

1 Article

# 2 Bioavailability of Orally Administered Active Lipid 3 Compounds from four different Greenshell™ mussel 4 formats.

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16 **Abstract:** Greenshell™ mussel (GSM, *Perna canaliculus*) is New Zealand's most important  
17 aquaculture species. They are a good source of long chain-polyunsaturated fatty acids (n-3 LC  
18 PUFA). Beyond a traditional food product, GSM are also sold as mussel powders and oil extract  
19 formats in the nutraceutical markets. In this study, a four-sequence, single dose, randomized  
20 crossover human trial with eight evaluable healthy male participants was undertaken to determine  
21 the bioavailability of the n-3 LC PUFA in four different GSM formats (oil, powder, food ingredient  
22 and half-shell unprocessed whole mussel) by measuring area under the curve (AUC) and maximal  
23 concentration (C<sub>Max</sub>). Blood samples were collected at baseline and up to 48 hours after initiation of  
24 product consumption in each administration period. There were minor differences between the  
25 bioavailability of FA between the different GSM formats. Eicosapentaenoic acid (EPA) peak  
26 concentrations and plasma exposures were significantly lower with GSM oil compared to GSM half-  
27 shell and GSM powder formats, which resulted in AUC<sub>0-48</sub> for the intake of GSM half shell mussel  
28 and GSM powder being significantly higher than that for GSM oil ( $p=0.013$ ,  $f=4.84$ ). This equated to  
29 a 20.6% and 24.3% increase in the amount of EPA present in the plasma after consumption of half-  
30 shell mussels and mussel powder respectively compared to GSM oil. GSM oil produced the shortest  
31 median time to maximal plasma n-3 LC PUFA concentration of all evaluated products demonstrated  
32 by a shorter maximum measured plasma concentration (T<sub>Max</sub> = 5 h). Docosahexaenoic acid (DHA)  
33 and n-3 LC PUFA plasma exposure parameters were statistically comparable across the four GSM  
34 products evaluated.

35 **Keywords:** eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), Green lipped mussels, *Perna*  
36 *canaliculus*, pharmacokinetics,  
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## 38 1. Introduction

39 The endemic Greenshell™ mussel (GSM), *Perna canaliculus*, is New Zealand's most valuable  
40 aquaculture species in terms of both total value and scale of production. In 2019 the New Zealand  
41 GSM industry produced export revenue of NZ\$337M from 30,551 tonnes of exported product<sup>1</sup>. The  
42 majority of GSM export sales (>80%) are presently sold as frozen half-shell food products. However,  
43 there has been recent significant growth in nutraceutical powder and oil product production, with  
44 sales of NZ\$45.7 M in 2018 equivalent to a 334% increase in the last 8 years. These nutraceuticals  
45 command high prices, with oil extracts selling for ca. NZ\$1,900 per kg (in 2018) as opposed to freeze-

46 dried mussel powders ca. NZ\$45 per kg. Furthermore, the industry is actively working to develop  
47 novel food ingredients for use in a variety of different food formats, to reach markets where live and  
48 frozen GSM cannot penetrate.

49 To the best of our knowledge GSM oil is the most valuable marine oil (by price) in the world,  
50 sitting presently at over \$1300 USD/kg<sup>1</sup>. The GSM lipid fraction contains a high proportion of omega-  
51 3 long-chain ( $C \geq 20$ ) polyunsaturated fatty acids (n-3 LC PUFAs), predominantly (DHA, 22:6n-3) and  
52 eicosapentaenoic acid (EPA, 20:5n-3), which are split between the triacylglycerol (TAG) and polar  
53 lipid (PL) classes<sup>2,3</sup>. These GSM products have been extensively tested in pre-clinical animal studies  
54<sup>4,5</sup>. A recent review has identified a gap in the literature for GSM active ingredients regarding their  
55 pharmacokinetics and identification of the bioactive factors responsible for each observed therapeutic  
56 effect<sup>4</sup>.

57 The health benefits of n-3 LC PUFA, such as DHA and EPA, have been extensively studied and  
58 found to include anti-inflammatory activity, maintenance of normal heart function and lipid profile,  
59 and hypertriglyceridemia management<sup>6-9</sup>. Due to these health benefits, n-3 LC PUFA enriched  
60 materials have been widely used in functional foods and nutritional supplements<sup>10-12</sup>. However, there  
61 are challenges in evaluating efficacy of n-3 LC PUFA in delivering health benefits<sup>7</sup> as the digestion of  
62 lipophilic components of foods and nutritional supplements is subject to a complex series of processes  
63 including bile salt emulsification and pancreatic lipase hydrolysis<sup>13</sup>. Account must be taken of the  
64 considerable variability in the absorption of different n-3 LC PUFAs, as well as the format they are in  
65 (i.e. lipid class) which may have a direct impact on their bioactivity<sup>12,14</sup>.

66 Nutraceutical products from GSM (both oil and powders) have been shown to have benefits in  
67 human clinical trials on the alleviation of osteo-/rheumatoid-arthritis<sup>15-17</sup>, asthma<sup>18,19</sup>, and adverse  
68 effects of exercise and/or inflammation<sup>20,21</sup>. With some new animal studies showing novel activity in  
69 protecting against cartilage damage in both early-stage and late-stage metabolic osteoarthritis<sup>5</sup>,  
70 increasing lean mass accrual in diet-driven obesity, and reducing loss of bone mineral density in  
71 osteoporosis<sup>22</sup>. Due to the low lipid content of mussels (~2%), nutraceutical oils from GSM are  
72 produced on an industrial scale either by solvent extraction or by supercritical CO<sub>2</sub> extraction. Freeze-  
73 and spray-drying are the most commonly utilised methods for producing GSM powders, which are  
74 utilised as nutraceuticals and are being developed as food ingredients. It has been identified that in  
75 these GSM nutraceutical studies using animal and clinical models there were inconsistencies in the  
76 degree of benefit provided, which could be attributed to the different dosages used and the different  
77 types of GSM extracts used<sup>4</sup>. In turn, these differences could affect the concentrations of key  
78 bioactives present.

79 The bioavailability and pharmacokinetics (PK) of a drug are key determinant factors in the  
80 ultimate manifestation of efficacy in clinical practise. The same applies to bioactives from natural  
81 sources that deliver health benefits such as n-3 LC PUFA. Critically important parameters are the  
82  $C_{Max}$  (Maximum measured plasma concentration from time),  $T_{Max}$  (Time of the maximum measured  
83 plasma concentration), elimination  $t^{1/2}$ , and AUC (incremental area under the plasma concentration-  
84 time curve) data after single and multiple doses, which are used to determine optimum dosing  
85 regimens. Various biomarkers (such as EPA and DHA) have been used in recent studies on the  
86 bioavailability of n-3 LC PUFA, but their effectiveness has not been systematically compared nor has  
87 their use been fully justified<sup>10,11,14,23-25</sup>. In addition, there is growing evidence that the bioavailability  
88 of n-3 LC PUFAs varies depending upon their derived sources, such as krill oil, fish oil, mussel or  
89 algal oils<sup>10-12</sup>, and is further dependent on the fat composition and content of the diet and the food  
90 preparation methods employed<sup>10,26</sup>. This is due to the food structure or format of the n-3 LC PUFAs  
91 of the difference sources. The difference in the bioavailability of n-3 LC PUFAs when eaten as food  
92 product, functional food, freeze dried powder and/or extracted oil is yet to be established and we  
93 hypothesise that may differ as food structure and therefore digestion may be effected by format. In  
94 this paper, pharmacokinetic parameters to determine bioavailability ( $C_{Max}$ ,  $T_{Max}$  and AUC) of n-3 LC  
95 PUFA were measured for different formats of GSM following single dose intakes, as a first step in  
96 gaining a better understanding of the pharmacokinetics of key active ingredients.

