Original Article

Fatty acid and sterol composition of frozen and freeze-dried New Zealand Green Lipped Mussel *(Perna canaliculus)* from three sites in New Zealand

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In view of previously reported anti-inflammatory bioactivity of the New Zealand Green Lipped Mussel (NZGLM), the overall lipid profile and fatty acid and sterol composition of the NZGLM from various sites in New Zealand (Hallam Cove, Port Ligar, Little Nikau) were investigated using thin layer chromatography (TLC) and gas liquid chromatography (GLC). Samples were either frozen (F) or freeze-dried (FD) soon after collection. It was also thought prior to the study, there may be differences in the dietary sources of phytoplankton between the sites, responsible for the bioactivity, however data collected in New Zealand reported no difference in the type of phytoplankton, but a difference in the quantity. There were no major significant differences in the major compo-nents of the lipid, fatty acid and sterol composition between FD or frozen samples, nor were there any significant differences in the major composition between sites. The only major difference was between total lipid composition of the freeze-dried and frozen samples due to the removal of water during freeze-drying. Total lipid content on a dry weight basis in FD samples was 8.4 g/100g tissue and was significantly higher than frozen samples ($P < 0.05$) and there was no significant site variation. The lipid class content between sites was also not significantly different as judged by TLC. Triglyceride (TG) lipid fraction appeared to be the most prominent in the frozen and FD samples. The free fatty acid (FFA) band was the next most prominent band and was visually more prominent in the frozen samples. Sterol esters (SE) were detected in higher amounts in the frozen samples compared with the FD samples. Phospholipid (PL) and sterols (ST) were distributed throughout all samples. Polyunsaturated fatty acids (PUFA) were the main group of fatty acids in both FD and frozen samples (45-46%), most of which were omega-3 (n-3) fatty acids (40-41%). Saturated fatty acids (SFA) accounted for approximately one quarter of total fatty acids, with little variation between FD and frozen samples. The major fatty acids of the NZGLM were docosahexaenoic acid (DHA; 22:6n-3) (19% in both FD and frozen samples), eicosapentaenoic acid (EPA; 20:5n-3) and palmitic acid (16:0) (15% in both FD and frozen samples). Cholesterol was the most prominent sterol (31% of total sterols). Other major sterols included desmosterol/ brassicasterol (co-eluting), 24-methylenecholesterol, *trans*-22-dehydrocholesterol, 24-nordehydrocholesterol and occelasterol. This study is unique as it compares the lipid composition of the NZGLM from three sites in New Zealand with the additional effect of processing. This is the second comparative study investigating the lipid, fatty acid and sterol composition of the NZGLM with added interest in the effect of freeze-drying on the lipid content of the mussel. This study showed that there were no major significant differences in lipid, sterol and fatty acid composition between the FD and frozen samples of the NZGLM for three sites in New Zealand. Food chain studies and further research is warranted to investigate the presence and role of major and minor lipid components of the NZGLM.

Key Words: fatty acid, sterol, cholesterol, 24-methylenecholesterol, brassicasterol, phospholipid, triglyceride, free fatty acid, docosohexaenoic acid, eicosapentaenoic acid, anti-inflammatory, thin layer chromatography, gas liquid chromatography.

Introduction

Lipids from marine sources are heterogenous and can contain unusual fatty acids such as non-methylene interrupted (NMI) fatty acids and fatty aldehydes.[1,2] The lipid, fatty acid and sterol composition has been classified for a number of crustacea including abalone, crabs, clams and some molluscan species. However, limited research has focussed on *Perna canaliculus*, the NZGLM. Molluscs are the second largest phylum of animals in terms of named species. They are divided into seven classes, four of the main being bivalvia, polyplacophora, cephalopoda and gastropoda, all of which include snails, clams, scallops, oysters, cuttlefish, and octopus.[3] Molluscs are bilaterally symmetric, they have a visceral mass covered with epithelium and a muscular foot and digestive, reproductive and excretory organs are found within the viscera.[3] The NZGLM, not to be confused with

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Perna viridis the greenshell mussel found in the region of Hong Kong,[4] is a bivalve native marine mussel of New Zealand and is generally found in a variety of habitats including rock faces, algal holdfasts and deep-sea beds over mud or sand in central and the North Island waters of New Zealand.[5] The green shell and green lip of the mussel is easily distinguished from other mussel species including the blue or black mussel *Mytilus edulis*. The external shape of the NZGLM is similar to other mussels and can sometimes exceed 240 mm in shell height.[5]

Past research has reported lipids in the NZGLM comprise around 2% of the wet weight[6-8] of which the predominant lipid class is PL (57-79%) followed by 10-20% TG[6,9] and the presence of FFA (7-12%). As the mussels are sedentary animals, total lipid content and composition of the lipid classes may vary due to several factors. During various stages of the mussel's lifecycle, particularly female mussels, there is considerable synthesis of 16:0 and stearic acid (18:0) and their derivatives *de novo*.[10] Other biochemical changes including variation in total lipid and fatty acid content in the mussel may vary resulting from variability in metabolic activity, intra-species seasonal variation, location and climate, stage of the lifecycle, sex, spawning and dietary intake.[6,11-14] It is not clear whether the presence of FFA is an artifact due to post-harvest changes. The method of collection, transport and storage could lead to high levels of FFA, due to the action of lipolytic enzymes as seen in mussels reported previously by Jeong *et al.*, Jeong and Murphy *et al.*[6,15,16]

The lipid and compositional classes including fatty acids and sterols in general vary considerably from marine animals and terrestrial organisms. Terrestrial organisms consume a diet rich in omega-6 (n-6) PUFA such as linoleic acid (18:2 n-6). In contrast, marine organisms consume diets rich in n-3 PUFA, and the lipids of the animals can contain up to 50% unsaturated fatty acids, with five or six double bonds, including 22:6 n-3 and 20:5 n-3.[13,17] Animal oils generally contain fatty acids with up to four double bonds and are also rich in SFA. The fatty acid and sterol profile of the lipids in marine vertebrates and invertebrates reflect fatty acid and sterol profiles in zooplankton, dinoflagellates and algae.[6,13,18,19] *Perna canaliculus* like many other marine organisms consume a diet rich in phytoplankton and algae which are rich sources of n-3 PUFA (18:3 n-3, 18:4 n-3, 20:5 n-3, 22:6 n-3 and 28:8 n-3).[6,13,20]

