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Differentiation of Fish Oils According to Species by ^{13}C-NMR Regiospecific Analyses of Triacyglycerols

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Abstract The aim of this study was to use ^{13}C-nuclear magnetic resonance (NMR) regiospecific analyses of triacylglycerols to distinguish fish oils from different fish species for authentication purposes. ^{13}C-NMR data of muscle lipids from Atlantic salmon (*Salmo salar* L.), mackerel (*Scomber scombrus*) and herring (*Clupea harengus*) were obtained, and the distribution of omega-3 polyunsaturated fatty acids between the sn-1,3 and sn-2 glycerol chains calculated from the carbonyl region. The results show that there were significant differences in the sn-2 position specificity of the fatty acids 22:6n-3, 20:5n-3 and 18:4n-3 among the species investigated. The most pronounced difference was that herring had a higher proportion of its 22:6n-3 in the sn-2 position compared to the two other species. Principal component analysis of data points in the carbonyl-region showed that there were also differences in the level and regiospecific distribution of monounsaturated/saturated fatty acids, which made it possible to distinguish oils of these three species solely from the carbonyl region of ^{13}C-NMR spectra.

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Introduction

The demand for fish oil, rich in the valuable long-chain n-3 polyunsaturated fatty acids docosahexaenoic acid (22:6n-3) and eicosapentaenoic acid (20:5n-3), is increasing, while the production of such oils has been rather constant for the last few years [1]. Fish oil is used increasingly as an n-3 fatty acid (omega-3) source for humans in the form of food ingredients/functional food, as dietary supplements and as pharmaceuticals [2]. In Norway, sources of high quality fish oils includes pelagic fish species such as herring, blue whiting, and mackerel in addition to cod livers and cut-offs from aquaculture. There is a potential for increasing the production of omega-3 oils by utilising more of the by-products from fillet-production of fatty fish species such as Atlantic salmon (*Salmo salar* L.), mackerel (*Scomber scombrus*) and herring (*Clupea harengus*) [2]. For example, the fish oil production from pelagic by-products today is approximately 20,000 tonnes, but the potential is between 30,000 and 75,000 tonnes [2].

However, differences in price between oils of different origins and qualities, may lead to mislabelling and adulteration, and it is therefore important, with well-functioning methods, to verify the origin of such oils, and the species used. Traditional methods for species authentication of fish, such as DNA- or protein analyses may not be applicable for fish oil since DNA/proteins may only be present at levels below the detection limit [3].

Fish oils are mainly composed of triacylglycerols (TAGs) and the pattern of TAGs is characteristic, and more or less unique, for different types of natural fat. Analyses of TAGs may therefore be used to study authenticity and adulteration, as has previously been shown for fish oils [4], milk fat [5], animal fat [6] and olive oils [7, 8]. Although fatty acid composition of fish tissues varies according to factors such as season, age, diet, and environmental factors, there are genetically determined differences between species. Both the fatty acid composition [9] and the stereospecific structure of TAGs vary among fish species [10–12]. Knowledge of TAG structure has also become increasingly important since the stereospecific structure influences the lipid metabolism [13, 14] and bioavailability of fatty acids.

Traditional methods to determine the distribution of fatty acids in acylglycerols are usually based on laborious and time-consuming chromatographic/enzymatic methods [15, 16]. High resolution ^{13}C nuclear magnetic resonance (NMR) has arisen as a valuable tool in the analysis of lipids, including fish lipids. In addition to fatty acid composition and lipid classes, the technique renders information on the regiospecific distribution of fatty acids in TAGs [10, 11] and phospholipids [17]. The technique is also well suited for studying esterification processes and if chemical modifications have been done to the natural fish oil [4, 18]. ^{13}C NMR, in combination with multivariate analysis, has allowed discrimination between wild and farmed fish [19, 20], and fish oil from different species and composition [4].

The European pharmacopoeia on salmon oil includes the regiospecific distribution (sn-2 position specificity) of fatty acids in TAGs by ^{13}C-NMR spectroscopy to minimise the risks of blends and adulteration [21]. Quantitative analysis of the distribution of fatty acids in TAGs by ^{13}C NMR is possible due to the different chemical shifts of fatty acids in sn-1,3 and sn-2 positions. The carbonyl region (172–174 ppm), olefinic signals (126–134 ppm), glycerol region (74–60 ppm), and the aliphatic region (19–35 ppm) of ^{13}C-NMR spectra have been used in regiospecific analysis [8, 10, 11, 22–28].

