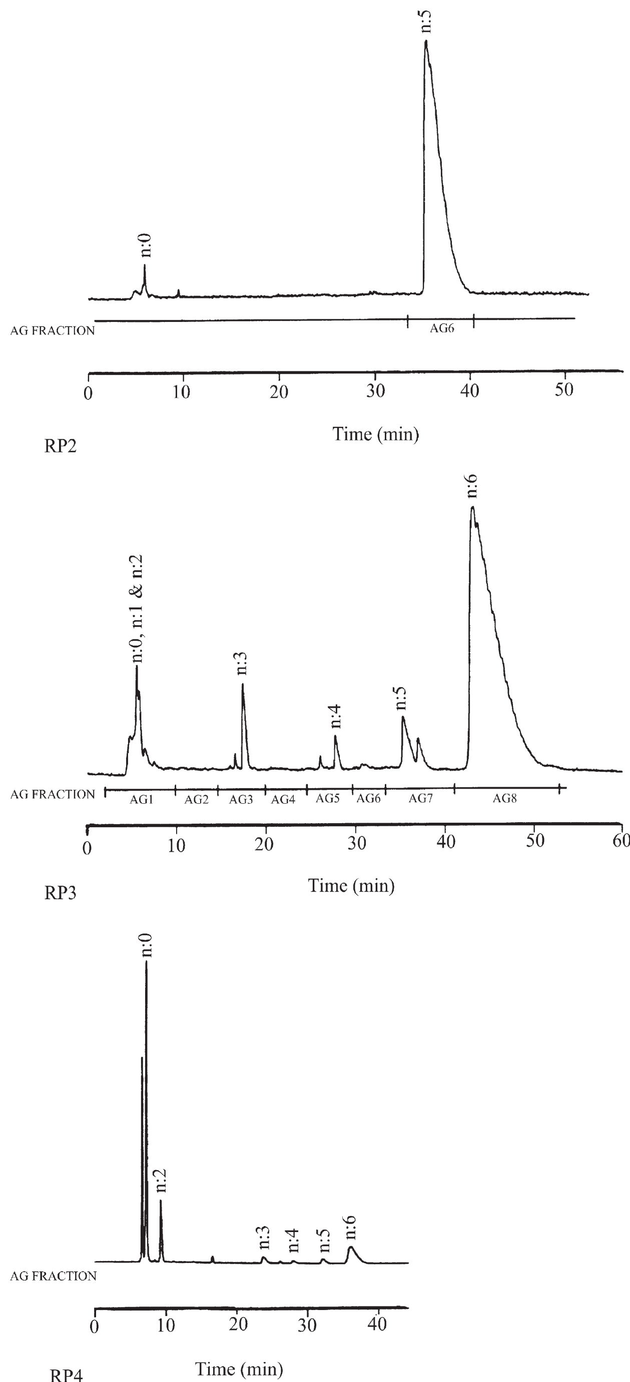

Fig. 4. Semi-preparative Ag–HPLC chromatogram of the bioactive RP–HPLC fractions RP2, RP3 and RP4. Chromatographic conditions: Chrompack Chromosphere, silver C18 semi-preparative column, using the multi-solvent system shown in Table 3, and a flow rate of 1.5 mL/min with 1 mg of each applied. Light scattering detector settings: ESV +70 °C, gas pressure 14 psi. The identified peaks represent the fatty acid methyl esters, with varying degree of unsaturation indicated (n, denoting the number of double bonds).

representative chromatogram of each in Fig. 4. The assignment of the degree of unsaturation was obtained by comparing the retention times with commercial standards.

The fraction RP2 (300 mg) was applied to the Ag–HPLC and showed one predominant peak (Fig. 4). This peak, which contained five double bonds, was collected and coded "$AG6_1$" and was analysed for bioactivity by the *in vitro* assay. Fraction RP3 (387 mg) was applied to the system, and the fractions were collected according to the established time windows as shown in Fig. 4. Fractions 2 and 4 contained no material and therefore were not further analysed. The remaining fractions, coded "AG1", "AG3", "AG5", "AG6", "AG7" and "AG8" were screened for bioactivity by the *in vitro* assay. Finally RP4 was subjected to the Ag–HPLC and a similar chromatogram to RP3 was produced (Fig. 4).