97 There are 40 years of scientific literature connecting GSM extracts with health benefits; however,  
98 there are major knowledge gaps preventing validated health claims, particularly around consistent

99 efficacy and proven bioavailability. This study was intended to evaluate comparative  
100 pharmacokinetics and bioavailability of orally administered bioactive compounds from whole  
101 mussels, nutraceutical products and a novel functional food ingredient. A four-sequence, single dose,  
102 randomized crossover study in healthy adults was undertaken to determine the bioavailability of n-  
103 3 LC PUFAs, EPA and DHA in different GSM formats (Frozen half-shell GSM, GSM Food ingredient,  
104 GSM Powder and GSM Oil extract). Blood samples were collected pre-dose and up to 48 hours with  
105 2-hour intervals in the initial 8 hours and 4-8 hour intervals in the later stage after initiation of product  
106 administration. The aim of this study was to assess the uptake and retention of the n-3 LC PUFA by  
107 healthy adults from the four different formats.

## 108 2. Results

109 A total of twelve subjects were screened for the study. One subject failed screening based on an  
110 exclusionary BMI. Two further subjects met eligibility criteria at screening but subsequently  
111 withdrew consent prior to study enrolment.

112 Nine subjects were randomized into the study and were included in the safety population. This  
113 included one subject (subject 04) who withdrew consent prior to completing PK sampling for  
114 Administration Period 1; a replacement subject (subject 14) was randomised into the study, to ensure  
115 eight evaluable subjects.

116 All subjects in the safety analysis set identified their ethnicity as European (7 New Zealand  
117 European, 1 Spanish, 1 European). The mean age of subjects was 23 years (range 20 – 30 years), with  
118 mean BMI 24.4 kg/m<sup>2</sup> (range 21.5-30.7 kg/m<sup>2</sup>).

119 The mean plasma concentrations of EPA, DHA and total n-3 PUFA (all omega 3 fatty acids  
120 including  $\alpha$ -linolenic acid (ALA, 18:3n-3), stearidonic acid (SDA, 18:4 n-3), eicosatetraenoic acid (ETA  
121 20:4, n-3), EPA, docosapentaenoic acid (DPA, 22:5 n-3) & DHA) after a single dose of the four GSM  
122 products are shown in figures 1-3 respectively. Two distinct periods identified and analysed for AUC,  
123 C<sub>Max</sub> and T<sub>Max</sub> which were the periods between 0-12 h and the whole period (0-48h) (Tables 1- 3). The  
124 values in table 1-3 are calculated from the raw plasma concentrations which are not corrected for  
125 wide variations in the baseline values which are reported in Figure 1-3. Figures 1-3 which have been  
126 baseline adjusted.

127 There were significantly ( $p=0.013$ ,  $f= 4.84$ ) higher AUC in EPA concentrations with the GSM half  
128 shell mussel and GSM powder compared to the GSM oil over the 48-hour period (Table 1). However,  
129 there were no differences in the EPA in the shorter 0-12h range. In the DHA and the n-3 LC PUFA  
130 there were no statistical differences between treatments in the AUC over either of the different time  
131 periods.

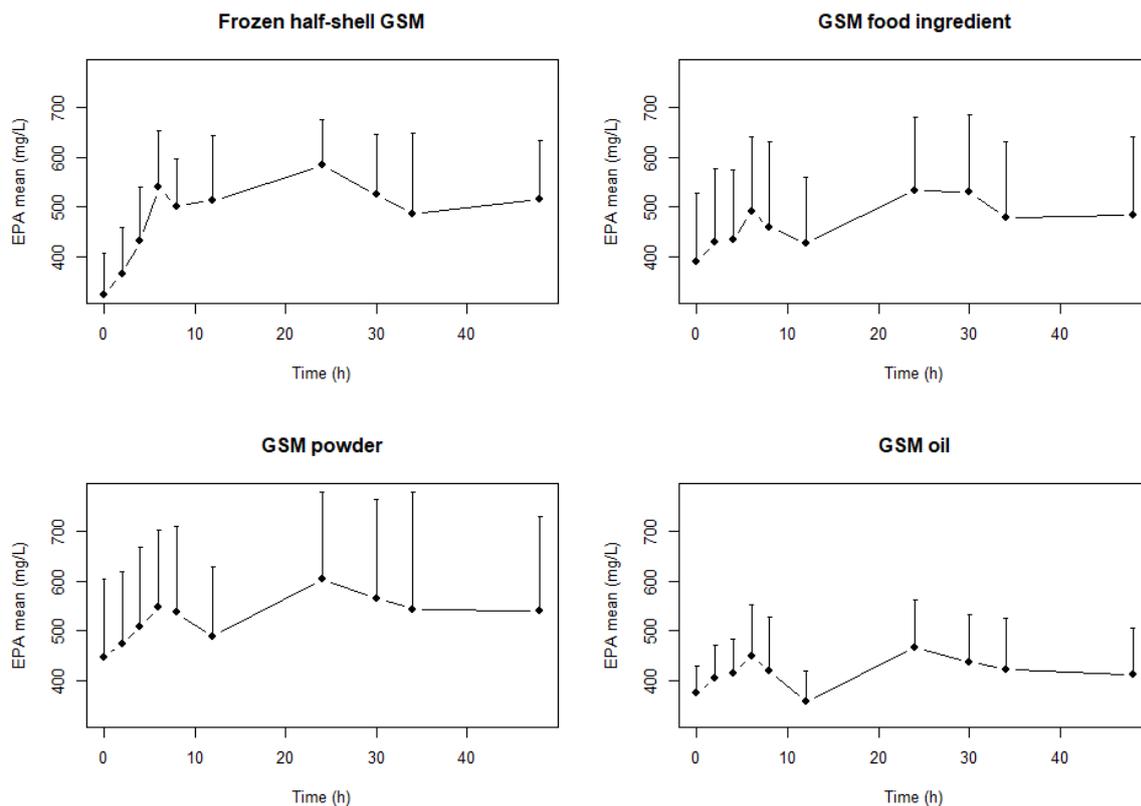
132 The C<sub>Max0-12h</sub> and C<sub>Max0-48h</sub> were significantly ( $p=0.011$ ,  $f= 5.05$  &  $p=0.004$ ,  $f=6.39$  respectively) higher  
133 in the EPA of the GSM half shell compared with GSM food ingredient and GSM oil over both time  
134 periods. Further, the C<sub>Max0-12h</sub> and C<sub>Max0-48h</sub> of the GSM powder were significantly different ( $p=0.011$ ,  $f=$   
135  $5.05$  &  $p=0.004$ ,  $f=6.39$  respectively) between the GSM powder and the GSM oil.

