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ORIGINAL PAPER

Discrimination of Cod Liver Oil According to Wild/Farmed and Geographical Origins by GC and ^{13}C NMR

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Abstract The objective of this study was to test the possibility of using lipid profiles obtained by gas chromatography (GC) and ^{13}C nuclear magnetic resonance (NMR) in authentication of cod liver oils according to wild/farmed and geographical origin. GC and ^{13}C-NMR data of cod liver oil from wild and farmed fish from different locations in Norway and Scotland were obtained, and analyzed by principal component analysis (PCA) and linear discriminant analysis (LDA) to test if it was possible to differentiate oil from wild and cultured cod (*Gadus morhua* L.), and to further elucidate differences between fish from the different farms/catch area. Cod liver oils of wild and farmed origin were clearly separated in the PCA score plot both from GC and NMR data. From NMR data it was also possible to observe groupings based on geographical origin (farm/catch area) of the different samples. Using LDA with cross validation the wild/farmed classification rates were 97% for GC data and 100% for NMR data. In the classification of cod liver oils according to geographical origin (38 samples from six different farms/catch area), the correct classification rate was 63% for GC data and 95% for NMR data.

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Introduction

Cod liver oil is considered health promoting because of the content of the long chained n-3 polyunsaturated fatty acids (PUFAs) eicosapentaenoic acid (20:5n-3) and docosahexaenoic acid (22:6n-3), in addition to vitamin A and D [1]. Raw material for the production of cod liver oil has traditionally been wild-caught cod. However, due to overexploitation of the natural stocks, global catches have declined dramatically, from over 3 million tonnes in 1970 to around 850,000 tonnes during recent years [2]. The increasing amount of farmed cod gives rise to a new source of the health promoting n-3 fatty acids. The production of farmed cod, with its large livers and traditionally higher fat content, could provide a valuable source of raw material for cod liver oil. Since the production of cod liver oil could take place directly after slaughtering, the product is expected to have low quantities of oxidation products and generally better stability (freshness) than traditional cod liver oil. The price and nutritional value of cod liver oils is determined by factors such as: quality of raw material (varies according to wild/farmed and geographical origin); fish species used, and processing history from extraction to processing and storage.

From a nutritional point of view, oil from cultured fish, should be similar to the corresponding oil from the wild counterpart, especially with regards to the content of health promoting n-3 PUFAs 20:5n-3 and 22:6n-3. In order to ensure the quality of medicinal cod liver oil, specific monographs have been defined [3]. In addition to fatty acid

composition, which alone would be easy to manipulate, this monograph contains specification of positional distribution of fatty acids in triacylglycerols, in addition to several other quality tests.

There are methods for general quality control to map the safety (contents of toxins etc.) and nutritional value of marine oils, however food authorities have expressed a need for suitable standard methods to verify data such as the species used, geographical origin and process history of these kinds of products. New EC regulations (Commission Regulation 1662/2006) bring fish oils within the scope of the fishery products definition, adding new requirements on fishery products under Regulation 853/2004. The new regulations require that "raw material used for the preparation of fish oil for human consumption must derive from fishery products deemed fit for human consumption, be prepared in an approved establishment or vessel and transported and stored in a hygienic condition". This implies that there will be an increasing focus on traceability and authenticity of fish oils.

Fatty acid composition is most commonly determined by gas chromatography (GC). Chemometric treatment of GC data on fatty acid composition of various fish organs and tissues has previously allowed discrimination between wild and farmed fish [4, 5] between two different species of redfish [6] and two stocks of cod reared under identical conditions [7]. However, this technique requires pretreatment of the lipid sample. An alternative to GC is ^{13}C nuclear magnetic resonance (NMR) spectroscopy which is a non-destructive technique in the analysis of lipids. Another advantage of ^{13}C-NMR spectroscopy is that it provides multi-component information. In addition to fatty acid composition of fish [8], the techniques render information about lipid classes [9, 10], the positional distribution of PUFAs in triacylglycerols and phospholipids [9, 11, 12], and content of other compounds such as cholesterol and wax esters [13]. The technique has also been shown to contain information about the process history of marine oils, i.e., extraction, processing and storage [14, 15]. Recently, ^{13}C- and ^{1}H-NMR spectroscopy has been used to detect and identify a wide range of compounds in encapsuled cod liver oil, and natural sources of cod liver oils could be distinguished from those subjected to chemical modifications [15].