Table 5
Percent inhibition of the production of LO metabolites by AG fractions

Code	Dilution	Inhibition (%)[1]			
		6-*trans* LTB_4	6-*trans*,12-*epi* LTB_4	LTB_4	5-HETE
AG1	1:100	41±13	34±10	8±6	29±5
	1:1000	28±16	27±21	29±17	23±14
	1:10,000	25±17	26±13	30±16	23±15
AG3	1:100	65±8	59±9	51±9	54±9
	1:1000	3±15	2±13	0±9	NT[2]
	1:10,000	10±19	11±21	0±14	20±14
AG5	1:100	82±13	70±12	64±20	71±15
	1:1000	0±25	0±20	4±11	10±3
	1:10,000	0±18	0±23	0±8	6±5
AG6	1:100	48±14	46±13	47±9	44±5
	1:1000	0±5	0±3	11±12	8±2
	1:10,000	0±15	0±18	2±4	11±17
AG7	1:100	25±10	21±10	3±4	40±5
	1:1000	0±15	0±10	1±7	4±7
	1:10,000	0±12	0±13	0±5	0±11
AG8	1:100	74±9	77±1	91±2	72±0[3]
	1:1000	41±10	35±14	37±13	23±14
	1:10,000	0±18	0±17	15±10	14±18
$AG6_1$	1:100	59±3	56±4	27±16	50±4
	1:1000	22±22	22±17	16±25	39±25
	1:10,000	16±11	19±12	4±12	17±2
EPA	1:100	72±10	80±11	39±14	44±12
	1:1000	32±7	32±6	16±6	24±8
	1:10,000	31±17	31±16	110±27	13±15

[1]Control values were, typically (ng/10^6 cells, mean±s.d, n=4): 6-*trans* LTB_4, 39.9±5.0; 6-*trans*,12-*epi* LTB_4, 39.0±5.8; LTB_4, 20.1±1.7; 5-HETE, 134±8.6. The activity of the test samples is expressed as the % inhibition of control production of LO metabolites (mean±s.d, n=4). [2]NT (not tested). [3]n=1 replicate.

3.7. Leukotriene in vitro assay of Ag–HPLC Fractions

The *in vitro* assay was performed on fractions $AG6_1$, AG1, AG3, AG5, AG6, AG7, and AG8 in addition to the eicosapentaenoic acid (EPA) commercial standard (Table 5).

Table 6
Preliminary identification by GC of the major components in fractions AG5 and AG6

Retention time (min)	Compound	% of total fraction	
		AG5	AG6
27.34	Eicosapentaenoic acid	0.1	–
26.00	Arachidonic acid	8.3	0.2
29.85	Unknown C21:5	0.1	–
27.03	Unknown C20:4	67.9	73.0
24.48	Unknown C19:4	2.8	1.0
23.47	Unknown C18:4	7.4	9.9

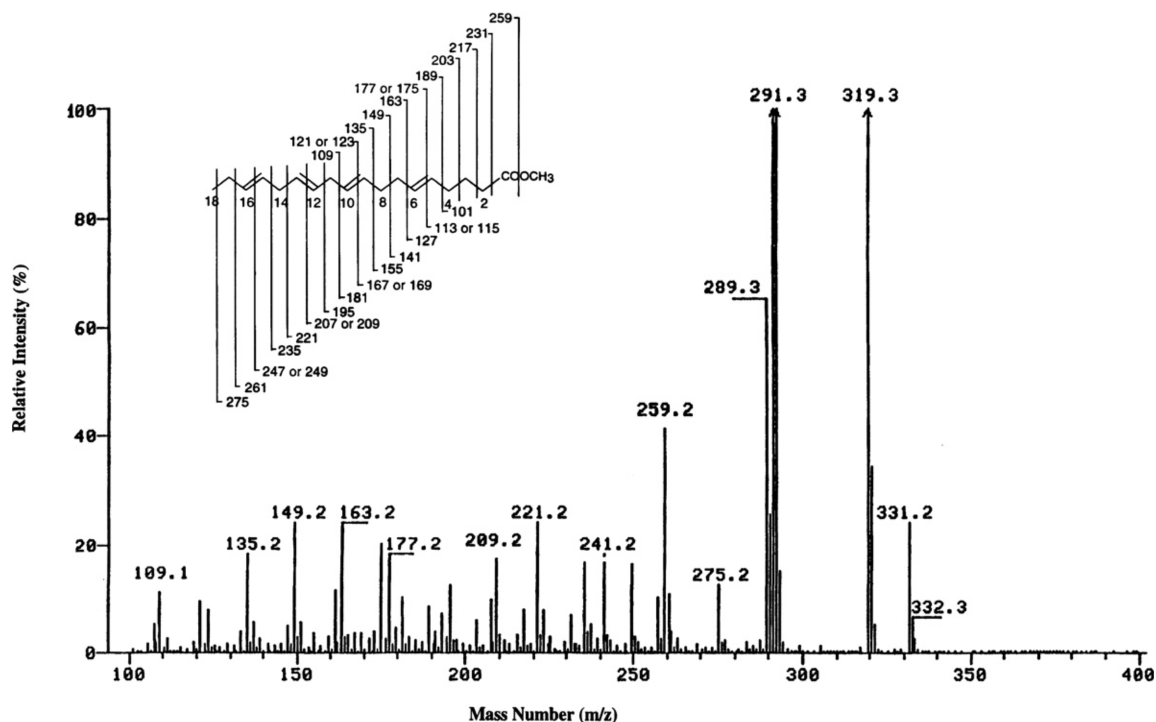

Fig. 5. Chemical ionisation mass spectrum of the methyl ester of the C18:4 FA in bioactive fraction AG5. Operating parameters: methane reactant gas, scan range 50–500 Da at 0.6 s/scan, interface temperature 280 °C, injector temperature 250 °C, and the ion source temperature 180 °C.