136 There were no statistical differences determined in the C<sub>Max0-12h</sub> and C<sub>Max0-48h</sub> in DHA and n-3 LC  
137 PUFA concentrations during the study.

138 The T<sub>Max0-48h</sub> was significantly ( $p=0.04$ ) lower (5h) in the GSM oil than the other three formats (in  
139 the 24-27 range) but this was not evident in the shorter T<sub>Max0-12h</sub> period. Two peaks are identified  
140 (figure 1-3) in the concentration-time profiles which led to two time periods analysed for maximum  
141 measured plasma concentration (0-12h and 12-48h). There were no statistical differences determined  
142 in the T<sub>Max0-12h</sub> and T<sub>Max0-48h</sub> in EPA and DHA concentrations during the study.

143 All evaluated GSM food platforms were well tolerated. A total of three subjects (33.3%)  
144 experienced a total of three adverse events (AEs). Two AEs (dry throat and upper respiratory tract  
145 infection) were considered unrelated to test product; the remaining AE of a headache was considered  
146 by the Investigator to have a remote relationship to test product. The three reported AEs were graded  
147 as mild in severity, required no specific intervention, and resolved spontaneously. No deaths, serious  
148 AEs, or AEs resulting in discontinuation of study product were reported.

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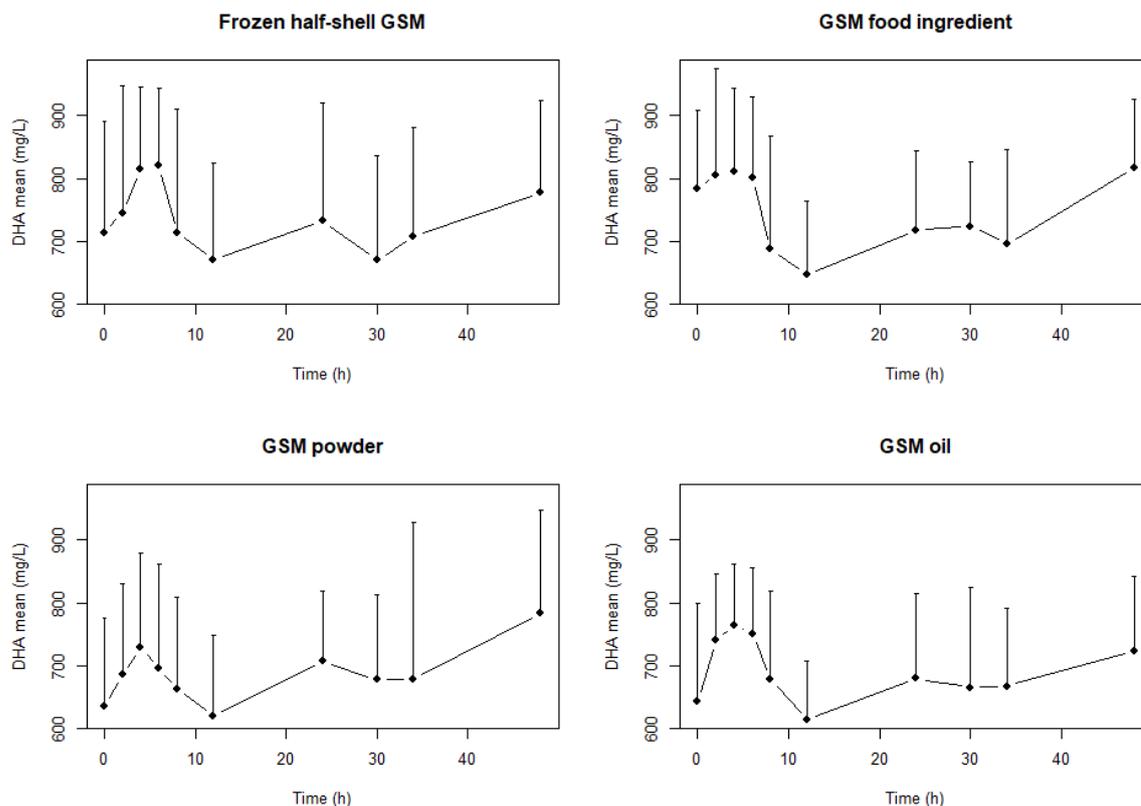
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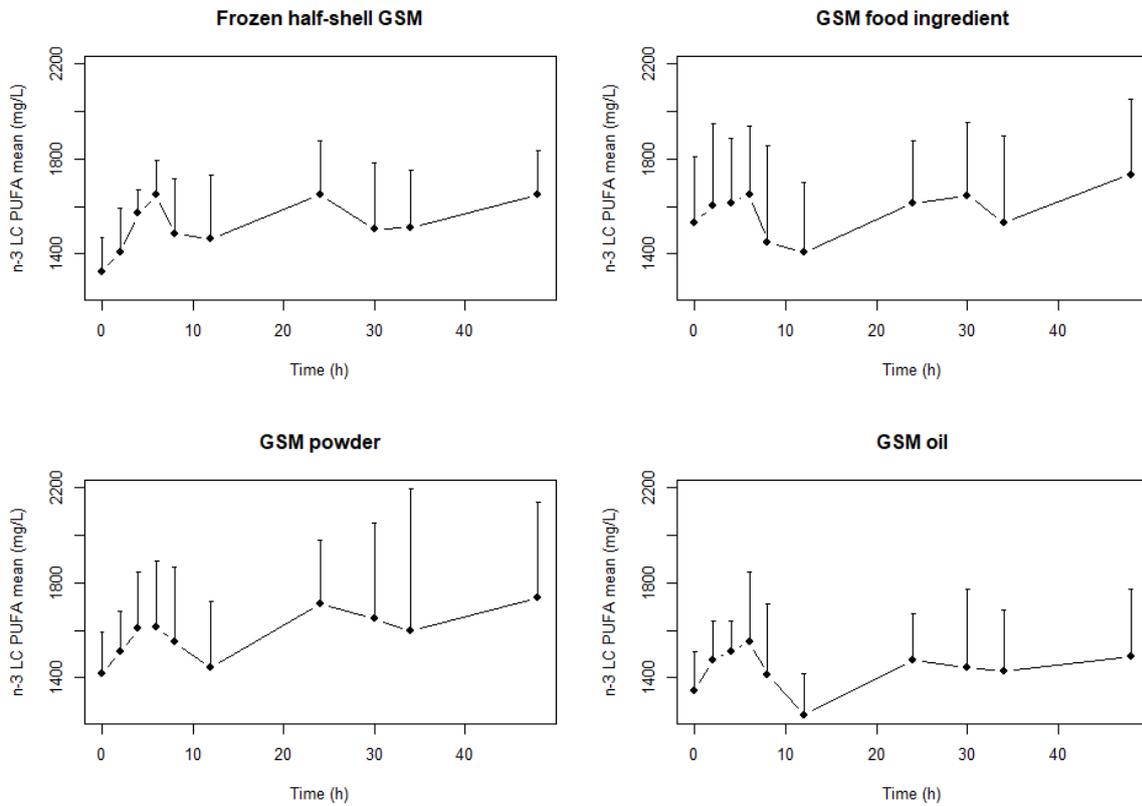
**Figure 1.** Mean (+SD) eicosapentaenoic acid (EPA) concentration-time profiles (baseline-adjusted change) after a single dose of Frozen half-shell GSM, GSM Food ingredient, GSM Powder and GSM Oil extract with matching levels of lipid.