Previous regiospecific ^{13}C-NMR studies reported the composition of lipids in TAGs of tuna oil [28], and differences between cod liver-, harp seal- and Atlantic salmon oil [11], and between menhaden-, Chilean-, anchovy- and cod liver oil [10]. The positional distribution of fatty acids in lipids from skin, white and dark muscle of mackerel [29], and differences between fish oils [30] menhaden and salmon oil [12] have been studied by chromatographic methods. However, to our knowledge, such data on positional distribution of fatty acids in triacylglycerols have not previously been used for authentication purposes. This study was undertaken to determine differences in the sn-2 position specificity of fatty acids in triacylglycerols of Atlantic salmon (*S. salar* L.), mackerel (*S. scombrus*) and herring (*C. harengus*). Further, it was tested if it is possible to use the characteristic ^{13}C-NMR carbonyl profile as a fingerprint for authentication of species of fish oils.

Materials and Methods

High Resolution ^{13}C-NMR Analyses

Lipids were extracted from white muscle of individuals ($n = 5$) of wild Atlantic salmon (*S. salar* L.), mackerel (*S. scombrus*) and herring (*C. harengus*) by a modified Bligh and Dyer procedure [31]. Approximately 90 mg of the extracted lipids was transferred to 5-mm NMR tubes and diluted with 0.6 mL deuterated chloroform ($CDCl_3$). The ^{13}C-NMR spectra were run semi-quantitatively, with short relaxation times, and a high number of scans, to achieve sufficient S/N ratio to calculate the proportion at the sn-2 position in TAGs of individual fatty acids. Since the carbonyl carbons of interest have similar Nuclear Overhauser Effect (NOE) and the T1 values of these carbons are not particularly influenced by the position of the fatty acids in the glycerol molecule [11, 22–24] regiospecific distributions can be measured from semi-quantitative spectra [11]. ^{13}C-NMR spectra were obtained on a Bruker Avance DMX 600 instrument operating at 150.9 MHz for carbons, using a 5-mm BBO probe at 298 K. Power gated decoupling was applied to obtain decoupled spectra with NOE. The following acquisition parameters were used: time domain 64 k, ^{13}C exitation pulse 60°, sweep-width 200.8 ppm, acquisition time 1.08 s, relaxation delay 0.5 s, number of scans 16 k. Zero filling and exponential line broadening (0.15 Hz) was applied before Fourier transform. As the chemical shifts of carbonyl carbons show marked concentration dependence [32], the chemical shift scale is referred indirectly to TMS by the singlet arising from C1 of 22:6n-3 in the sn-2 position of triacylglycerols at 172.13 ppm. Assignments were made according to Aursand et al. 1995 and the fact that the distance between the sn-1,3 and sn-2 chains is approximately 0.4 ppm [10].

Calculation of the sn-2 Position Specificity of Fatty Acids

Peak fitting was applied to the carbonyl-region of the ^{13}C-NMR spectra, to facilitate integration of peaks arising from sn-1,3 and sn-2 positions of omega-3 fatty acids, and to reveal hidden/overlapping peaks. The processed ^{13}C-NMR data was converted to ASCII files, and the data in the carbonyl region (174–172) were selected (600 data points) and imported to PeakFit 4.12 (SeaSolve Software Inc., San Jose, CA). The AutoFit Residuals method was chosen as the peak-fitting method. This procedure initially

places peaks by finding local maxima in a smoothed data stream. Hidden peaks are then optionally added where peaks in the residuals occur (Peakfit 4.12). Peaks were fitted, assuming Lorentzian lineshape, with a linear baseline subtracted prior to fitting. Hidden residual peaks were added. The area of peaks arising from sn-1,3 and sn-2 positions of the fatty acids docosahexaenoic acid (22:6n-3, DHA), eicosapentaenoic acid (20:5n-3, EPA) and stearidonic acid (18:4n-3) were found, and the proportion in the sn-2 position (mol%) of these fatty acids calculated. Significant differences among species in the sn-2 specificity of each fatty acid were found by one-way analysis of variance (ANOVA) with a significance level of 0.01 (Excel, Microsoft XP).

Classification by Using Data Points in the Carbonyl-Region

The data points in the carbonyl region (ASCII file mentioned previously) were used in multivariate analysis, to evaluate if the information in the carbonyl region of the spectra were characteristic enough to distinguish the different species. The original 600 data points from the carbonyl-region, were reduced to 413 (by removal of regions without peaks) and exported for principal component analysis (PCA) [33, 34], a commonly used multivariate technique for dimension reduction and to observe groupings. PCA was implemented using Unscrambler (version 9.2, CAMO process AS, Oslo, Norway).