Omega-3 PUFA from marine sources have been widely investigated in terms of their beneficial effect on reducing certain risk factors for cardiovascular disease in humans such as decreasing platelet aggregation,[21] reducing plasma triacylglycerol[21] as well as alleviating the symptoms of inflammatory conditions such as arthritis, psoriasis, ulcerative colitis and inflammatory bowel disease.[22-26] Particular attention has been given to the NZGLM, as it may have therapeutic value in the treatment of inflammatory conditions with additional gastric mucosal protection. Whitehouse *et al.* investigated the anti-inflammatory activity of the oil extracted from the freeze-dried powder of the NZGLM (Lyprinol™) in adjuvant-induced polyarthritis or collagen-induced arthritis in rats.[27] Lyprinol™ inhibited the biosynthesis of leukotriene B_4 (LTB_4) from stimulated human neutrophils and inhibited prostaglandin E_2 (PGE_2) production from activated human macrophages *in vitro*. It is unknown where the anti-inflammatory activity resides in the NZGLM, however it has been hypothesised to reside in the protein fraction of the mussel,[28] the polysaccharide fraction,[29] or the lipid fraction, in PUFA with four, five and six double bonds.[27] To our knowledge limited research has been conducted on the lipid composition of the NZGLM. Murphy *et al.* presented a comparative study on the lipid, fatty acid and sterol composition of the NZGLM and the Tasmanian BM (TBM) using Thin Layer Chromatography-Flame Ionisation Detection (TLC-FID) and Gas chromatography-Mass Spectrometry (GC-MS).[6] The aim of the present study was to determine the fatty acid and sterol composition of from three sites in New Zealand and to determine if freeze drying affected the total lipid profile.

Materials and methods

Sample collection, preparation and lipid extraction

Frozen and freeze-dried NZGLM were collected by the Cawthron Institute, Nelson, New Zealand on behalf of McLab NZ Ltd. (Nelson, New Zealand), in March 2000. Three locations were selected for harvesting of mussel species, Port Ligar, Little Nikau and Hallam Cove. Approximately 40 individual mussels were collected from each site and cooled on ice. Twenty mussels were allocated from each site for either freeze-drying or freezing (Fig.1). Shells were removed and the flesh of the allocated 20 mussels from each site were then combined, crushed and stored at -20°C.

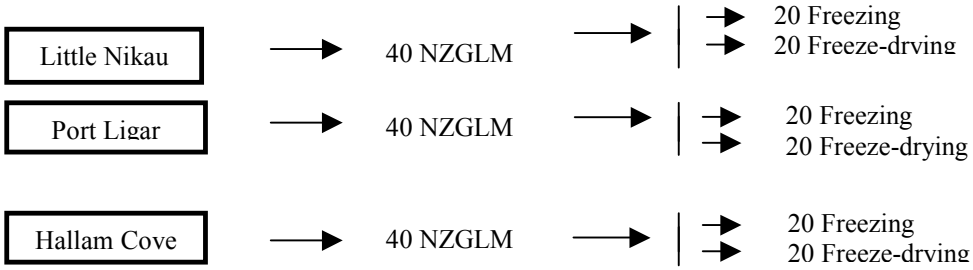

Figure 1. Collection of mussels and respective allocation for processing. A total of 120 mussels were collected; 40 from each site (Port Ligar, Little Nikau, Hallam Cove). Twenty mussels from each site were allocated for freeze-drying or freezing.

Mussels for freeze-drying were stored for no more than 24 hours at 4°C and were then freeze-dried. Mussels for freezing were collected and stored for no more than 24 hours at 4°C and were then frozen at -20°C. Frozen and FD samples were shipped frozen to RMIT University and stored at -20°C for three to five days until analysis. Data relating to water temperature and water quality is unknown, however it is assumed that all conditions were standard to aquaculture farms. Data reporting food sources and availability was collected during March 2000 by the Cawthron Institute. The following species of phytoplankton were common throughout sites; *Chaetoceros species (spp.), Leptocylindricus spp.* and *Skeletonema spp.*, however the availability and quantity of phytoplankton varied between sites. Table 1 lists the total amount of phytoplankton (cells/L) for the three sites. In general, there were less phytoplankton at Hallam cove compared with the other two collection sites.

Table 1. Total phytoplankton numbers collected (cells/L) at three sites (Port Ligar, Little Nikau, Hallam Cove) where the New Zealand Green Lipped Mussel was harvested.[1]

Phytoplankton	Sites of collection (New Zealand)		
Date	Port Ligar	Little Nikau	Hallam Cove
6/3/00	93,600 [2]	49,600	5,800
13/3/00	59,000	67,000	49,200
20/3/00	16,400	20,400	1,400
27/3/00	15,600	84,000	2,000
Average	46,150	55,250	14,600

[1]Information gathered by the Cawthron Institute, New Zealand from 6/3/00 to 27/3/00. [2] cells/L.

Approximately 20 grams (gm) of the combined flesh of the frozen samples was removed and minced using an electric blender to increase the surface area for lipid extraction. Similarly, 3 gm of the combined FD mussel samples were removed and extracted. All samples were extracted using a modification of the Bligh & Dyer chloroform:methanol extraction technique.[30] Briefly, mussels were extracted overnight in chloroform:methanol (Merck, Germany) (1:1, by vol.) containing 0.05% butylated hydroxytoluene (BHT, Sigma Chemical Co, St Louis, USA). Following extraction, the solvent was made up to a ratio of 2:1 chloroform: methanol (Merck, Germany) (by vol.) and partitioned overnight using 0.9% saline. After phase separation, the lipid phase was removed and the total solvent extract was evaporated *in vacuo* at 40°C. Lipid content to a constant weight was determined under a stream of nitrogen gas and transferred into a known volume of chloroform and stored at -20°C until analysis (no more than 4 days).

Lipid fraction

A portion of the total lipid extract for each mussel sample was analysed by TLC, using silica gel 60G (Merck, Germany) applied to 20 cm x 20 cm glass plates. Separation was achieved using a polar solvent system of petroleum ether:diethyl ether:glacial acetic acid (90:10:1, by vol.) to resolve non-polar components including SE, TG, FFA, ST and PL. A standard mixture of TLC lipid classes was used for identification (Nuchek Prep Inc, USA). The TLC plates were sprayed with copper sulphate in 8% orthophosphoric acid (BDH Laboratory Supplies, Poole, England) and methanol ($CuSO_4$; Univar, AJAX chemicals, Australia), following the development and then heated in an oven at 105°C for 30 minutes. This procedure resulted in lipid fractions charring and the plates were then able to be photo-copied for a permanent record.