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**Figure 2.** Mean (+SD) docosahexaenoic acid (DHA) concentration-time profiles (baseline-adjusted change) after a single dose of Frozen half-shell GSM, GSM Food ingredient, GSM Powder and GSM Oil extract with matching levels of lipid.



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**Figure 3.** Mean (+SD) n-3 LC PUFA (>18 carbon length) concentration-time profiles after a single dose of Frozen half-shell GSM, GSM Food ingredient, GSM Powder and GSM Oil extract with matching levels of lipid

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**Table 1.** Plasma content of the pharmacokinetic parameter, AUC (area under the plasma concentration-time curve) for eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), and long chain-polyunsaturated fatty acids (n-3 LC-PUFA) after a single dose of Frozen half-shell GSM, GSM Food ingredient, GSM Powder and GSM Oil extract with matching levels of lipid at 12 and 48 hour time points. <sup>a,b</sup>Geometric means values across the column not sharing a common superscript were significantly different ( $p < 0.05$ ) as determined by Fisher's protected LSD

<b>AUC<sub>0-12h</sub></b>												
		AUC <sub>0-12h</sub> mg/L	95.0% Lower CL for Mean	95.0% Upper CL for Mean		AUC <sub>0-12h</sub> mg/L	95.0% Lower CL for Mean	95.0% Upper CL for Mean		AUC <sub>0-12h</sub> mg/L	95.0% Lower CL for Mean	95.0% Upper CL for Mean
Frozen half-shell GSM	EPA	5407.0	4375.7	6681.5	DHA	8819	7457	10429.9	n-3 LC PUFA	17880.3	16250.7	19673.4
GSM Food ingredient		5046.2	3722.2	6841.2		8840.1	7570.1	10323.2		18071.9	15091.8	21640.5
GSM Powder		5821.7	4395.8	7710.3		7947.8	6771.1	9328.9		18180.9	15760.3	20973.3
GSM Oil extract		4886.9	4137.2	5772.3		8340.8	7350.8	9464.0		17305.3	15516.0	19300.8
F		-	-	-		-	-	-		-	-	-
<i>p</i> -value		-	-	-		-	-	-		-	-	-

<b>AUC<sub>0-48h</sub></b>												
		AUC <sub>0-48h</sub> mg/L	95.0% Lower CL for Mean	95.0% Upper CL for Mean		AUC <sub>0-48h</sub> mg/L	95.0% Lower CL for Mean	95.0% Upper CL for Mean		AUC <sub>0-48h</sub> mg/L	95.0% Lower CL for Mean	95.0% Upper CL for Mean
Frozen half-shell GSM	EPA	23963.6 <sup>b</sup>	19489.7	29464.6	DHA	34167.4	28805.8	40526.9	n-3 LC PUFA	73793.4	65863	82678.8
GSM Food ingredient		22138.7 <sup>a,b</sup>	17126.5	28617.8		34549.4	30361.7	39314.6		74412.4	63442.4	87279.2
GSM Powder		24683.2 <sup>b</sup>	18464.3	32996.6		32574.5	27579.2	38474.6		75639.8	62800.7	91103.7
GSM Oil extract		19858.4 <sup>a</sup>	16821.8	23443.2		32194.5	28017.1	36994.9		68033.5	60115.2	76994.7
F		4.840	-	-		-	-	-		-	-	-
<i>p</i> -value		0.013	-	-		-	-	-		-	-	-

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**Table 2.** Plasma content of the pharmacokinetic parameter  $C_{Max}$  (Maximum measured plasma concentration over the time span specified) for eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), and long chain-polyunsaturated fatty acids (n-3 LC-PUFA) after a single dose of Frozen half-shell GSM, GSM Food ingredient, GSM Powder and GSM Oil extract with matching levels of lipid for 12 and 48 hour time points. <sup>a,b,c</sup> Geometric mean values across the column not sharing a common superscript were significantly different ( $p < 0.05$ ) as determined by Fisher's protected LSD.

		$C_{Max0-12h}$										
		$C_{Max0-12h}$ mg/L	95.0% Lower CL for Mean	95.0% Upper CL for Mean	$C_{Max0-12h}$ mg/L	95.0% Lower CL for Mean	95.0% Upper CL for Mean	$C_{Max0-12h}$ mg/L	95.0% Lower CL for Mean	95.0% Upper CL for Mean		
Frozen half-shell GSM	EPA	546.6 <sup>c</sup>	441.4	676.9	DHA	851	747.8	967.3	n-3 LC PUFA	1694	1578.5	1818.5
GSM Food ingredient		474.4 <sup>a,b</sup>	359.7	625.7		834	724.1	959.3		1681	1439.6	1961.9
GSM Powder		539.0 <sup>b,c</sup>	405.7	716		736	640.2	845.5		1659	1434	1918.5
GSM Oil extract		450.4 <sup>a</sup>	372.1	545.1		780	711.2	855.6		1604.5	1414.2	1820.9
F		5.045				-				-		
<i>p</i> -value	0.011			-			-					
		$C_{Max48h}$										
		$C_{Max0-48h}$ mg/L	95.0% Lower CL for Mean	95.0% Upper CL for Mean	$C_{Max0-48h}$ mg/L	95.0% Lower CL for Mean	95.0% Upper CL for Mean	$C_{Max0-48h}$ mg/L	95.0% Lower CL for Mean	95.0% Upper CL for Mean		
Frozen half-shell GSM	EPA	594.7 <sup>c</sup>	499.8	707.5	DHA	857	759.2	968.4	n-3 LC PUFA	1772	1623.6	1934
GSM Food ingredient		536.8 <sup>a,b</sup>	421.5	683.7		852	749.1	968		1782	1509.3	2103.4
GSM Powder		593.3 <sup>b,c</sup>	445.5	790.2		835	703.6	992		1832	1504.6	2230.6
GSM Oil extract		477.4 <sup>a</sup>	395.1	576.8		806	713.9	909.2		1634	1431.5	1864.7
F		6.388				-				-		
<i>p</i> -value	0.004			-			-					

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**Table 3.** Plasma content of the pharmacokinetic parameter  $T_{Max}$  (Time of the maximum measured plasma concentration over the time span specified) for eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), and long chain-polyunsaturated fatty acids (n-3 LC-PUFA) after a single dose of Frozen half-shell GSM, GSM Food ingredient, GSM Powder and GSM Oil extract with matching levels of lipid for 12 and 48 hour time points. <sup>a,b</sup> Arithmetic mean values across the column not sharing a common superscript were significantly different ( $p < 0.05$ ) as determined by Friedman's Two-Way Analysis of Variance by Ranks

		$T_{Max12h}$										
		$T_{Max}$ (0-12h) 25 percentile	$T_{Max}$ (0-12h) Median	$T_{Max}$ (0-12h) 75 percentile	$T_{Max}$ (0-12h) 25 percentile	$T_{Max}$ (0-12h) Median	$T_{Max}$ (0-12h) 75 percentile	$T_{Max}$ (0-12h) 25 percentile	$T_{Max}$ (0-12h) Median	$T_{Max}$ (0-12h) 75 percentile		
Frozen half-shell GSM	EPA	6	6	8	DHA	2	4	6	n-3 LC PUFA	6	6	8
GSM Food ingredient		6	6	6		2	3	5		3	5	6