Results and Discussion

^{13}C-NMR Spectra

^{13}C-NMR spectroscopy of fish oil provides a fingerprint of the sample analysed. A typical ^{13}C-NMR spectrum of salmon oil is shown in Fig. 1a. Different carbon atoms give signals in different regions of the ^{13}C-NMR spectrum. The region where carbonyl carbons (C1 atoms) give signals (between 173.4–172.0 ppm) is shown in Fig. 1b. Assignments of resonances are shown in Table 1 and are made according to previous studies on fish lipids [11]. In general, the chemical shift of carbonyl carbons of fatty acids in triacylglycerols depend on the regiospecific position (whether the fatty acid is a sn-1,3 or sn-2 chain), and for carbonyl carbons of unsaturated fatty acids, the position and number of double bonds in the chain [11, 23].

Figure 2 shows the carbonyl region of oils extracted from salmon, mackerel and herring muscle respectively. There are clear differences in the relative intensities of resonances between the different fish species, the most pronounced differences are marked by arrows. From this

Table 1 Chemical shift values (ppm) and assignments of resonances in the carbonyl region (C1) of salmon, mackerel and herring oils

Assignment	δ (ppm)
SFAs/MUFAs/other FAs (sn-1,3)	173.22–173.29
20:4n-3 (sn-1,3)	173.20
22:5n-3 (sn-1,3)	173.16
18:4n-3 (sn-1,3)	173.07
20:5n-3 (sn-1,3)	173.01
SFAs/MUFAs/other FAs (sn-2)	172.82–172.88
20:4n-3 (sn-2)	172.80
22:5n-3 (sn-2)	172.76
18:4n-3 (sn-2)	172.67
20:5n-3 (sn-2)	172.61
22:6n-3 (sn-1,3)	172.53
22:6n-3 (sn-2)	172.13

FAs fatty acids, *SFAs* saturated fatty acids, *MUFAs* monounsaturated fatty acids

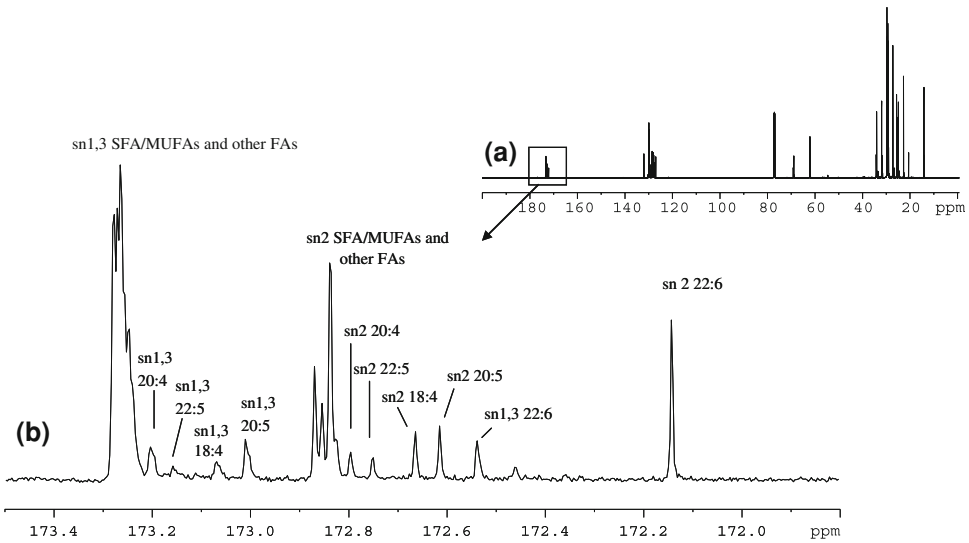

Fig. 1 600 MHz ^{13}C-NMR spectra of salmon oil (**a**), with the carbonyl region (172.0–173.4 ppm) enlarged (**b**). *FAs* fatty acids, *SFAs* saturated fatty acids, *MUFAs* monounsaturated fatty acids