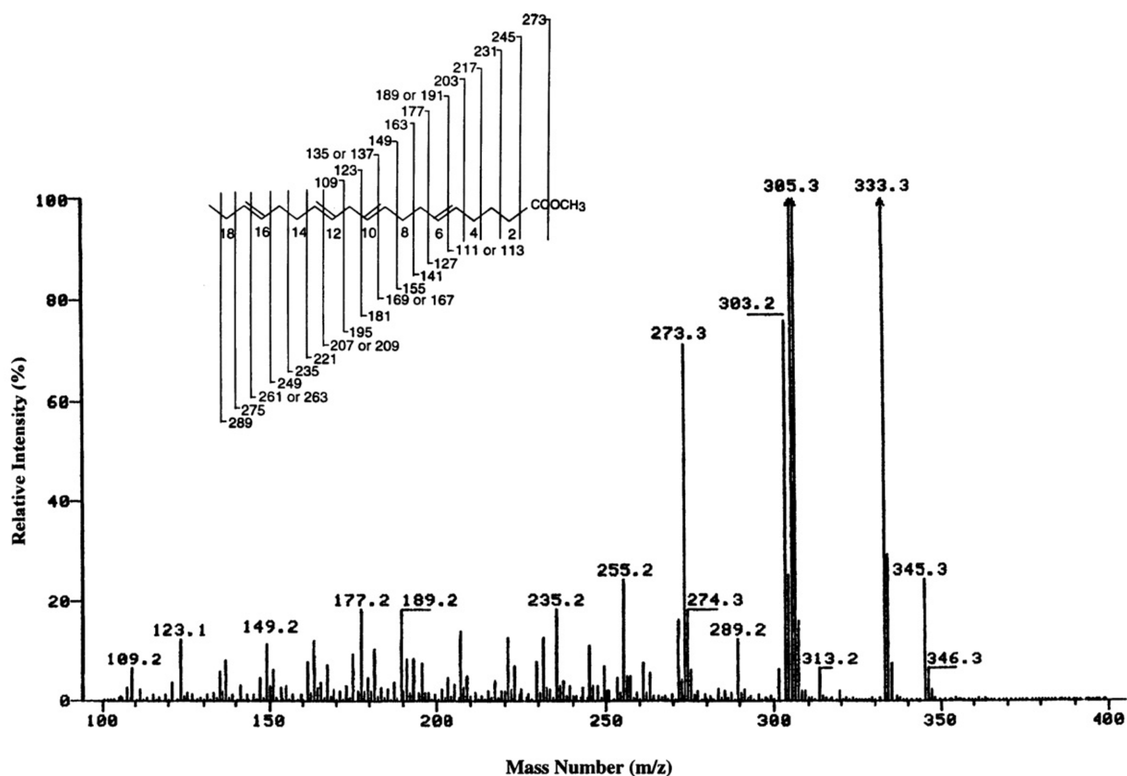

Fig. 6. Chemical ionisation mass spectrum of the methyl ester of the C19:4 FA in bioactive fraction AG5. Operating parameters: methane reactant gas, scan range 50–500 Da at 0.6 s/scan, interface temperature 280 °C, injector temperature 250 °C, and the ion source temperature 180 °C.