GSM Powder		5	6	7		4	4	6		4	5	7
GSM Oil extract		5	6	6		4	4	5		4	5	6
F												
<i>p</i> -value												

		<b>T<sub>Max48h</sub></b>														
		T <sub>Max</sub> (0-48h) 25 percentile	T <sub>Max</sub> (0-48h) Median	T <sub>Max</sub> (0-48h) 75 percentile				T <sub>Max</sub> (0-48h) 25 percentile	T <sub>Max</sub> (0-48h) Median	T <sub>Max</sub> (0-48h) 75 percentile				T <sub>Max</sub> (0-48h) 25 percentile	T <sub>Max</sub> (0-48h) Median	T <sub>Max</sub> (0-48h) 75 percentile
Frozen half-shell GSM	EPA	12	24	24	DHA	2	4	6	n-3 LC PUFA	12	24 <sup>b</sup>	48				
GSM Food ingredient		24	30	30		2	4	6		15	27 <sup>b</sup>	48				
GSM Powder		24	24	30		4	29	48		24	30 <sup>b</sup>	41				
GSM Oil extract		5	15	24		4	4	18		4	5 <sup>a</sup>	16				
F																
<i>p</i> -value														0.04		

### 3. Discussion

In this study we measured the pharmacokinetic parameters (AUC,  $C_{Max}$  and  $T_{Max}$ ) for the bioactive n-3 LC PUFA in human subjects fed different forms of GSM. Two time periods were analysed as there were two distinct peaks in the bioavailability of these bioactive lipids (Figure 1-3). These two distinct periods have been previously reported in a high PL n-3 LC PUFA bioavailability study<sup>27</sup>. The whole 48h period pharmacokinetic parameters were determined as well as the shorter 0-12h (table 1-3). These two time periods allowed for good comparison with published literature results (most of which were over the 0-12 h period) and also extrapolation with longer-term studies. This research addresses the essential step in understanding the bioavailability of key bioactives (n-3 LC PUFA) in GSM formats. The lack of bioavailability data in the literature for GSM, in both animal and clinical models, has enhanced inconsistencies with the efficacy data and makes comparisons between trials/formats difficult.

The results showed differences only in the bioavailability of EPA in the GSM half shell mussel and GSM powder compared to the GSM oil over the 48-hour period with significant ( $p = 0.013$ ,  $f = 4.84$ ) differences in the  $AUC_{0-48t}$  (table 1). This corresponds to a 20.6% and 24.3% increase in the amount of EPA present in the plasma after consumption of half-shell mussels and mussel powder respectively. The GSM food ingredient had a similar bioavailability of EPA to the GSM half shell and GSM powder products but was not significantly different to that of the GSM oil. In the DHA and n-3 LC PUFA there were no differences determined between the four different GSM formats.

The  $C_{Max}$  of EPA was significantly lower for the GSM oil than the GSM half-shell and GSM powder formats in both the 0-12h and 0-48h time frames (table 2). Further there was a significant difference in  $C_{Max}$  between the GSM half shell mussels and the GSM food ingredient for both time periods. This relates to a 22-25% increase in EPA in the plasma if delivered as a GSM half shell and GSM powder compared to extracted GSM oil depending on the timeframe calculated. The extracted oil (GSM oil) had a significantly shorter time (5 h) to reach the time of the maximum measured plasma concentration  $T_{Max}$  of n-3 LC PUFA than the other three formats, which were between 24-30h only when assessed over the 48h period (table 3).

The data (shown in the tables) were not baseline-adjusted due to the fact that many of the adjusted AUCs were negative for all formats and therefore it was not possible to analyse these with the transformed ANOVA models. However, the figures were all adjusted to account for differences in baseline levels of omega 3. Further we intended to calculate half-life ( $t^{1/2}$ ) of the n-3 LC PUFA but the plasma levels did not equalise to baseline in 48h and stayed high for the duration of the study, and therefore the calculation was not possible. The  $t^{1/2}$  is an important kinetic parameter and is worthy of mention due to the longevity of the key actives in the plasma, which could indicate one or two feeds a week would provide elevated levels of n-3 LC PUFA.

Different food formats will deliver n-3 LC PUFA in different molecular forms. In this study, we attempted to match the lipid content ( $2.2 \pm 0.1$  g/100g) across the four formats with only the food ingredient (2.05 g/100g) not meeting that requirement (table 4). However, the lipid classes and the amount of n-3 LC PUFA were different. The GSM oil and frozen half shell mussels were predominately in the form of PL (64% and 68% respectively), whilst the food ingredient and the mussel powder were mainly TAG (97% and 94% respectively). EPA was consistent across the four diets ( $341 \pm 15$  mg/100g); however, the DHA content was markedly different. The frozen half shell mussels had the highest content of DHA (311 mg/100g) followed by the oil (267 mg/100g), then the two powders (174 & 189 mg/100g for the food ingredient and mussel powder respectively). These differences in content contributes uncertainty to our results but using natural and food products as a part of a clinical trials it is hard to get interventions that are uniform in composition due to natural variation in these products.

The GSM oil and particularly the GSM half shell mussel formats were abundant in these phospholipids (identified in this paper as PL), but there has yet to be a lipidomic profile determining the phospholipid profile of GSM published. PL has the capability to act as an emulsifier, enhancing the formation of lipid extract emulsion in the digestion. In general, in marine oils the more unsaturated

fatty acids are most likely to attach to the sn2-position. It has been shown in GSM that EPA is more abundant in the TAG fraction than the PL fraction when extracted chemically (the opposite was shown in supercritical extracted fraction albeit the PL fraction was a very minor part of that oil)<sup>2</sup>. However there was no statistical difference in the concentration of DHA across the non-polar and polar fractions<sup>2</sup>. The PL fraction has been reported to be the predominant lipid across 12 months of sampling in the GSM production areas, and n-3 PUFAs were consistent across the seasons with 35-38% of all fatty acids primarily made up of EPA and DHA<sup>3</sup>. Further analysis has shown that there are differences in the concentrations of EPA (but not DHA) dependent on gender (with females having 43% more EPA than males) as well as significant differences in the lipid classes (with PL lower and TAG greater in female mussels)<sup>28</sup>. In this study we only use female mussels to reduce the impact of these gender differences. Both the GSM powder and GSM food ingredient have been processed to form these products (e.g. homogenising, drying etc.) and through this process the PL fraction of the lipid profile was minor (see table 4). Therefore, the bioavailability of EPA and DHA in GSM, independent of format, may be affected by factors such as season, extraction method, processing method and gender of mussels due to structural and concentration changes of the bioactive FA and these may account for some of the differences we have shown in this study as well as discrepancies in the published literature.