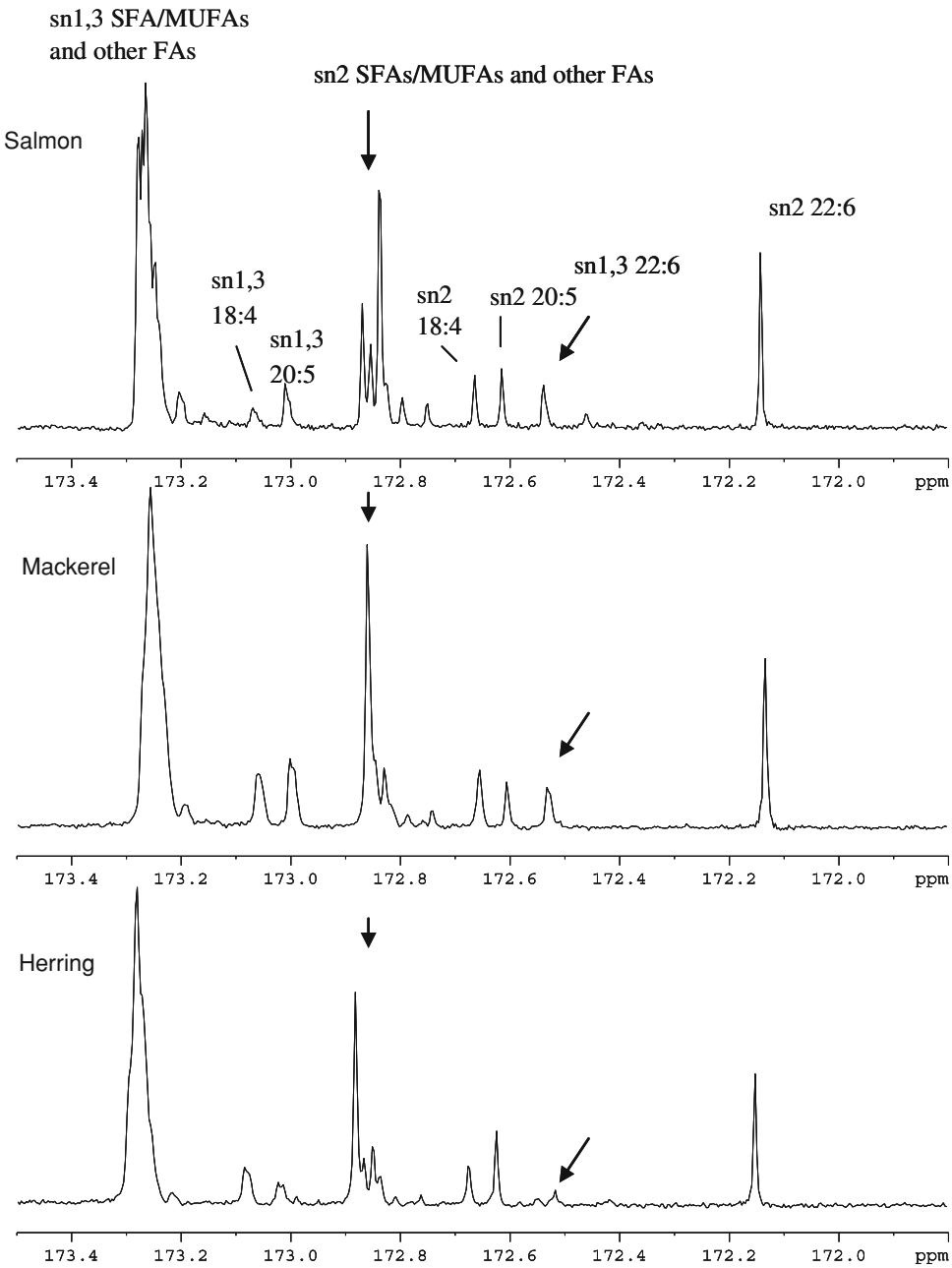

Fig. 2 600 MHz ^{13}C-NMR carbonyl region of muscle lipids extracted from wild salmon, mackerel and herring. The most pronounced differences among the species are highlighted with *arrows*. *FAs* fatty acids, *SFAs* saturated fatty acids, *MUFAs* monounsaturated fatty acids

figure, one can see that herring has a lower distribution of 22:6n-3 in the sn-1,3 position (lower intensity at 172.53 ppm) compared to the two other species and that salmon differs from mackerel and herring in the distribution of signals in the region 172.88–172.82 ppm. Peaks in this region are attributed sn-2 carbonyl carbons in (in decreasing chemical shift order) saturated- and monounsaturated fatty acids (SFAs/MUFAs) in addition to (but less abundant) other fatty acids with the first double bond in the acyl chain further away from the carbonyl carbon than at C8 (i.e. 18:2n-6, and 18:3n-3) [28]. It is also observed that herring and mackerel spectra show poor resolution of peaks in the area 173.2–173.4 ppm, where carbonyl carbons in the sn-1,3 position of SFAs/MUFAs give signals. This may be attributed to sample characteristics, but more peaks may be revealed in this region by improving digital resolution (and applying a higher field), as is currently being investigated.