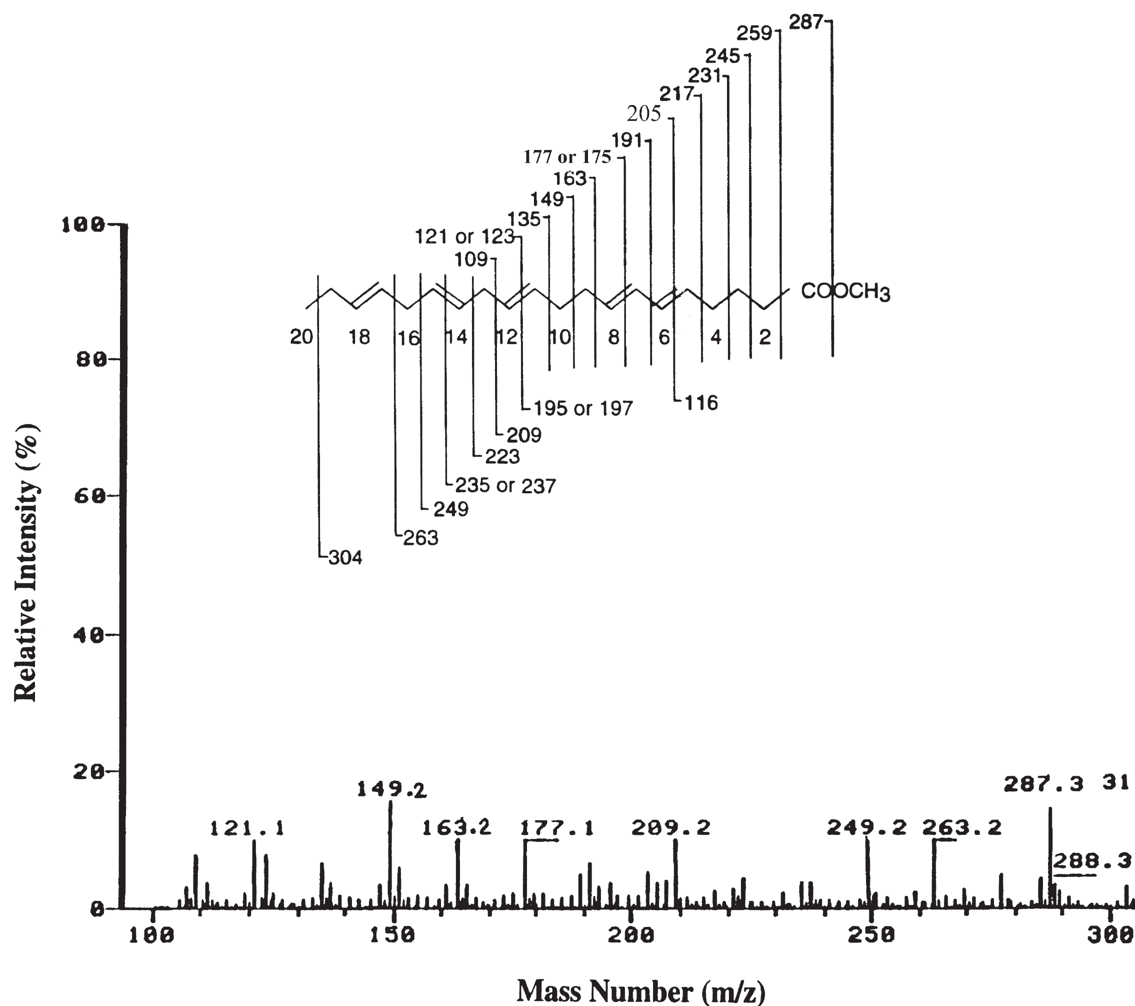

Fig. 7. Chemical ionisation mass spectrum of the methyl ester of the C20:4 FA in bioactive fraction AG5. Operating parameters: methane reactant gas, scan range 50–500 Da at 0.6 s/scan, interface temperature 280 °C, injector temperature 250 °C, and the ion source temperature 180 °C.

The results demonstrate that fractions AG5 and AG8 had the highest activities (1:100 dilutions).

3.8. Analysis of bioactive Ag–HPLC fractions by GC

Fractions which were shown to be bioactive from the *in vitro* neutrophil assay were analysed by GC, which indicated fractions AG1 and AG3 contained known unsaturated FFA including EPA. Fractions AG5 and AG6 however contained several novel compounds, and their percentage abundance are shown in Table 6. Fraction AG5 contained four unknown compounds in addition to EPA and AA. Fraction AG6 contained three unknown compounds and a trace of AA. Fraction AG8 was found by GC analysis to exclusively contain docosahexaenoic acid (DHA). Consequently, fractions AG5 and AG6 were further characterised by GC-MS.

3.9. GC-MS of active fractions from Ag–HPLC separation

The GC-MS analyses of fractions AG5 and AG6 were performed in both the CI and EI modes. Figs. 5–8 show the PICI mass spectra of the novel compounds and the fragmentation proposed to support the structural assignments. While neutral loss and impact fragmentation from protonated parents is a less energetic approach to EI of the same analytes, polyunsaturated compounds still show some propensity for rearrangement. This novel use of full scan PICI mass spectrometry was of benefit in that it provided for bioprospective examination of fractions on the basis of carbon number, degree of unsaturation and unstable substitution while also providing a population of intact and diagnostic high mass fragments that would be atypical in EI mode. Interpolation of structure from the similar fragmentation of known standards, such as EPA, AA and DHA, that contained common structural elements allowed the tentative assignment of structure that were then able to be confirmed by the same technique against known reference compounds available by independent routes.