Fatty acids

An aliquot of the total lipid extract (including internal standard C23:0 methyl ester; Nuchek Prep Inc, USA), was taken and dried under a stream of nitrogen, then hydrolysed to FFA using 7.9% potassium hydroxide (KOH) (Univar, AJAX chemicals, Australia) in methanol (Merck, Germany). The internal standard was added to aid in the identification of the fatty acid methyl esters (FAME). Samples were cooled and converted to FAME using 20% boron trifluoride in methanol (Merck, Germany). Gas Chromatographic analyses of the FAME were performed using a Shimadzu Gas Chromatograph (GC) 17A (Shimadzu Corporation, Kyoto, Japan) fitted with a flame ionisation detector (FID) and using a 50 m BPX70 cross-linked 70% Cyanopropyl Polysilphe-nylene-siloxane capillary column (0.32 mm ID and 0.25 µm film thickness, Alltech, Australia). Samples were injected at 125°C and held for 1.0 minute. The oven temperature was set to increase by 5ºC/minute to 170ºC and held for four minutes, then by 0.5ºC/minute to 175ºC and 4ºC/minute to a final temperature of 220ºC that was held for three minutes. The injector and detectors were maintained at 260ºC and helium was used as the carrier gas. Peak areas and fatty acid percentages were quantified on an IBM compatible work-station using Shimadzu software. All samples were analysed in duplicates. A standard mixture of FAME (Nuchek Prep Inc, USA) was used to calibrate the GC and to determine response factors. Results are expressed as percent of total fatty acids.

Sterols

A portion of the total lipid extract was taken and dried under a stream of nitrogen. Sterols were obtained by alkaline saponification[6] and were then converted to their corre-sponding tri methylsilyl ethers (OTMSi) with N,O-Bis (trimethylsilyl) trifluoroacetamide (BSTFA; Alltech, Austra-lia) for 15 minutes at 70ºC. Gas Chromatographic analyses were performed using a Shimadzu GC 17A (Shimadzu Corporation, Kyoto, Japan) fitted with a FID and a 50 m BPX5 5% Phenyl Polysilphenylene-siloxane capillary column (0.32 mm ID and 0.25 µm film thickness, SGE, Australia). Samples were injected at 200°C and held for one minute. The oven temperature was set to increase by 20ºC/minute to 340ºC and held for 30 minutes. The injector, detector and carrier gas conditions were the same as described above for

fatty acid analysis and sterol percentages were quantified on an IBM compatible workstation using Shimadzu software. All samples were analysed in duplicates. Sterol peaks were identified using a standard mixture of sterols (cholesterol, β-sitosterol, 24-methylenecholesterol, stigmasterol and dihydro-brassicasterol; Sigma Chemical Co, St Louis, USA). Results are expressed as percent of total sterols.

Statistical analysis

Statistical analyses were conducted using Minitab Version 12.0 for Windows (Minitab Inc, PA, USA). Comparison of FD and frozen samples were analysed using Two-Sample T-tests. Sites could not be compared statistically, as the total mussel number (n) was too small. Data is reported as mean ± SD (data is the mean of duplicates taken from approximately 20 combined individual animals). Significance was accepted at $P < 0.05$ unless otherwise stated.

Results

Total lipid

Total lipid on a wet weight basis for the frozen NZGLM was 1.6% (SD 0.5) (Table 2). The lipid content on a dry weight basis (FD) was 8.4% (SD 0.8) and was significantly higher than the frozen NZGLM ($P = 0.0012$). Visual qualitative TLC analyses showed there were seven bands in all samples analysed. The most prominent band was TG, followed by FFA, ST, PL, SE (two bands) and a minor band just below the PL. Triglyceride was the major fraction in frozen and FD samples irrespective of harvest site. Phospholipid and ST levels also appeared not to be affected by harvest site.

Table 2. Total lipid content (%) for freeze-dried and frozen samples of the New Zealand Green Lipped Mussel and sites (g/100 g wet weight)

	New Zealand Green Lipped Mussel
	g/100 g tissue[a]
Freeze-dried	
Port Ligar	8.3
Little Nikau	9.3
Hallam Cove	7.7
Mean (%)	8.4 ± 0.8*
Frozen	
Port Ligar	1.3
Little Nikau	2.2
Hallam Cove	1.2
Mean (%)	1.6 ± 0.5

[a]Values for each species are the Mean ± SD of approximately 20 combined individual animals.
* $P = 0.0012$, freeze-dried significantly higher than frozen, based on two-sample t-tests.

Sterol ester was not present in all mussel samples at all sites and was only observed at low levels (Fig. 2). Free fatty acids appeared to be more prominent in the frozen samples and Hallam Cove appeared to have the highest level of FFA, while Little Nikau appeared to have the least amount of FFA.

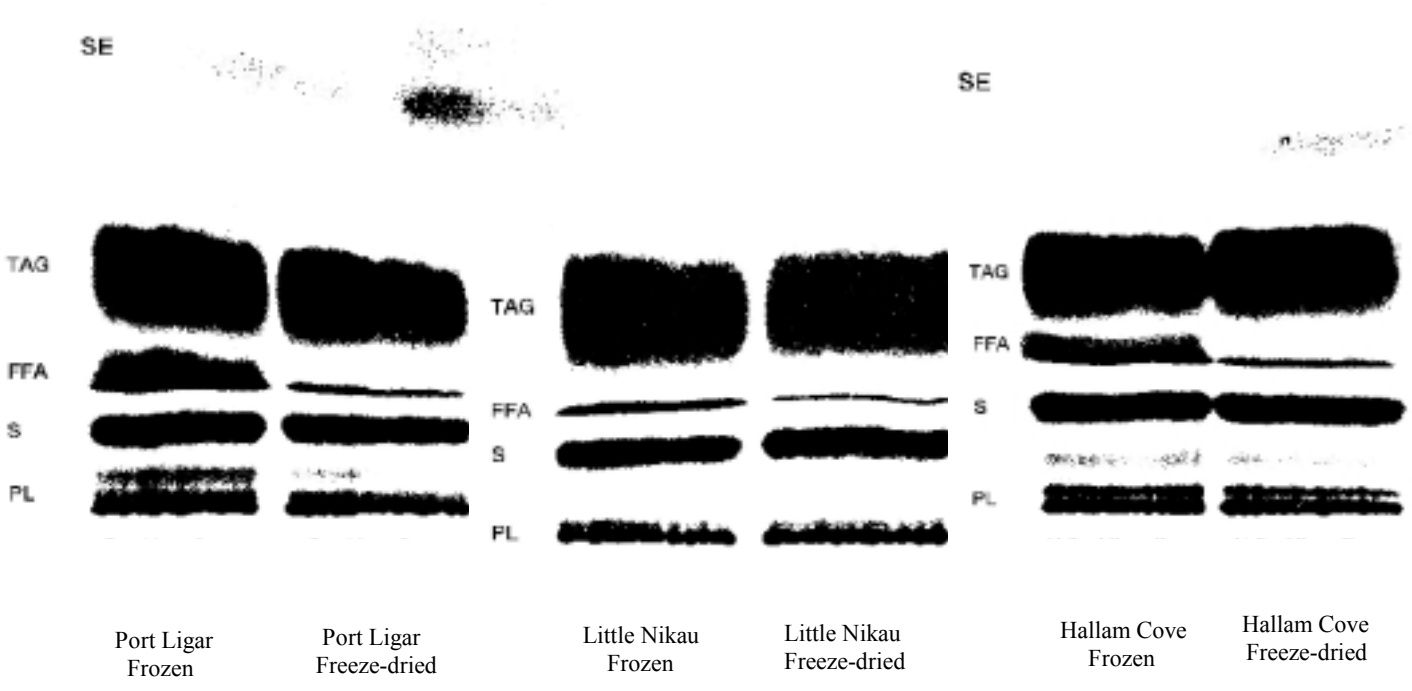

Figure 2. Thin Layer Chromatography plate of Port Ligar, Little Nikau and Hallam Cove site for New Zealand Green Lipped Mussel, using solvent system of petroleum ether:diethyl ether:glacial acetic acid (90:10:1, by vol.).