sn-2 Position Specificity of Fatty Acids

The areas under peaks from carbonyl carbons of 22:6n-3, 20:5n-3 and 18:4n-3 were found by peak fitting, and the sn-2 position specificity of these fatty acids in triacylglycerols

was calculated (mol%). A random distribution corresponds to a sn-2 position specificity of 33% (and 67% in sn-1,3). The results are given in Table 2, and show that 22:6n-3 was preferentially esterified to the sn-2 position in all species investigated with mean values: 76% for salmon, 75% for mackerel, and approximately 90% for herring. 20:5n-3 was less esterified in the sn-2 position, with 47, 30 and 68% for salmon, mackerel and herring, respectively. 18:4n-3 was close to randomly distributed in herring and mackerel, while salmon had a relatively high percentage of this fatty acid in the sn-2 position (60%).

The values are in accordance with previous results on fish lipids, showing that the long-chain polyunsaturated fatty acids (PUFAs) tend to be preferentially esterified to the sn-2 position in fish triacylglycerols [10, 28, 29, 35], in contrast to in mammals such as seal [11].However, a recent study on sea bass [36] reported a markedly lower sn-2 position specificity of 22:6n-3 (sn-1,3:sn-2 ratio of 1.7 and thereby sn-2 position specificity of 37%) compared to results from other fish species investigated to date. The limits set in the European pharmacopoeia for farmed Atlantic salmon oil on the proportion of 22:6n-3, 20:5n-3 and 18:4n-3 in sn-2 position are 60–70, 24–35 and 40–55% respectively. Our results on wild Atlantic salmon are slightly higher than these limits (76, 47 and 60% for 22:6n-3, 20:5n-3 and 18:4n-3 respectively). However, previous ^{13}C-NMR regiospecific analysis on farmed salmon also reported higher values than the limits specified in this monograph [11]. Our results on 22:6n-3 and 20:5n-3 (76 and 47% in the sn-2 position respectively) are also somewhat higher than literature values on farmed salmon (73 and 62–68% for 22:6n-3, and 40 and 34–36% for 20:5n-3 [11, 26]. This implies that there is a degree of natural variation in sn-2 position specificity within species. Previous studies suggested a reduced percentage of 22:6n-3 at sn-2 position at elevated temperature [26], however it remains to be studied to what extent diet and other environmental factors affect the sn-2 position specificity of fatty acids. The sn-2 position specificity of 18:4n-3 for salmon in this study (60%) is in the range of previously reported values 73 and 42–46% [11, 26] for the sn-2 position.

The results from herring in the present study are well in accordance with previous studies where the proportions of 22:6n-3 and 20:5n-3 in the sn-2 position were 87 and 62% respectively [30]. The results from mackerel are similar to previous studies on mackerel white muscle, in that the sn-2 position is the preferred position for 22:6n-3, while 20:5n-3 is more randomly distributed [29].

When it comes to differences among species, ANOVA calculations ($P = 0.01$) showed that herring displayed a significantly higher proportion of its 22:6n-3 in the sn-2 position than the other two species. The sn-2 position specificity of 20:5n-3 was significantly different among salmon, mackerel and herring. In addition, salmon displayed a higher proportion of 18:4n-3 in the sn-2 position (60%) than mackerel and herring (39%, 40%).

Table 2 Proportion in sn-2 position (mol%, with standard deviations for each species) of the fatty acids 22:6n-3 (DHA), 20:5n-3 (EPA) and 18:4n-3 in triacylglycerols of salmon, mackerel and herring respectively

	22:6n-3	20:5n-3	18:4n-3
Salmon	76 ± 2	47 ± 3	60 ± 6
Mackerel	75 ± 1	30 ± 5	40 ± 1
Herring	>90[a]	68 ± 5	39 ± 1

[a] sn-2 22:6n-3 for herring set to approximately 90%, since no detectable amounts in sn-1,3-position