The CI-MS of C18:4 in bioactive fraction AG5 is shown in Fig. 5. The proposed structure of this compound as the methyl ester is indicated, with the fragmentation shown for $M^{+}+H=291$ and a molecular formula of $C_{19}H_{30}O_2$. The compound was identified as having the bond positions of 5, 9, 12 and 15. The

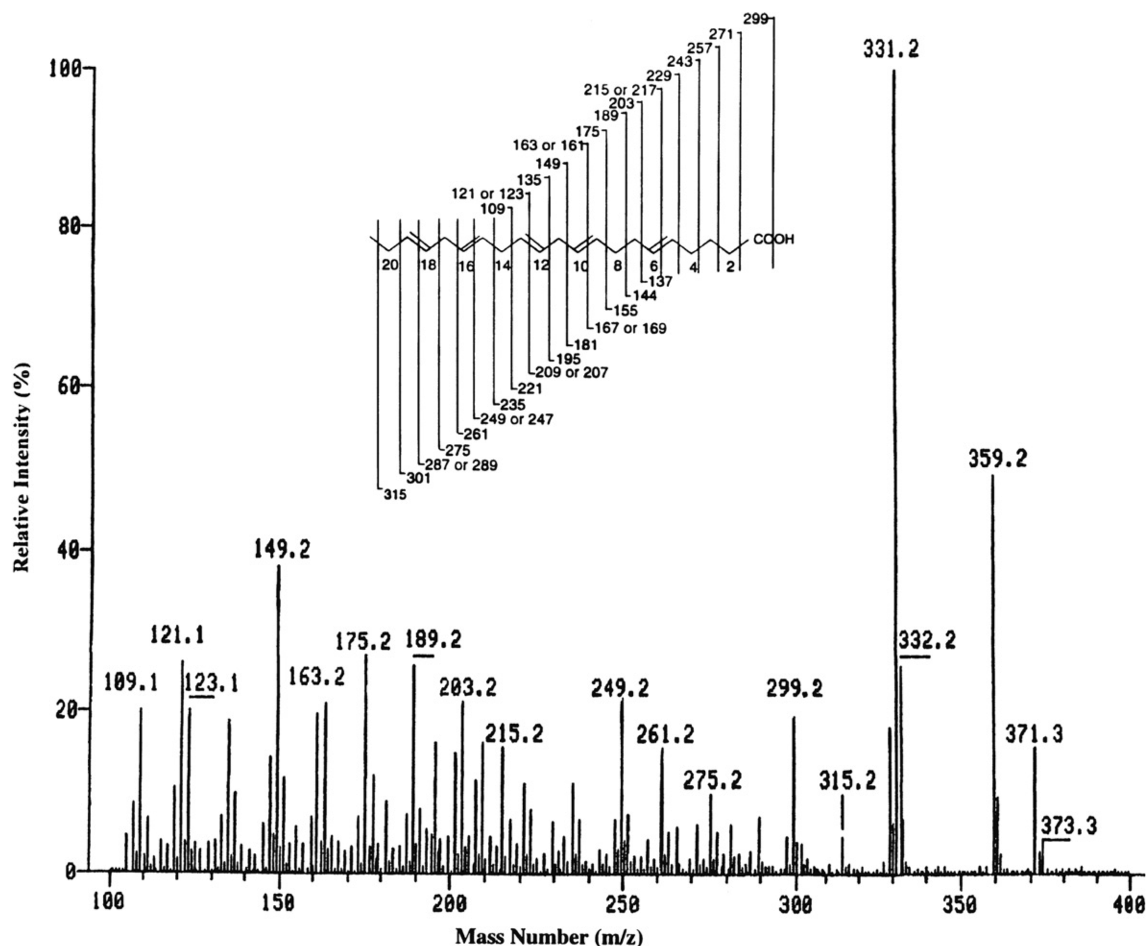

Fig. 8. Chemical ionisation mass spectrum of the methyl ester of the C21:5 FA in bioactive fraction AG5. Operating parameters: methane reactant gas, scan range 50–500 Da at 0.6 s/scan, interface temperature 280 °C, injector temperature 250 °C, and the ion source temperature 180 °C.

unsaturated centre C5/C6 position was indicated by the gap between m/z 189 and 163, positions C9/C10 by the gap between m/z 135 and 109, positions C12/C13 by the gap between m/z 221 and 195, positions C15/C16 by the gap between m/z 261 and 235.

The CI-MS of C19:4 in bioactive fraction AG5 is shown in Fig. 6 and the structure proposed to be consistent with the methyl ester with $M^{+}+H=305$ and $C_{20}H_{32}O_{2}$. The compound was identified as having the bond positions of 5, 9, 12 and 16. With an additional methylene group in the chain, the unsaturated centre C5/C6 position was indicated by the gap between m/z 203 and 177, positions C9/C10 by the gap between m/z 149 and 123, positions C12/C13 by the gap between m/z 221 and 195, positions C16/C17 by the gap between m/z 275 and 249.