Fatty acids

Polyunsaturated fatty acids were the dominant fatty acid in both FD and frozen samples (45-46%, respectively) of total fatty acids, most of which were n-3 PUFA (40-41% of total PUFA) (Table 3). Saturated fatty acids were the next most common fatty acids (26% in the FD and 25% in the frozen NZGLM). Monounsaturated fatty acids (MUFA) were present at 23% in the FD and frozen NZGLM. Omega-6 fatty acids accounted for 5% of total PUFA.

There were 30 individual fatty acids identified, while one fatty acid (A) could not be identified but was present in all mussel samples (Fig. 3). It is possible that this fatty acid is an isomer of 20:1 fatty acid. The dominant fatty acids in frozen and FD samples were 22:6 n-3 (up to 19%) and 20:5 n-3 (15%) (Fig. 4, Table 4). Palmitic acid was present at 15% in frozen and FD samples, palmitoleic acid (16:1 n-7c) was present up to 7%, while stearic acid (18:0), heptadecenoic acid (17:1) were present up to 4% in the frozen and FD NZGLM. Octadecatetraenoic acid (18:4 n-3) was present at 3% while oleic acid (18:1 n-9) was found in all samples at approximately 2% of total fatty acids. The FD NZGLM had greater levels of dodecenoic acid (12:1; $P = 0.0046$) and myristic acid (14:0; $P = 0.033$) compared with the frozen NZGLM. The unidentified fatty acids (UI) could not be identified with GLC alone and requires further GC-MS analysis.

Table 3. Percent composition (% of total fatty acids) of fatty acid type for freeze-dried and frozen samples of the New Zealand Green Lipped Mussel

	Percent composition † NZGLM	
	Freeze-dried	Frozen
SFA	25.6 ± 1.1	25.4 ± 0.8
MUFA	22.6 ± 1.2	23.1 ± 0.8
PUFA	45.2 ± 0.8	45.9 ± 0.7
Total n-3 PUFA	40.4 ± 0.7	40.7 ± 1.1
Total n-6 PUFA	4.8 ± 0.3	5.3 ± 0.4
Total A‡	0.6± 0.1	0.7 ± 0.1
Unidentified	6.3 ± 1.5	4.9 ± 0.6

†Values for each species are the Mean ± SD of approximately 20 combined individual animals.
‡ Represents peak labelled A in figure 5, which is common in all samples and could not be identified using BPX70 GLC.

Sterols

Eighteen sterols were evident in the NZGLM (Fig. 5), five of which could not be identified without GC-MS and were classed as unidentified. Cholesterol was the dominant sterol in the frozen and FD NZGLM samples (31%) (Fig. 6, Table 5). Other major sterols included desmosterol/brassicasterol (co-eluting) (21-23%), 24-methylenecholesterol (14%), *trans*-22-dehydrocholesterol (10%), 24-nordehydrocholesterol (5%) and occelasterol (4%). There were site variations in the proportions of some of the sterols. However, as noted earlier, the number of samples analysed at each site was insufficient for statistical analysis. There were higher proportions of cholesterol and 24-methylcholesterol for both FD and frozen mussels collected from Little Nikau site. There were no significant differences found for sterol composition between the frozen and FD NZGLM, nor between sites.

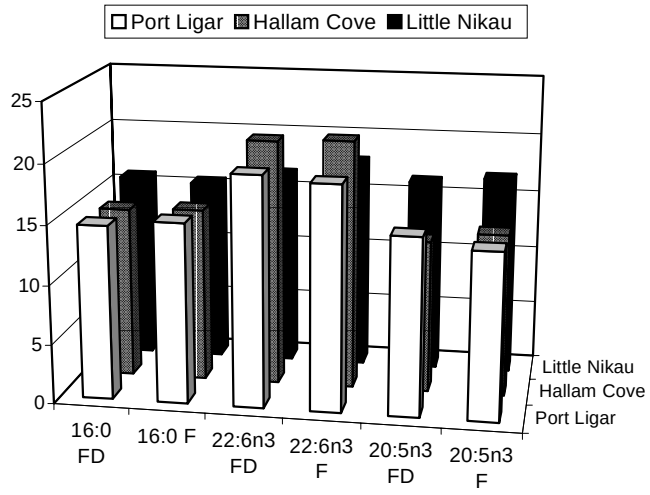

Figure 4. Content of the three major fatty acids; palmitic acid (16:0) docosahexaenoic acid (22:6 n-3) and eicosapentaenoic acid (20:5 n-3) (% of total fatty acids) for freeze-dried (FD) and frozen (F) samples of the New Zealand Green Lipped Mussel, at Port Ligar, Hallam Cove and Little Nikau.

Discussion

Lipid composition

In view of the reported anti-inflammatory bioactivity of the lipids from the NZGLM, the aim of the study was to compare and contrast the lipid, fatty acid and sterol composition of the NZGLM from three sites in New Zealand and following either freezing or freeze-drying. Lipid content of the frozen NZGLM (2%) was similar to values reported by Murphy *et al.* and Gordon investigating the lipid, fatty acid and sterol composition of the NZGLM and TBM and other marine invertebrates.[6,7] Murphy *et al.* found the lipid content of the NZGLM to be 1.8% and the TBM 1.2%.[6] Gordon has also reported the BM to contain around 1.8% lipid in comparison to clams (1-2%) and cockles (0.9-2%) and oysters (2.5-3%).[7] Perry investigated the lipid content of various molluscs and found that lipid content varied from 1% in a female mollusc *(Subninella undulata)* to 4% in *Cellana tranoserica*.[12] Total lipid in the FD samples of the NZGLM was 8.4% on a dry weight basis. There were differences in the lipid contents from mussels collected at each site (7.7 to 9.3 for FD NZGLM; 1.2 to 2.2 for F NZGLM).