^{13}C NMR gives a fingerprint of the sample analyzed. Since ^{13}C-NMR spectra contain a large amount of information about the lipid profile, the resulting spectra may become quite complex, consisting of many hundred peaks. Due to this complexity, various multivariate methods are frequently applied to study differences among NMR spectra [16, 17]. Aursand et al. [17] used the ^{13}C-NMR technique (in conjunction with chemometrics) to classify salmon oil according to wild/farmed. Commercial fish oil has been classified according to nature/composition and refinement of the products [14]. However, to our knowledge, no one has applied the ^{13}C-NMR technique to classify cod liver oils according to wild/farmed and geographical origin of the raw material.

The aim of this study was to demonstrate the potential of lipid profiles to distinguish cod liver oil of wild and farmed fish and to discriminate among cod liver oil of different geographical origins (farms/catch area). Results from the standard technique in the analysis of fatty acid composition, GC, were compared with the non-destructive technique ^{13}C NMR.

Materials and Methods

Materials

Wild cod were obtained from Scotland (group SW, $n = 5$) and Norway (group NW, $n = 8$). Farmed cod were obtained from two different Scottish sea farms (group SF1, $n = 5$ and SF2, $n = 5$) and from a Norwegian sea farm on two different years (group NF1, $n = 10$ and group NF2, $n = 5$). In total, 38 fish were analyzed, a sufficient number to establish feasibility of classification.

Lipid Extraction

Lipid was extracted from cod liver according to the method of Bligh and Dyer [18]. Before analyzing the lipid extract by NMR, parts of the chloroform phase were removed by evaporation.

Gas chromatography

The lipids were first transesterified with boron trifluoride-methanol and 0.5 M methanolic sodium hydroxide, and then the fatty acid methyl esters (FAMEs) were extracted into hexane (AOCS Method CE 2-66). An internal standard 21:0 methyl ester was added to the extracted sample prior methylation. FAMEs were analysed on a Fison 8160 (Fisons Instruments S.pA. Milan, Italy) capillary gas chromatograph equipped with capillary cold on-column injector, a fused silica capillary column, Omegawax 320 (30 m, 0.32 mm id, 0.25 μm film thickness; Supelco Inc, Bellefonte, PA, USA) connected to a flame ionisation detector (FID). The FID was connected to a computer implemented with Chrom-card for Windows 1.21 software. The gas chromatograph was provided with an AS800 autosampler. The oven temperature was increased from 80 to 180 °C at 25 °C min^{-1} and held for 2 min. Then the temperature of oven was increased by 2.5 °C min^{-1} to

205 °C (held for 8 min) and up to 215 °C min^{-1} and held for 3 min. The temperature of the detector was 250 °C. Hydrogen was used as the carrier gas at a flow rate of 1.6 ml min^{-1}. Free fatty acid methyl esters were identified by the comparison of their retention times with those of a reference solution (Nu-Chek-Prep, Elysian, MN, USA) chromatographed under identical GC conditions. Analyses were performed in duplicate. Fatty acid compositions are presented as the percentage of total fatty acids. About 26 fatty acids were quantified, the 15 most abundant fatty acids are shown in Table 1.