The CI-MS of C20:4 in bioactive fraction AG5 is shown in Fig. 7. Fragmentation supports the assignment of the methyl ester with $M^{+}+H=319$ and molecular formula $C_{21}H_{34}O_{2}$. The compound was identified as having the bond positions of 7, 11, 14 and 17. The unsaturated non-skipped double bond centre C7/C8 position was assigned on the basis of the strong allylic fragment at m/z 149 and the fragment at m/z 163. Further assignment for the C7 double bond produced complex fragmentation resulting from bond migration for the isolated carbon double bond. Comparison of the spectra of the methyl esters of AA and EPA with that of the FAME at retention peak 27.03 min supports the assignment of this PUFA as 7, 11, 14, 17-eicosatetraenoic acid.

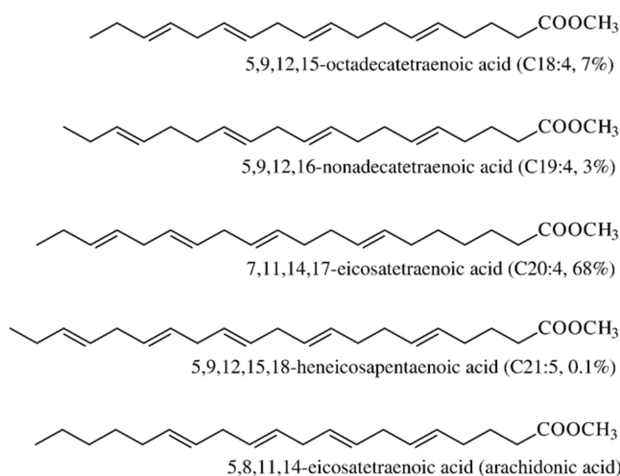

Fig. 9. The structures of ω-6 AA, and the novel bioactive ω-3 FAME from AG5 (as identified by GC-MS) and their percent concentration of the AG5 fraction. The novel compounds were identified as C18:4, C19:4, C20:4 and C21:5, and are an homologous series where the second double bond is separated from the first by more than one methylene group.

The CI-MS of C21:5 in bioactive fraction AG5 is shown in Fig. 8 and the data support the assignment as the methyl ester with $M^{+}+H=331$ and molecular formula $C_{22}H_{34}O_2$. The compound was identified as having the bond positions of 5, 9, 12, 15 and 18. The unsaturated centre C5/C6 position was identified by the gap between m/z 229 and 203, positions C9/C10 by the gap between m/z 175 and 149, positions C12/C13 by the gap between m/z 135 and 109, positions C15/C16 by the gap between m/z 261 and 235, and the position C18/C19 by the gap between m/z 275 and 301.

The novel compounds identified were all ω-3 FAME in an homologous series (Fig. 9). The FAME showed structural similarity in the occurrence of the second double bond from the proximal end (carboxyl end) of each acid being separated from the first by more than one methylene group. The compounds were identified as 5, 9, 12, 15-octadecatetraenoic acid (C18:4); 5, 9, 12, 16-nonadecatetraenoic acid (C19:4); 7, 11, 14, 17-eicosatetraenoic acid (C20:4) and 5, 9, 12, 15, 18-heneicosapentaenoic acid (C21:5). The predominant compound was the C20:4 fatty acid which is a structural isomer to AA.

4. Discussion

The potential AI activity of lipids isolated from the green-lipped mussel of New Zealand, *P. canaliculus*, was investigated in this study. The tartaric acid-stabilised freeze dried powder of the mussel was subjected to supercritical-CO_2 fluid treatment, and the resulting oil was fractionated for its lipids using standard methodology. The SFE process utilises liquefied CO_2 to avoid the use of any chemical solvents that could create health or acceptability concerns for human applications.

The FFA class was purified from the mussel extract and then rigorously fractionated in order to isolate and identify the bioactive constituents. The group purification was carried out by two means, namely column chromatography and NP–HPLC. The column chromatography was performed to remove the highly polar mussel lipids and offered a reproducible means of separation. However, large quantities of solvents were consumed. Group purification by NP–HPLC provided a fast and efficient separation technique that was comparable to conventional open column technique. While the entire FFA class was not completely resolved by the NP–HPLC, the unsaturated FFA portion was relatively free from other lipid classes. Fractionation of the FFA group of the mussel extract utilised two complementary systems, namely RP–HPLC and Ag–HPLC. The RP–HPLC served as a preliminary fractionation stage in which the elution sequence of the fatty acids was governed by chain length and the presence of double bonds. The Ag–HPLC was carried out using $AgNO_3$–impregnated silica columns, which offer a sensitive means of separation. In the presence of silver ions, the separation was determined by the number of double bonds in the FAME, with FAME with *cis* double bonds eluting slower than those with double bonds in the *trans* configuration (Nikolova-Darnyanova, 1992).