There have been minimal and conflicting studies comparing the bioavailability of EPA and DHA when delivered in different food formats, e.g. oil in a capsule verses fish as a meal. Visioli et al.<sup>29</sup> showed increased levels of plasma EPA and DHA when they fed salmon compared to capsules but attributed their finding to the larger fat content of the meal. They concluded that fish is more effective than capsules in providing n-3 LC PUFAs. Elvevoll et al.<sup>30</sup> showed fish consumption is more effective in increasing serum EPA and DHA than supplementing the diet with fish oil. In contrast, Stonehouse et al.<sup>31</sup> showed salmon oil or fillets were equally effective in delivering n-3 LC PUFA; however, there was additional benefit of increased levels of plasma selenium in the healthy volunteers who ate the fish. Finally, Harris et al.<sup>32</sup> determined there was no difference between oily fish (2 servings of oily fish e.g. salmon and albacore tuna per week) and fish-oil (1-2 capsules per day) on enriching blood lipids with n-3 FA.

Our GSM formats were all consumed with a low-fat soup meal (see section 4.1) to reduce influence of breakfast/food has on the intake of n-3 LC PUFA. It has been shown that the lipid content of a meal in postprandial studies such as a high fat breakfast can influence plasma omega 3 levels<sup>33</sup>. Diet was restricted of n-3 LC PUFA and standardised over during the clinic visits with further diet, medication and lifestyle restrictions that applied over the duration of the study (see section 4.3 for details).

Our results show that there is a slight but significant GSM format-dependent increase in AUC<sub>0-48h</sub>, C<sub>Max</sub> and T<sub>Max0-48h</sub> for EPA but not DHA or n-3 LC PUFAs. Our results do not conclusively answer the differences in food formats on the bioavailability of EPA and DHA and acknowledge the format of the EPA (whether on the position on the glycerol backbone or in the lipid class) may play a vital role in its bioavailability. We provided the food in a form of a soup to reduce the effect of a large fat content of the meal as a previous study<sup>29</sup> indicated this may be a confounding factor. However due to the nature of all the food products delivering n-3 LC PUFA through different lipid classes (e.g. TAG or PL), metabolic fates would be different, and comparisons are not identical.

There are limitations of the trial which are worthy of discussion. Firstly, participants were not screened for intake of n-3 LC PUFA prior to the trial and therefore bioavailability results may be different in individuals who have a high baseline n-3 LC PUFA status. Sample size is also a limitation however this is an exploratory study and the small sample size is sufficient to assess these fatty acid pharmacokinetic parameters. Future studies may want to consider a longer wash out period between administration and a study length of greater than 48 hours. A two week wash out would be preferable as some LC-PUFA, particularly as DHA concentrations appear to be still increasing at 48 hours and the 14-day wash will achieve a better chance to return to baseline.

Reviews of short-term post-prandial studies have reported that the type of lipid has implications on the bioavailability<sup>33, 34</sup>. The bioavailability of EPA and DHA in the ethyl ester form (EE, a synthetic form commonly used for nutraceutical lipids and particularly used in concentrated delivery systems)

were significantly lower than in TAG<sup>29</sup>. Further complexity occurs when comparing PL and TAG forms, as the literature suggests that these lipid classes are metabolised via different mechanisms and possibly have different fates. It has been shown that labelled <sup>13</sup>C DHA in the PC form has a markedly different kinetics and metabolic fate compared to TAG<sup>35</sup>. A recent review of human clinical trials found no conclusive evidence that the bioavailability of n-3 LC PUFA was greater from PL versus TAG sources; however there was some evidence of increase bioavailability of n-3 LC PUFA delivered as PL in animal models<sup>34</sup>.

In this trial the different delivery formats had differing proportions of TAG and PL. Both the GSM oil (94%) and GSM half shell (68%) were predominantly PL, whereas the opposite was true for the GSM powder (92% TAG) and GSM food ingredient (95% TAG). However, the differences we demonstrated in the EPA in AUC, T<sub>Max</sub> and C<sub>Max</sub> were seen between the GSM oil and GSM half shell, which had similar lipid class profiles. Further, the GSM powder and GSM oil, which have opposite lipid class profiles, had significantly different pharmacokinetic profiles in regard to EPA. This evidence suggests that lipid class can play a role in the bioavailability of FA from GSM.

Experimental and human studies with GSM now span some 40 years, and although animal veterinary and clinical trials have been conducted in the last 15 years there is still considerable inter-study variation in the ingredients and dosages used. Sound QA on raw materials and formulations of GSM ingredients have been lacking as well as poor understanding of absorption, bioavailability, distribution, metabolism, and excretion of key active ingredients. As our understanding on the key active ingredients in GSM extracts improves, analytical methodology can be used to assess the concentration of these bioactives in GSM foods, extracts, and final product. This will open up new research avenues as more pharmacokinetic studies become a possibility. It will also allow GSM breeding programmes to select for mussel families that produce higher levels of the bioactives of interest, with good bioavailability yielding a more potent feedstock for therapeutic usage in animals and humans alike. This single dose trial has provided early insights which will need to be complemented by multi-dose trials in clinical settings to further establish the bioavailability and pharmacokinetics of GSM bioactive components.

In summary, the AUC, C<sub>Max</sub> and T<sub>Max</sub> of EPA were affected by the delivery format. This may be related to EPA's positions on the glycerol backbone as well as the processing procedures. The evidence suggests that lipid class (PL or TAG format) plays a role in the bioavailability of EPA. EPA has previously been shown to be more bioavailable in terms of T<sub>Max</sub> than DHA<sup>12</sup>. T<sub>Max</sub> for EPA and DHA is in the range 2 h-24 h, which may be related not only to their molecular chains and bonding structure, but also to the format by which they were delivered. In our study we were able to show there were no differences in DHA or n-3 LC PUFA in terms of AUC, C<sub>Max</sub> and T<sub>Max</sub> in any of the four GSM formats.

## 4. Materials and Methods

### 4.1 GSM food formats

Four food formats were prepared to match in lipid content (table 4)

1. Frozen half-shell GSM - 125 g Sanford (Blanched GSM, Orvida brand, Sanford, Havelock NZ, Lot # M177812907, production date 9 May 2018)
2. GSM Food ingredient - 22.5 g Sanford (Batch L001, production 25 March 2018)
3. GSM Powder - 22.5 g Enzaq (Batch L457-2, production 08 July 2018)
4. GSM Oil extract - 2.3 g Pernatec oil (Waitaki Bioscience, production date 5 May 2018)

All samples were aliquoted and kept frozen until administration during the trial period of 31 July – 24 September 2018. The food ingredient is an activated GSM powder product developed to be utilised in the incorporation of functional foods.

To control for the effects of whole-meal components, all four GSM products were served in the same format, a leek and potato soup (204.3g per portion) comprised of the ingredients; 600g Leeks, 250, Onions, 5g Garlic, 30g unsalted butter, 350 g Potato (agria), 600 g Chicken stock, 2g Bay leaf, 0.2g Thyme, 1 tsp Iodised salt, Ground black pepper and 200ml low fat milk. The study was conducted from

31 July – 24 September 2018 at the Christchurch Clinical Studies Trust Ltd (CSST) site in Christchurch, New Zealand. The study was approved from the Central Health and Disability Ethics Committee (18/CEN/120) and conducted in accordance with ICH GCP and local ethical and regulatory requirements.

**Table 4.** Composition of the four samples (Frozen half-shell GSM, GSM Food ingredient, GSM Powder and GSM Oil extract) fed to clinical trial participants.