Qualitative TLC analyses visually showed the dominant lipid in both frozen and FD samples of the NZGLM was TG followed by FFA. Quantitative data has shown the most common lipid class in molluscs is PL, up to nearly 80% in the common blue mussel and 62% in the NZGLM.[6-9,12]

The present study visually assessed the lipid classes whereas past research has used TLC-FID to determine lipid class composition of the mussels.[6] An explanation may be that since PL do not spread on the TLC using this solvent system compared with the TG band (Fig. 2), the apparent intensity of the TG band appears greater than that of the PL band. In contrast the Iatroscan TLC-FID relies on the quantity of carbon in each band. Comparing the FD and frozen samples, some obvious differences were associated with the freeze-drying process. Free fatty acid appeared to be more prominent in frozen samples compared with the FD samples. Molluscs have been reported to undergo various changes in their lipid composition possibly due to the presence of lipases responsible for enzymatic hydrolysis. Lovern, Levinton *et al.* and Gardner *et al*. have reported the main lipid fractions of fish are TG followed by PL.[13,31,32]

Our data contradicts this finding, but is similar to what was found in a previous study.[6] The most likely explanation is due to the method of collection, transport and storage time, which affects lipid composition, particularly PL, TG and FFA. Past lipid class composition studies on molluscs have shown little or no presence of FFA.[32] In a study by Jeong *et al.* and Jeong, which investigated the effect of storage and temperature on oxidation and lipid composition in the scallop, storage time was indicative of the action of several lipolytic enzymes due to the high level of PL and FFA and lower level of TG seen in samples.[15,16] Similarly mussels from the previous study[6] found high levels of FFA (7-12%) while PL was the main class of lipid. However, the role of other factors may also explain lipid class variations. Jeffs *et al.* has reported the NZGLM to inhabit waters with varying depths and temperatures, such as 5°C in the South Island waters and 27°C in North Island waters.[5] These mussels which inhabit greater depths reduce oxygen uptake by over half, due to a reduction in aerial exposure, thus decreasing food intake and basal metabolism.

Possibly a cooler habitat or climate could result in mussels with higher levels of storage lipid such as TG, instead of FFA or PL. Dembitsky *et al.* has reported variations in total lipid composition of various organs in *Mytilus galloprovincials*.[33] The gills contained 1.7 mg/g wet weight (w/w) total lipid, the mantle 3 mg/g (w/w) and the digestive gland 10 mg/g (w/w). This was also reported in the species *Octopus dofleini*, where up to 39 mg/g (w/w) of total lipid was present in the hepatopancreas while only 5 mg/g (w/w) was present in the tentacle. Similarly Johns *et al.* has reported changes in n-3 PUFA concentration in various organs depending on the season and annual cycle of gastropods.[18] Omega-3 PUFA appeared to rise, while SFA and monoenoic fatty acids decreased.

Geographical location is also thought to influence lipid content and lipid classes of mussel species. Mussels are sedentary animals that feed on drifting phytoplankton, thus differences in location and algal bloom could quite easily influence the lipid composition seen in the present study. Murphy *et al.* have suggested the lifecycle of the mussel, particularly the development of the gonads may affect total lipid and lipid class composition.[6] Gonadal development like growth rate, is also dependent on food availability and temperature, where development only occurs at temperatures greater than 11°C[5] and in addition n-3 PUFA concentration in varies during gonadal development. The sex of the mussels in the present study was not determined which could have also been another factor causing the variations seen between FFA presence between the frozen and FD samples. Female mussels in particular appear to have a lower level of total

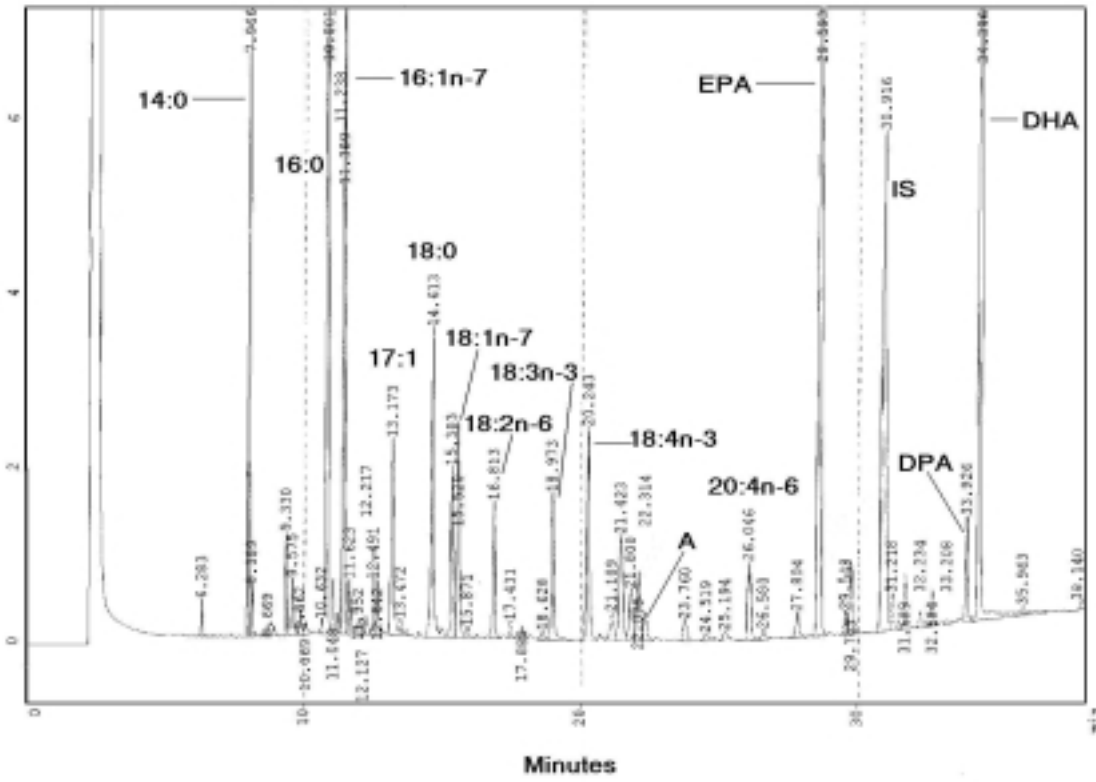

Figure 3. Typical fatty acid methyl ester profile of New Zealand Green Lipped Mussel. Separated on a 50 m BPX70 cross-linked 70% Cyanopropyl Polysilphenylene-siloxane capillary column using a Shimadzu GC17A with FID. IS; Internal Standard (23:0 methyl ester). Fatty acid A may be 20:1 isomer.