^{13}C Nuclear Magnetic Resonance

Approximately 70 mg of the oil samples were transferred to 5-mm NMR tubes and diluted with 0.5 ml CDCl3. The spectra were obtained at 297 K on a Bruker AM500 spectrometer, operating at 125.25 MHz for carbons. A pulse program with inverse-gated decoupling of protons and a pulse angle of 30° were applied, 512 free induction decays of an accumulated spectral width of 25 kHz were collected into 10,1006 data points. The 1D ^{13}C spectra were run in a semi-quantitative manner, due to the fact that quantitative measurements require a significantly longer experimental time. The acquisition time was 2 s, resulting in a total repetition time of 4.5 s. The ^{13}C spectra were obtained by Fourier transformation of the resulting FID after applying an exponential line broadening function of 0.1 Hz. Zero filling to 26,2144 points, phasing and baseline correction were applied. The chemical shift scale is referred indirectly to TMS by the triplet of CDCl3 at 77.00 ppm. Maximum peak height (except for the solvent peak) was set to 100 for each spectrum. Peak positions and intensities were obtained for resonances greater than 1% of the maximum peak intensity within each spectrum. The resulting peak list was exported for manual alignment (necessary because of small variations in chemical shift between samples) and multivariate data analyses. The resulting data matrix consisted of 123 variables for the 38 samples investigated. The aligned chemical shift intensities or principal component scores were used as input for the multivariate data analysis.

Multivariate Analysis

Principal component analysis (*PCA*) [19, 20] was applied as an unsupervised multivariate technique. The PCA has frequently been applied to spectral data for dimensionality reduction, to identify outliers and to classify samples. In PCA the original variables are transformed into new, uncorrelated variables called principal components, which retains as much as possible of the information present in

Table 1 Relative amounts (weight % with standard deviations) of fatty acids in cod liver oil of wild fish from Scotland (SW) and Norway (NW), and farmed fish from Scotland (SF1 and SF2) and Norway (NF1 and NF2)

Fatty acid	SW $n = 2$	NW $n = 8$	SF1 $n = 5$	SF2 $n = 5$	NF1 $n = 10$	NF2 $n = 5$
C14:0	4.1 ± 1.4	4.0 ± 0.9	3.5 ± 0.5	4.1 ± 0.6	3.0 ± 0.6	3.6 ± 0.4
C16:0	13.1 ± 0.5	11.2 ± 1.8	11.6 ± 0.9	12.2 ± 0.7	11.3 ± 0.7	13.2 ± 0.6
C16:1n-7	3.0 ± 3.1	6.1 ± 0.7	4.7 ± 2.5	1.8 ± 2.1	5.6 ± 0.9	6.6 ± 0.6
C18:0	4.0 ± 1.1	2.7 ± 1.1	4.4 ± 0.8	3.0 ± 0.6	4.3 ± 0.7	4.5 ± 1.2
C18:1n-9	16.6 ± 3.1	16.7 ± 3.7	19.5 ± 0.8	19.5 ± 0.7	21.5 ± 1.3	19.7 ± 1.4
C18:1n-7	5.1 ± 2.1	4.0 ± 1.3	6.0 ± 0.3	5.0 ± 0.5	5.5 ± 0.4	5.3 ± 0.4
C18:2n-6	1.5 ± 0.5	1.9 ± 0.2	7.4 ± 0.4	6.5 ± 0.4	7.3 ± 0.3	5.8 ± 0.3
C18:3n-3	0.6 ± 0.2	1.4 ± 0.3	1.3 ± 0.1	1.2 ± 0.1	1.2 ± 0.1	0.9 ± 0.1
C18:4n-3	2.2 ± 1.4	2.1 ± 0.6	1.7 ± 0.2	1.7 ± 0.2	1.6 ± 0.3	1.7 ± 0.1
C20:1n-9	8.4 ± 4.2	9.8 ± 3.7	7.1 ± 0.4	10.4 ± 0.7	10.0 ± 1.2	8.3 ± 0.6
C20:4n-6	1.2 ± 0.4	0.8 ± 0.4	0.5 ± 0.1	0.4 ± 0.1	0.3 ± 0.0	0.5 ± 0.1
C20:5n-3	9.7 ± 0.9	7.2 ± 1.3	9.1 ± 0.9	7.5 ± 1.2	8.2 ± 0.9	11.4 ± 0.6
C22:1n-11	7.9 ± 5.4	8.5 ± 3.3	4.1 ± 0.4	7.3 ± 1.1	5.7 ± 1.4	4.1 ± 0.5
C22:5n-3	2.4 ± 2.0	2.0 ± 0.4	2.2 ± 0.3	1.8 ± 0.3	1.1 ± 0.2	1.3 ± 0.1
C22:6n-3	16.7 ± 3.3	18.8 ± 2.4	14.0 ± 1.3	14.8 ± 2.2	11.1 ± 2.0	10.9 ± 0.4
∑SFA	21.3 ± 1.0	18.0 ± 1.9	19.6 ± 0.7	19.4 ± 1.0	18.7 ± 0.7	21.4 ± 0.9
∑MUFA	42.7 ± 3.6	46.3 ± 2.8	42.5 ± 2.1	45.2 ± 1.5	49.4 ± 2.3	45.0 ± 0.7
∑PUFA	35.7 ± 3.9	35.7 ± 3.1	37.6 ± 1.7	35.4 ± 1.3	31.9 ± 2.6	33.7 ± 1.6
∑n-3	32.4 ± 3.4	32.5 ± 3.1	29.1 ± 1.7	28.0 ± 1.7	23.9 ± 2.5	26.9 ± 1.3
∑n-6	2.9 ± 0.9	2.8 ± 0.4	8.2 ± 0.3	7.1 ± 0.5	7.7 ± 0.3	6.6 ± 0.3
∑n-3/∑n-6	11.6 ± 2.6	11.7 ± 2.2	3.6 ± 0.2	4.0 ± 0.5	3.1 ± 0.3	4.1 ± 0.0