	GSM Oil extract	Frozen half-shell GSM	GSM Food ingredient	GSM Powder
Sample size (g)	2.3	125	22.5	22.5
<b>Proximate composition</b>				
	Amount per serving (g)			
Fat	2.30	2.25	2.05	2.23
Ash	0.00	1.75	4.82	3.94
Crude Protein	0.00	17.79	11.12	10.44
Carbohydrate	0.00	5.25	3.85	5.06
<b>Fatty acid profile</b>				
	Amount per serving (mg)			
C14:0 myristic acid	111.3	97.5	81.8	87.1
C16:0 palmitic acid	326.2	289.4	308.0	348.4
C16:1 palmitoleic acid	146.5	128.1	137.5	151.5
C18:0 stearic acid	84.0	88.6	69.6	92.8
C18:1n7 vaccenic acid	58.6	50.4	50.5	64.4
C18:1n9c oleic acid	48.8	21.7	24.4	24.6
C18:2n6c linoleic acid	84.0	39.5	40.0	32.2
C18:3n3 alpha linolenic acid (ALA)	25.4	26.1	27.8	17.6
C18:3n4 octadecatrienoic acid	23.4	21.0	2.6	2.5
C18:4n3 stearidonic acid (SDA)	48.8	58.0	47.0	32.2
C20:1 gadoleic acid	37.1	31.2	36.5	41.7
C20:4n6 arachidonic acid (AA)	33.2	27.4	29.6	26.5
C20:5n3 eicosapentaenoic acid (EPA)	355.5	350.0	339.4	320.0
C22:5n3 docosapentaenoic acid (DPA)	25.4	23.6	24.4	28.4
C22:6n3 docosahexaenoic acid (DHA)	267.6	311.1	174.0	189.3
∑SFA	554.1	509.8	493.4	567.8
∑MUFA	291.0	234.7	251.5	285.3
∑PUFA	879.5	880.5	718.3	684.8
∑Omega 3	727.9	776.0	622.2	595.7
∑Omega 6	140.6	91.5	77.4	69.7
<b>Lipid class</b>				
	Amount per serving (mg)			
Polar lipids (PL)	1463.6	1530.0	14.3	46.8
Sterols	29.9	104.3	43.0	82.4
Triacylglycerols (TAG)	801.9	622.5	1951.3	2051.5

$\Sigma$ SFA includes C15:0 pentadecanoic acid, C17:0 heptadecanoic acid, C20:0 arachidic acid and C21:0 heneicosanoic acid.  $\Sigma$ PUFA include C16:2n4 hexadecadienoic acid, C18:3n6 gamma linolenic (GLA), C20:3n3 cis-11, 14, 17-eicosatrienoic acid, C20:3n6 cis-8, 11, 14-eicosatrienoic acid and C20:4n3 eicosatetraenoic acid..

#### 4.2 Clinical study

The study was designed as an open-label, randomized, four-sequence crossover trial in eight evaluable healthy adult male participants. Administration occurred over the course of four administration periods, with a wash-out of at least seven days between test products.

The open-label, randomized cross-over design was utilised to enable both intra-subject and inter-subject comparison. No formal sample size calculations were made for this study, however the sample size of eight was expected to be adequate for evaluation of the parameters of interest. Single exposure pharmacokinetics allowed adequate characterization of the performance of each product and was therefore appropriate in meeting the study objective.

The dose of each product was selected to have similar lipid content ( $2.2 \pm 0.1$ g). Products were tested by the accredited Cawthron food testing laboratory, to determine accurate lipid and fatty acid content. The one-week washout interval utilised in the current trial was based on a previous study<sup>36</sup>.

Key study inclusion criteria limited the study to healthy adult males aged 18 – 45 years, body mass index (BMI) 18 to 32 kg / m<sup>2</sup> inclusive, with no clinically significant medical conditions. The study population was selected to reduce inter-subject variability and is considered appropriate for a non-therapeutic trial assessing pharmacokinetic parameters.

All subjects were provided with written and oral information about the study prior to the screening visit. Subjects were required to sign and date the most current Independent Ethics Committee (IEC)-approved written informed consent form before any study specific assessments or procedures were performed.

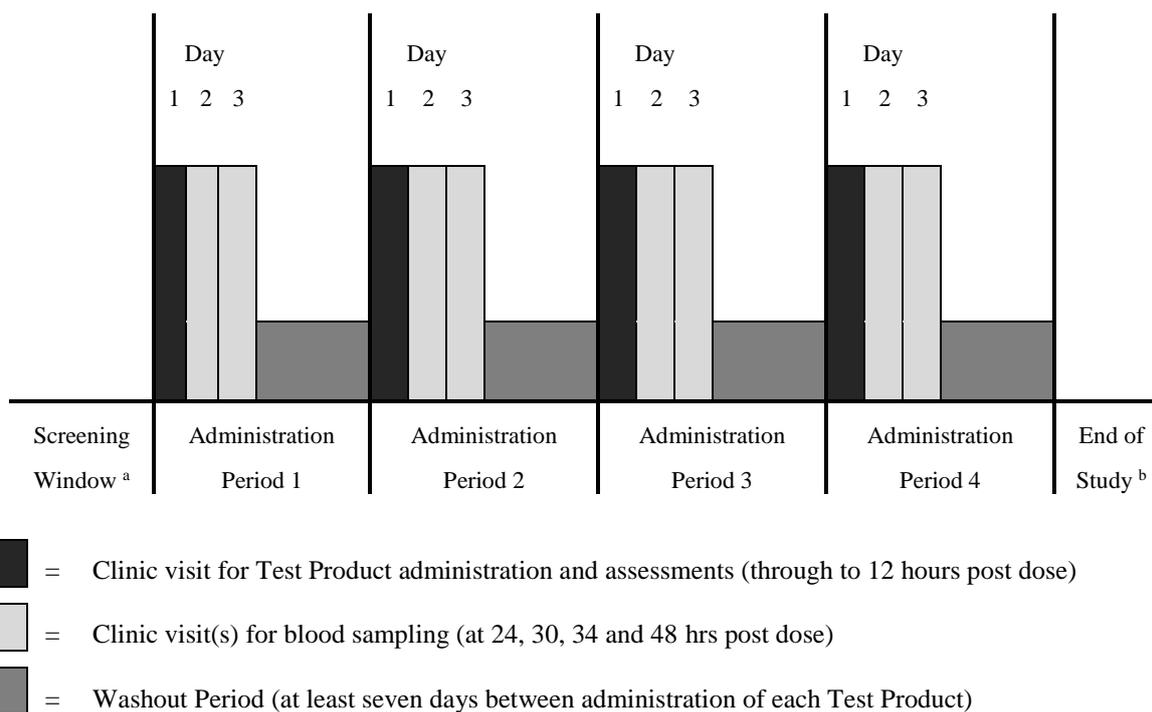
Potential subjects underwent screening procedures up to 28 days before the Day 1 of the first assessment period, to evaluate their eligibility for the study. Demographics, relevant medical history, and medication history were recorded. Subjects underwent a complete physical examination. Body weight, height (and derived BMI), vital signs and a single 12-lead electrocardiogram (ECG) were recorded. Blood and urine samples were collected for laboratory safety tests (haematology, biochemistry, urinalysis). A urine drug screen and breath alcohol tests were performed.