The FFA fractions obtained from the RP procedures were subjected to a proven *in vitro* assay for AI activity that uses a rapid and sensitive approach for the quantification of leukotrienes released by stimulated neutrophils (Cleland et al., 1990). The leukotriene assay monitors the production of four of the LO pathway products, namely LTB_4, 6-*trans* LTB_4, 6-*trans* 12 *epi* LTB_4, and 5-HETE. The formation of these LO products was significantly reduced by the *P. canaliculus* FFA fractions. The RP–HPLC fractions (more specifically fractions RP2 to RP5) demonstrated higher bioactivity than the entire FFA class.

Analysis by GC of the bioactive fractions was employed to ascertain whether any of the FFA fractions contained novel compounds. It was found that three of the bioactive RP–HPLC fractions, namely RP2, RP3 and RP4, contained unusual fatty acids. These fractions were carried through to the subsequent purification stage of Ag–HPLC. The results of the *in vitro* analysis of the Ag–HPLC fractions showed that fraction AG8 had high bioactivity and was identified by GC as 4, 7, 10, 13, 16, 19-docosahexaenoic acid (i.e. DHA), a known long chain ω-3 PUFA. The AI activity of DHA is believed to be due to a substrate substitution effect which results in a reduction of the relative concentration of AA, thereby diminishing the production of pro-inflammatory eicosanoids (Calder, 2006).

It has been reported that other bivalves which do not possess the marked AI activity of *P. canaliculus*, contain considerable DHA in their lipid fraction (Ackman, 1990; Couch et al., 1982). Consequently, it is possible that the AI activity of *P. canaliculus* is not due entirely to the presence of DHA, but also to other PUFA. Indeed, other Ag–HPLC fractions exhibited marked bioactivity in the *in vitro* assay, i.e. fractions AG5 and AG6. Analysis by GC of fractions AG5 and AG6 showed that they contained several novel compounds. These novel compounds were identified by GC-MS as four ω-3 PUFA compounds, namely C18:4, C19:4, C20:4 and C21:5. The GC-MS was performed in the EI and CI modes. In the EI mode, the long chain carboxylic acids and their esters undergo considerable skeletal rearrangement and bond migration. Therefore, it is not possible to determine the exact position of the double bonds in these long chain unsaturated compounds from the EI mass spectra. This novel application of positive ion chemical ionisation mass spectrometry provided data that was truly complimentary to the more energetic impact technique both because it was lower in energy and diagnostic fragments were sourced from protonated parents thereby altering the thermodynamics influencing their formation. Using PICI in a bioprospective approach against external standards allowed assignment of the bond positions in the bioactive compounds of fractions AG5 and AG6. The structures of the unusual FFA of *P. canaliculus* were found to be an homologous series, and a possible AI mechanism can be inferred from a comparison of these structures with AA.

The inflammatory precursor AA is an ω-6 PUFA of 20 carbons in length and has 4 unsaturated double bonds (positions 5, 8, 11 and 14) with each double bond being separated by one methylene group. The predominant bioactive PUFA of *P. canaliculus* identified in this study is similar to AA in that it also possesses 20 carbons with four double bonds. However, the first double bond is located at the seventh position, and the second double bond is interrupted from the first by two methylene groups resulting in the double bonds at positions 7, 11, 14 and 17. A similar pattern is shown for the other three novel compounds identified, whereby the second double bond is separated from the

first by more than one methylene group. The interrupted bond positioning of these structural analogues of AA may account for their AI behaviour, by competitively inhibiting the active site of enzymes which use AA as a substrate, i.e., LO and COX, thereby reducing the production of leukotriene (LT) and prostaglandin (PG) metabolites.

We have previously demonstrated strong inhibition of the LT and PG metabolites of AA metabolism, as well as production of alternate LT and PG metabolites, for a variety of PUFA found in Lyprinol® (McPhee et al., 2001; McPhee et al., 2007). It is not yet known whether the novel homologous series of ω-3 PUFA isolated in this study are substrates for the AA metabolising enzymes; nevertheless, these compounds might not be converted to the endogenous eicosanoids, and consequently the inflammatory processes promoted by increased LT and PG levels would be diminished. In addition, inhibition of both COX and LO may also reduce the incidence of the side effects that occur when only PG production is inhibited (Charlier and Michaux, 2003).