Table 4. Fatty acid composition (% of total fatty acids) for freeze-dried and frozen samples of the New Zealand Green Lipped Mussel

	Percentage of total fatty acids† NZGLM	
Fatty acid	Freeze-dried	Frozen
12:1	0.3 ± 0.1 *	0.1 ± 0.1
14:0	3.7 ± 0.2 *	3.1 ± 0.2
14:1	0.1 ± 0.1	0.1 ± 0.1
15:0	0.7 ± 0.1	0.7 ± 0.1
15:1	0.6 ± 0.1	0.8 ± 0.1
16:0	15.0 ± 0.7	15.1 ± 0.4
16:1 n-7t	0.2 ± 0.1	0.2 ± 0.1
16:1 n-7c	7.0 ± 1.1	6.7 ± 1.0
17:0	1.0 ± 0.1	1.0 ± 0.1
17:1	3.6 ± 0.6	3.3 ± 0.5
18:0	3.9 ± 0.4	4.4 ± 0.4
18:1 n-9	1.7 ± 0.4	2.0 ± 0.3
18:1 n-7	2.4 ± 0.3	2.5 ± 0.3
18:2 n-6	1.8 ± 0.1	1.9 ± 0.1
18:3 n-3	1.9 ± 0.2	1.9 ± 0.1
18:4 n-3	2.8 ± 0.3	2.7 ± 0.2
20:1 n-11	0.7 ± 0.1	0.7 ± 0.1
20:1 n-9	2.2 ± 0.1	2.4 ± 0.2
20:1 n-7	0.9 ± 0.1	0.9 ± 0.2
20:1 n-5§	1.6 ± 0.2	1.9 ± 0.6
20:2 n-6	0.4 ± 0.1	0.5 ± 0.1
20:3 n-6	0.2 ± 0.1	0.2 ± 0.1
20:4 n-6	1.6 ± 0.3	1.8 ± 0.3
20:3 n-3	0.2 ± 0.1	0.2 ± 0.1
20:4 n-3	0.4 ± 0.1	0.5 ± 0.1
20:5 n-3	14.7 ± 1.8	14.9 ± 1.7
22:0§	0.5 ± 0.1	0.6 ± 0.2
22:2§	1.3 ± 0.1	1.4 ± 0.1
22:4 n-6	0.6 ± 0.1	0.6 ± 0.1
22:5 n-6	0.3 ± 0.1	0.3 ± 0.1
24:0	0.5 ± 0.1	0.5 ± 0.1
22:5 n-3	1.4 ± 0.1	1.3 ± 0.1
22:6 n-3	18.9 ± 2.2	19.2 ± 1.6
A	0.6 ± 0.1	0.7 ± 0.1
UI‡	6.3 ± 1.5	4.9 ± 0.6

† Values for each species are the Mean ± SD of approximately 20 combined individual animals.
‡ UI, unidentified fatty acids, could not be identified using BPX70 GLC.
§ Identity of these fatty acids are tentative only, due to lack of standards and GC-MS.
* $P < 0.05$, freeze-dried significantly higher than frozen, based on two-sample t-tests

lipid and FFA.[20] Spawning and larval development has also been reported to influence the total lipid, lipid class and fatty acid composition of marine invertebrates.[13, 34]

Fatty acids

The main purpose of the present study was to eliminate differences in food intake of the mussels affecting the fatty acid composition in the NZGLM. This study achieved this by sampling NZGLM from three sites. The NZGLM in the present study contained a similar profile to that of the NZGLM and TBM in a recent study by Murphy *et al.*[6] The majority of marine species are rich sources of long chain PUFA, particularly 22:6 n-3 and 20:5 n-3. The New Zealand mollusc *Mytilus canaliculus* for instance contains a similar profile to the mussels being rich in 22:6 n-3 and 20:5 n-3.[12] In the present study the dominant n-3 PUFA was 22:6 n-3 in both frozen and FD samples. This differed from a past study in which Murphy and colleagues found 20:5 n-3 to be the most dominant n-3 PUFA in the NZGLM (18%) while 22:6 n-3 was the highest n-3 PUFA in the TBM (21%).[6] The present study identified around 19% of 22:6 n-3 in the frozen and FD samples of the NZGLM which was higher than the 22:6 n-3 content of the NZGLM reported by Murphy *et al.*[6] Perry reported other bivalvia species such as *Crassostrea gigas* to contain high levels of 20:5 n-3 and 22:6 n-3.[12] In the present study there were no significant differences between levels of 20:5 n-3 or 22:6 n-3 or within species, but 22:6 n-3 was the most dominant PUFA. This result probably reflects the dietary sources available to each species of mussel. Various algal species are known to be rich sources of 22:6 n-3, including dinoflagellates, cryptomonads and zooplankton[35,36] and often when evaluating the composition of lipids from fish and molluscs, transmission of n-3 PUFA from marine phytoplankton to zooplankton and eventually marine invertebrates needs to be taken into consideration.[37,38] The mussels in this study were feeding on various algal species (Table 1), which may have differed in 22:6 n-3 composition compared to the mussels reported by Murphy *et al.*[6] However the abundance of phytoplankton varied considerably between sites. Little Nikau had the greatest level of phytoplankton species (55,250 cells/L), followed by Port Ligar (46,150 cells/L) and Hallam Cove (14,600 cells/L).

The NZGLM contains a complex profile of fatty acids including novel long chain PUFA, NMI fatty acids (22:2 NMI) and eicosatetraenoic acids (ETAs).[6] Past research has reported 22:6 n-3 and 20:5 n-3 to be anti-inflammatory based on their ability to reduce LTB_4 synthesis and PGE_2 synthesis and these PUFA have been used in the treatment of arthritis and psoriasis.[25,26,39,40] The NZGLM have also been reported to possess anti-inflammatory activity as reported by Whitehouse *et al.*, Couch *et al*., Rainsford *et al.*, Gibson *et al*., Kosuge *et al*. and Emelyanov *et al.*[27,28,41-44] Whitehouse *et al.* showed that NZGLM oil at a dose rate of 20 mg/kg body weight/day in rats was more effective than fish oil (MaxEPA, NY, USA) at a dose rate of 1850 mg/kg body weight/day in reducing induced paw oedema.

Table 5. Sterol composition (% of total sterols) for freeze-dried and frozen samples of the New Zealand Green Lipped Mussel.

		Percent composition of total sterols†	
		NZGLM	
Sterol	Peak	Freeze-dried	Frozen
Unidentified‡	1	0.9 ± 0.7	0.4 ± 0.5
24-nordehydrocholesterol	2	4.5 ± 1.4	4.6 ± 0.7
C26 sterol	3	0.5 ± 0.2	0.6 ± 0.2
Occelasterol	4	3.8 ± 1.3	3.8 ± 1.4
trans-22-dehydrocholesterol	5	9.8 ± 1.4	9.6 ± 1.5
Cholesterol	6	31.2 ± 2.1	30.7 ± 1.8
Desmosterol/Brassica-sterol	7	20.6 ± 2.6	23.0 ± 2.7
24-methylcholesterol	8	2.6 ± 0.9	3.3 ± 1.2
24-methylenecholesterol	9	13.6 ± 2.0	13.9 ± 2.2
24-ethylcholesterol	10	1.0 ± 0.2	1.0 ± 0.1
β-sitosterol	11	3.4 ±0.1	3.5 ± 0.3
Isofucosterol	12	2.7 ± 0.4	3.1 ± 0.5
Dinosterol	13	2.1 ± 1.1	1.2 ± 0.3
Unidentified‡	14	0.2 ± 0.2	0.2 ± 0.1
Unidentified‡	15	0.1 ± 0.2	0.0 ± 0.0
4,23,24-Trimethyl-5α-choles-7en-3β-ol	16	2.3 ± 1.3	0.9 ± 0.9
Unidentified‡	17	0.4 ± 0.1	0.3 ± 0.3
Unidentified‡	18	0.4 ± 0.4	0.2 ± 0.2

†Values for each species are the Mean ± SD of approximately 20 combined individual animals; ‡ Unidentified, could not be identified using BPX5 GLC.