Following screening procedures, eligible subjects were enrolled into the study and completed four administration periods at the CCST research unit. For each subject, product administration order was determined by randomized allocation to one of four Test Product sequences.

Each administration period comprised a long clinic visit (from approximately 1½ hours prior to product administration through until completion of 12-hour post-administration assessments) and several short visits.

During each Administration Period PK samples were collected pre-dose and 2, 4, 6, 8, 12, 24, 30, 34 and 48 hours after initiation of product administration, for measurement of plasma EPA, DHA and n-3 LC PUFA. Blood samples were centrifuged at 4000 rpm (1520g) at 4°C for 10 min and the plasma was taken and stored at -80°C, cryopacked and shipped to Cawthron Institute for analysis.

Subjects were discharged from the study on completion of the final administration period assessments. Total study duration for each subject was up to approximately 90 days. Figure 4 gives an overview of the study design.



**Figure 4.** Study Design: (a) Up to 28 Days prior to Day 1 of Administration Period 1 (b) On satisfactory completion of all Administration Period 4 procedures

#### 4.3 Protocol Restrictions

Dietary, medication and lifestyle restrictions were applied during the study. At each visit subjects were reminded of the study restrictions and each subject's compliance was assessed. Restrictions were as follows:

- No prescription or over-the-counter medications, vitamins, minerals, or herbal supplements were permitted, from 2 weeks prior to first test product administration until study completion. Although medications required to treat adverse events were permissible with approval from the Investigator, no concomitant medications were used by any subjects during the study.
- Consumption of fish or other seafoods, or fish/seafood-containing products was not permitted, from two weeks prior to first test product administration until study completion.
- A fat-controlled diet was required, from 1 week prior to first test product administration until study completion.

Standardised meals were provided during long clinic visits, with no other food permitted.

- Alcohol was not permitted, from 48 hours prior to each test product administration until the final PK sample collection in each administration period. A negative alcohol breath test was required at screening and prior to test product administration.
- Consumption of cigarettes or other nicotine-containing products was not permitted, from screening until study completion.
- Recreational drug use was not permitted, from screening until study completion. A negative urine drugs of abuse screen was a requirement for study entry.
- Water was not permitted, for one hour pre- and post- test product administration. Water was otherwise permitted ad libitum.

#### 4.4 Assessment of Safety

Clinical laboratory tests and electrocardiograms were performed at screening only, to confirm study eligibility. Tolerability during the study was assessed by monitoring adverse events (AEs) and vital signs (recorded pre-dose and 12-hours post-dose in each Administration Period, and prior to discharge from the study).

#### 4.5 Lipid analysis

Plasma was defrosted (500 $\mu$ L aliquot) and 250 $\mu$ L cold isotonic saline (0.9%) and 1ml isopropanol (containing 0.005% BHA as an antioxidant) were added and vortexed thoroughly. Chloroform (2ml, containing 0.005% BHA as an antioxidant) was added and mixed thoroughly for 10 min. The samples were centrifuged at 3000 rpm (850g) at 4°C for 10min. The bottom organic phase was sampled and concentrated under nitrogen prior to methylation. The sample was trans-methylated in methanol: chloroform: hydrochloric acid (10:1:1, v/v/v) for 1 h at 100°C. After addition of water the mixture was extracted three times with hexane: chloroform (4:1, v/v) to obtain fatty acid methyl esters (FAME). Samples were made up with 200 $\mu$ L Hexane with an internal injection standard (C19:0 methyl nonadecanoate; NuCheck Elysian, MN, USA).

FAME samples were run in accordance to AOAC official methods 963.22 ("Methyl Esters of fatty acids in oils and fats"). In brief, FAME was analysed by gas chromatography (GC) performed using an Agilent 6890 with an Agilent SP-2560 silica capillary column (100m x 0.25 mm i.d., 0.2  $\mu$ m film thickness) and peak area determined by flame ionised detection (FID). Samples (1  $\mu$ L) were injected via a split injector at 260°C. The column temperature programme was: 220°C at 17 min, then raised by 2.8°Cmin<sup>-1</sup> to 240°C and held for 5 min. Fatty acids were identified to an external commercial fatty acid standard (Supelco 37 Component FAME Mix, Merck, Auckland, NZ) using ChemStation software (Agilent, Auckland, NZ). Nitrogen was the carrier gas.

#### 4.6 Pharmacokinetics calculation and statistical analyses

The following PK parameters were calculated for EPA, DHA, n-3 LC PUFA and EPA+DHA plasma concentrations out to 12 and 48 hours using a non-compartmental model.

- AUC<sub>0-t</sub>: The area under the plasma concentration-time curve, from time t=0 to 12 and 48 hours, calculated by the linear trapezoidal method.
- C<sub>Max</sub>: Maximum measured plasma concentration from time 0 to 12 and 48 hours.
- T<sub>Max</sub>: Time of the maximum measured plasma concentration to 12 and 48 hours.
- t<sup>1/2</sup>: A measure of elimination, half-life is the time necessary for the concentration in the plasma to decrease by half.

If the maximum value occurred at more than one time point, T<sub>Max</sub> was defined as the first time point with this value. The plasma concentrations were not baseline adjusted for the PK calculations as the concentration changes for all four analytes showed considerable variability after dosing with some participants increasing and other decreasing.

Arithmetic means and standard deviations were calculated for the AUC and C<sub>Max</sub> parameters and medians and inter-quartile ranges (IQR) for T<sub>Max</sub>. Additionally, geometric means and 95% confidence intervals were calculated for AUC and C<sub>Max</sub>.

ANOVA was performed for the AUC and C<sub>Max</sub> parameters to 12 and 48 hours for EPA, DHA, and n-3 LC PUFA. These parameters were ln-transformed prior to analysis. The ANOVA model included treatment and period (time) as fixed effects, and participant as a random effect. The ANOVA included calculation of least-squares means (LSM), and the differences between LSMs and the 95% confidence intervals for these differences. These differences and confidence intervals were back-transformed to produce geometric mean ratios and 95% confidence intervals.

The T<sub>Max</sub> parameters were compared between treatments using Friedman's non-parametric ANOVA with pair-wise comparisons between treatments undertaken when the Friedman's test indicated a significant difference amongst the treatments.

A two-tailed  $p$ -value  $<0.05$  was taken to indicate statistical significance. The multiple comparison strategy used an approach called Fisher's protected LSD, this means that only when the  $f$ -ratio from the ANOVA as significant were the pairwise tests undertaken.

## 5. Conclusions

Based on this exploratory study of EPA, DHA and n-3 LC PUFA pharmacokinetic parameters we conclude: EPA peak concentrations and plasma exposures were significantly lower with GSM oil compared with GSM half-shell and GSM powder products, DHA and n-3 LC PUFA plasma exposure parameters were statistically comparable across the four GSM products evaluated and GSM oil produced the shortest median time to maximal plasma n-3 LC PUFA concentration of all evaluated products. This was primarily due to maximal concentrations being reached during the first peak in plasma concentrations, in contrast with the other evaluated products. All products evaluated were well-tolerated, with no adverse effects assessed.

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