Classification from Data Points in Carbonyl Region

As mentioned previously, the ^{13}C-NMR carbonyl region also revealed differences among species in the region between 172.88 and 172.82 ppm (Fig. 2) where saturated- and monounsaturated fatty acids (SFA/MUFAs) dominate (other fatty acids with signals here are less abundant in fish oils). However, it was difficult to calculate the distribution of SFAs and MUFAs between the sn-1,3 and sn-2 chains, because the individual samples showed different numbers of overlapping peaks here. To evaluate if the fingerprint obtained in the carbonyl region is characteristic enough to separate fish species, the data points in this region were exported for multivariate analysis. Principal component analysis is a commonly used multivariate technique to observe groupings and to elucidate differences between groups [33, 34]. PCA of data points in the carbonyl-region is shown in Fig. 3. The first two principal components explained 49 and 27% of the variance in the dataset. Similar samples will be closely positioned in a PCA score plot. The PCA score plot (Fig. 3a) shows clear separation between the different species salmon (S), mackerel (M) and herring (H). The loading plot (Fig. 3b) illustrates to what extent individual variables participate in defining the principal components. Variables that do not contribute much are positioned close to the centre of the plot, while variables with the most impact are plotted close to the border of the plot. The chemical shifts where the sn-2 position of MUFAs and SFAs give signals were the variables with the most impact in the analysis (data point numbers 160–180 correspond to chemical shifts 172.9–172.8 ppm) (Fig. 3b).

In conclusion, these results add to the previous findings that analysis of lipids by ^{13}C NMR is a promising tool for authentication of marine oils [4, 20]. Previous ^{13}C-NMR studies showed that fish oils can easily be differentiated from

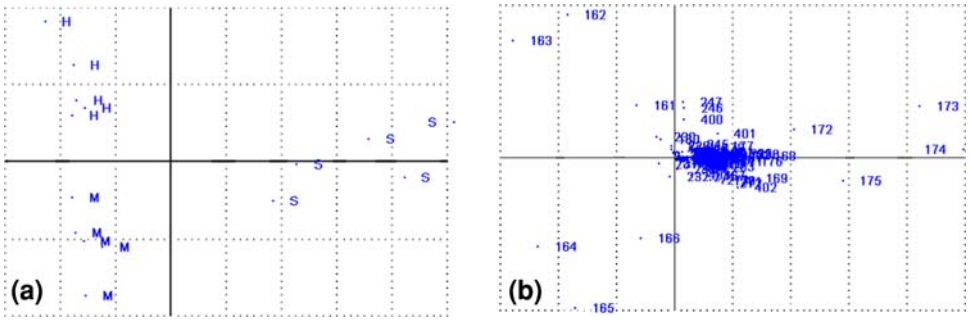

Fig. 3 PC1 vs. PC2 scores (**a**) and loadings plot (**b**) obtained from 400 data points in the carbonyl-region of the ^{13}C-NMR spectra of lipids from salmon (*S*), mackerel (*M*) and herring (*H*). The two-first principal components explained 49 and 27% of the variance in the dataset. Variable number 160–175 corresponds to the region 172.9–172.8 ppm where resonances from monounsaturated- and saturated fatty acids (MUFAs and SFAs) in the sn-2 position dominate

seal oil by regiospecific analyses of triacylglycerols [11]. Our results show that there were significant differences in the sn-2 position specificity of the fatty acids 22:6n-3, 20:5n-3 and 18:4n-3 among the species salmon, mackerel and herring. There were also differences in the level and distribution of monounsaturated/saturated fatty acids, which made it possible to distinguish lipids of these three species solely from the carbonyl region of ^{13}C-NMR spectra.

For authentication purposes of fish oils, regiospecific information is valuable, since this characteristic is more difficult to manipulate than pure fatty acid composition. The most important advantage of ^{13}C NMR in regiospecific analysis is that it is applied to intact lipids without enzymatic or chemical manipulation of the sample. The overall profile in the carbonyl region is likely to be characteristic enough to separate fish species, even though the results reported to date show that there is a degree of natural variation within species. However, these variances should be systematically investigated and characterised. It also remains to determine the detection limits of other oil as adulterant, and in this respect the full ^{13}C-NMR spectra would also provide important information to discover such fraud [4]. ^{13}C NMR is also a powerful tool to analyse the possible influence of environmental and dietary factors on regiospecific distributions of fatty acids in triacylglycerols. Moreover, the ^{13}C-NMR technique is well suited to studying the composition and quality of marine oils, since it provides information on esterification processes (lipid classes) and other compounds such as cholesterol and additives [4, 18].

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