Fatty acid profiles of other molluscs are usually dominated by SFA,[7,12,20] however our study only reported SFA present at 25-26% in the NZGLM. Myristic acid was lower in the frozen NZGLM, but 18:0 was higher in the frozen samples compared with the FD samples. The most dominant SFA was 16:0 present at 15% in both FD and frozen samples. The dominant SFA in the NZGLM was 16:0 followed by 18:0 and 14:0 which is consistent with results from past studies of molluscs.[6,12] Perry reported other molluscs such as the male and female mollusc *Subninella undulata* to contain 16:0 and 18:0 as the dominant SFA, similarly the mollusc *Austro-cochlea constricta* and the gastropod *Telescopium tele-scopium* (Telescopic creeper) were also dominated by 16:0.[12] These differences may be due to processing once again. These small differences may reflect variations in metabolism of precursor fatty acids in the mussel species. There may also be differences in dietary sources of these fatty acids as well as lifecycle and sex of the mussel and degradation of different SFA in the FD mussels compared to the frozen mussels or perhaps different rates of 16:0 synthesis *de novo* in species as reported earlier by Kluytmans.[10] Jeong *et al.* and Jeong reported harvesting and storage of fish, molluscs and other

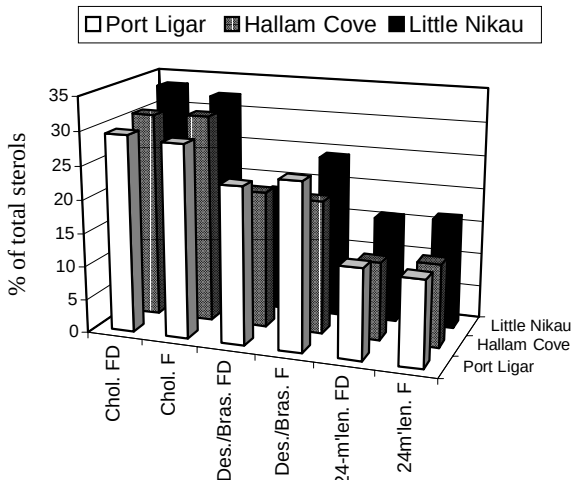

Figure 6. Content of cholesterol (chol), desmosterol/brassicasterol (Des/Bras) and 24-methylenecholesterol (24-m'len) content (% of total sterols) for the freeze-dried (FD) and frozen (F) New Zealand Green Lipped Mussel, at Port Ligar, Hallam Cove and Little Nikau

marine invertebrates, could in fact alter the total lipid content and class composition of an animal.[15,16] Canning, flesh extraction methods and pasteurisation can alter sterol levels, particularly cholesterol. Similarly a study by el-Shafei *et al* found freeze-dried milk powders had a higher percentage of fat compared to regular dry milks.[45]

A processing technique such as freeze-drying which uses a vacuum to remove oxygen and water and converting to a gas phase may affect the lipid compositional makeup of the fatty acid and sterol profile of the mussels. This processing could cause the disappearance of some volatile fatty acids and isomerisation of fatty acids, particularly unsaturated fatty acids.

Lema and colleagues reported a decrease in nutritive protein in *Mytilus edulis* after drying the mussels at a high temperature.[46] Similarly de Moura *et al.* reported a decrease in the amount of lipid extracted from sardines (*Sardinella aurita*), by nearly 1% after cooking.[47] These studies indicate that processing prior to lipid extraction may in fact cause compositional changes in total lipid extracted from the animals as well as the fatty acid and sterol composition.

Monounsaturated fatty acids accounted for approximately 23% in the NZGLM samples, of which the dominant MUFA were 16:1n-7c (7% in FD and 6.7% in frozen samples), irrespective of harvest site. The slight difference between the percentage of 16:1n-7c in the FD and frozen samples could possibly be explained by a slightly greater amount of isomerisation of precursor fatty acids of 16:1n-7c or the desaturation of 16:0 to 16:1n-7c. The identity of fatty acid A in all samples could not be identified by GLC, however it is thought it may be an isomer of 20:1.

Sterols

Both FD and frozen samples contained a similar sterol profile, with cholesterol dominating in all samples (31%). Cholesterol has also been reported in the NZGLM and TBM

at 30%[6] and 46% in the New Zealand species *Mytilus planulatus*.[12] Fourteen sterols were identified, while four could not be identified with the standard mixtures of sterols or without the availability of GC-MS analysis. Invertebrates such as mussels have a wide sterol profile other than cholesterol, which is probably the most common sterol identified in marine invertebrates, particularly in molluscs.[7] Cholesterol generally ranges from around 2-90% of total sterols in various molluscs, around 45% total sterols in clams[7,48] and can be as high as 90% in abalone[49] and around 50% of total sterols in zooplankton.[50] These include brassicasterol and desmosterol (co-eluting) 24-methylenecholesterol, *trans*-22-dehydrocholesterol, 24-nordehydrochoelsterol and occelasterol. This sterol profile is similar to that reported by Murphy *et al.* and to sterols in the BM, *Mytilus planulatus* reported by Perry.[12] Other species of molluscs such as scallops and abalone appear to have a higher cholesterol content (60% and 90%, respectively) than the mussels in the present study. Teshima *et al.* have reported that molluscs are able to synthesise cholesterol from precursor sterols, however is dependent on other factors such as lifecycle and sex. Various planktonic species have a high content of cholesterol.[11] It is reported that some species in Mexico have contain around 52% of cholesterol and in Australia around 59%.[12] It is therefore more likely that molluscs such as the abalone and scallops have the ability to synthesise cholesterol at a much higher rate and perhaps have a higher dietary intake of cholesterol precursors. It is difficult to accurately compare sterol profiles due to various techniques and analytical methods. Murphy *et al.* identified 20 sterols in the NZGLM using GC-MS. Perry identified eight sterols in *Mytilus planulatus*, which were also common sterols reported in the present study.[12] A similar profile was also seen in the study by Murphy *et al.* with slightly lower levels of cholesterol, 24-methylenecholesterol and sitosterol and higher levels of 24-methylcholesterol and *trans*-22-dehydrocholesterol.[6] The current study identified several additional sterols apart from cholesterol. The blue mussel *Mytilus planulatus* was reported by Perry to contain 46% cholesterol, 13% brassicasterol, 10% 24-methylenecholesterol, 10% *trans*-22-dehydrocholesterol, 6% desmosterol and 4% each of 24-methylcholesterol and sitosterol (% of total sterols).[12]