In this study we isolated four potentially AI ω-PUFA from *P. canaliculus* using various chromatography techniques. The compounds identified are biologically significant as AI agents, and are structural analogues forming an ω-3 PUFA homologous series. The structure proposed for the most prominent PUFA has double bonds at alternative positions to AA, the inflammatory pathway precursor. This implies that this prominent C20:4 and its homologues may act as inhibitors of LT and PG metabolite production in the inflammation pathways.

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References

Ackman, R.G., 1990. Marine, Biogenic Lipids, Fats and Oils. CRC Press, Boca Raton, USA, p. 84.

AOAC, 1995. Methyl esters of fatty acids in oils and fats–gas chromatography method. Official Methods of the Association of Official Analytical Chemists, Arlington, VA, Method 96322.

Bitman, J., Wood, L.D., 1982. An improved copper reagent for quantitative densitometric thin-layer chromatography of lipids. J. Liq. Chromatogr. 5, 1155–1162.

Bogatcheva, N.V., Sergeeva, M.G., Dudek, S.M., Verin, A.D., 2005. Arachidonic acid cascade in endothelial pathobiology. Microvasc. Res. 69, 107–127.

Calder, P.C., 2006. Polyunsaturated fatty acids and inflammation. Prostaglandins Leukot. Essent. Fatty Acids 75, 197–202.

Charlier, C., Michaux, C., 2003. Dual inhibition of cyclooxygenase-2 (COX-2) and 5 lipoxygenase (5-LOX) as a new strategy to provide safer non-steroidal anti-inflammatory drugs. Eur. J. Med. Chem. 38, 645–659.

Christie, W.W., 1982. Lipid Analysis, 2nd edn. Pergamon Press, Oxford, UK, p. 82.

Cleland, L.G., James, M.J., Gibson, R.A., Hawkes, J.S., Betts, W.H., 1990. Effect of dietary oils on the production of *n*-3 and *n*-6 metabolites of leukocyte 5-lipoxygenase in five rat strains. Biochim. Biophys. Acta 1043, 253–258.

Couch, R.A., Ormrod, D.J., Miller, T.E., Watkins, W.B., 1982. Anti-inflammatory activity in fractionated extracts of the green lipped mussel. NZ Med. J. 95, 803–806.

Dugas, B., 2000. Lyprinol® inhibits LTB_4 production by human monocytes. Allerg. Immunol. (Paris) 32, 284–289.

Gibson, S.L.M., Gibson, R.G., 1998. The treatment of arthritis with a lipid extract of *Perna canaliculus*: a randomised trial. Complement. Ther. Med. 6, 122–126.

Huang, A.S., Robinson, L.R., Gursky, L.G., Profita, R., Sabidong, C.G., 1994. Identification and quantification of SALATRIM 23CA in foods by the combination of supercritical fluid extraction, particle beam LC-mass spectroscopy, and HPLC with light-scattering detector. J. Agric. Fd. Chem. 42, 468–473.

Kosuge, T., Sugiyama, K., 1989. Stabilized mussel extract. United States Patent 4801453.

Macrides, T.A., Kalafatis, N., 2000. Super-critical lipid extract from mussels having anti inflammatory activity. United States Patent 6083536.

McPhee, S., Kalafatis, N., Wright, P.F.A., Macrides, T.A., 2001. The marine oil Lyprinol is a substrate for the 5-Lipoxygenase enzyme in porcine neutrophils. Proc. Aust. Soc. Clin. Exp. Pharmacol. Toxicol. 9, 95.

McPhee, S., Hodges, L.D., Wright, P.F.A., Wynne, P.M., Kalafatis, N., Harney, D.W., Macrides, T.A., 2007. Anti-cyclooxygenase effects of lipid extracts from the New Zealand green-lipped mussel, *Perna canaliculus*. Comp. Biochem. Physiol. Part B. 146, 346–356.

Morris, T., Rajakariar, R., Stables, M., Gilroy, D.W., 2006. Not all eicosanoids are bad. Trends Pharmacol. Sci. 27, 609–611.

Nikolova-Damyanova, B., 1992. Silver ion chromatography and lipids. In: Christie, W.W. (Ed.), Advances in Lipid Methodology, Part One, Ch 6. The Oily Press, Scotland.

Stamp, L.K., James, M.J., Cleland, L.J., 2005. Diet and rheumatoid arthritis: a review of the literature. Semin. Arthritis Rheum. 35, 77–94.

Still, W.C., Kahn, M., Mitra, A., 1978. Rapid chromatographic technique for preparative separations with moderate resolution. J. Org. Chem. 43, 2923–2925.

Whitehouse, M.W., Macrides, T.A., Kalafatis, N., Betts, W.H., Haynes, D.R., Broadbent, J., 1997. Anti-inflammatory activity of a lipid fraction (Lyprinol) from the NZ green lipped mussel. Inflammopharmacology 5, 237–246.