Some plant phytosterols have been reported to contain anti-inflammatory activity.[51-55] Plant sterols such as β-sitosterol and 24-ethylcholesterol (stigmasterol) have been reported to reduce induced ear oedema and myeloperoxidase activity (marker of neutrophil influx to the inflamed area) in mice, in a dose dependant manner.[52,54,57] Similarly a mixture of 24-methylcholesterol (campesterol), β-sitosterol and 24-ethylcholesterol have also been reported to reduce carageenan induced paw oedema and ear oedema in mice.[54] De La Puerta *et al*. have reported β-sitosterol from olive oil inhibits myeloperoxidase activity and reduces arachidonic acid-induced oedema in mice. Epidemiological studies have shown that plant sterols, including b-sitosterol from fruits and vegetables may potentially exhibit immuno-modulatory and anti-inflammatory activity *in vivo*. β-sitosterol appears to target type one and two T-lymphocytes (Th 1 and Th 2) and may reduce the overactive antibody response. In the present study, levels of these reported anti-inflammatory sterols were

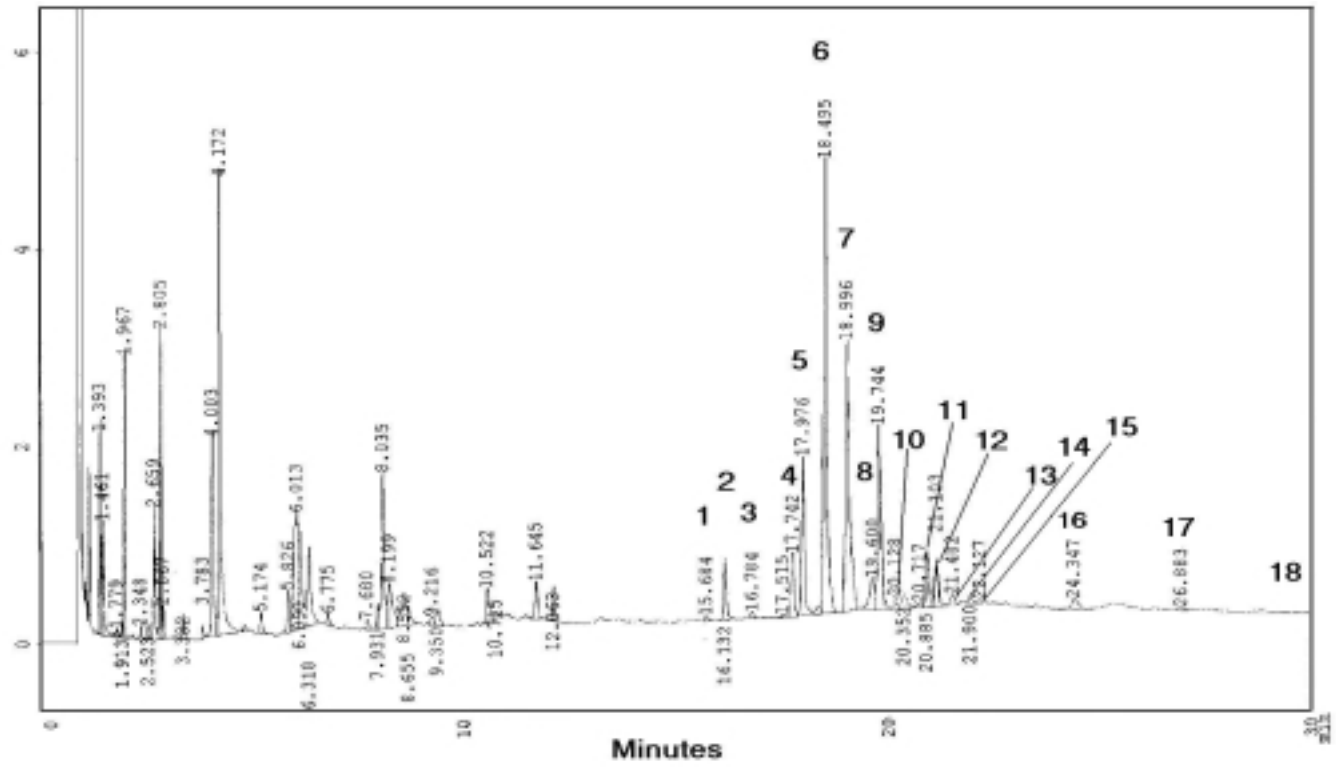

Figure 5. Typical sterol profile of New Zealand Green Lipped Mussel. Separated on a 50 m BPX5 5% Phenyl Polysilphenylene-siloxane capillary column using a Shimadzu GC17A with FID. Peaks from 0–15 minutes are fatty acids remaining after saponification.

were quite low: β-sitosterol 3.5%, 24-methylcholesterol 3%, 24-ethylcholesterol 1 %.

In conclusion, this is the second comparative study investigating the lipid, fatty acid and sterol composition of the NZGLM with added interest in the effect of freeze-drying on the lipid content of the mussel. Notably, there is limited literature on the correlation between dietary intake and lipid composition of molluscs. The total lipid composition of the frozen NZGLM differed from the FD NZGLM due to processing techniques and effects. Evidence from a number of studies[15,16,45-47] show that freeze-drying, storing and other methods of processing affect the total lipid, fatty acid and sterol content of the animals, particularly PL and FFA content which offers an explanation of the presence of FFA in all samples. There was little variation between the FD and frozen samples and between sites of harvest. The dominant fatty acid types were PUFA, particularly n-3 PUFA, of which 22:6 n-3 and 20:5 n-3 were the dominant forms. Omega-3 PUFA content was similar to that from past studies, however in the present study, 22:6 n-3 was in higher concentration in most samples whereas 20:5 n-3 was the highest in past studies.

The small variations seen between qualitative lipid class data and fatty acids could be attributed to dietary intake of the mussels harvested from three separate sites in New Zealand. Differences may also reflect seasonal variation, geographical location with respect to climatic temperature, inter and intra-species variation, the development and sex of the mussel. Dietary intake plays a major role in the composition of body oils from molluscs. Mussels are sedentary animals and are generally unable to migrate to feed on a variety of food sources. The marine phytoplankton, dinoflagellates and zoo-plankton that pass over them, the composition of the food will certainly reflect the fatty acid and sterol composition of the mussel oils. Food chain studies are necessary to provide a more complete understanding of the interrelationships within the marine environment. Further research is warranted to investigate the presence and role of major and minor lipid components of the NZGLM. Further research is also required to investigate the effect of different harvesting and processing techniques on the lipid composition and nutritive value of *Perna canaliculus* used in preparation for human consumption.

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