SFA saturated fatty acids, *MUFA* monounsaturated fatty acids, *PUFA* polyunsaturated fatty acids

the original data. Each principal component (PC) is a linear combination of the original variables. The scores of a subset of the principal components, can be used in subsequent multivariate analysis.

PCA on GC data was carried out (Unscrambler, 7.8, Camo) on the fatty acids with content over 1% of total fatty acids (15 variables) (mean-centered data). In the analysis of NMR data, the autoscaled data matrix of the 123 chemical shift intensities was used as input in the PCA analysis.

Variable selection. In the PCA of ^{13}C-NMR data, 123 chemical shifts were used as variables, and to find the most discriminating chemical shifts, variable selection was performed using the Partial Least Squares-Genetic Algorithm (PLS-GA). The top 10 most important chemical shifts were determined. PLS-GA has previously been successfully used to select the most informative variables [21].

Linear discriminant analysis (*LDA*) is a supervised pattern recognition technique, which is based on the assumption that samples of the same group are more similar than samples belonging to different groups. The technique seeks to find a linear transformation by maximizing the between-class variance and minimizing the within-class variance. Linear discriminant analysis has been widely used for pattern recognition and data analysis. In situations where the number of variables exceeds the number of objects, the principal components can be used as variables in the linear discriminant analysis [22]. In the LDA (Minitab, version 14), the sample scores on principal components (PC 1-3 for GC data and PC 1-2 for NMR data) in the previously mentioned PCA were used as input variables. Two analyses were performed for each dataset, one with samples assigned group 0 or 1 according to wild/farmed, and the second with sample grouping 1–6 according to geographical origin (SW, NW, SF1, SF2, NF1, NF2). Cross validation (CV) was used as validation method when LDA models were developed. In the leave one out (LOO) CV each sample is removed one at a time, the classification function is recalculated using the remaining data, and the omitted observation is classified.

Results and Discussion

Fatty Acid Composition by GC

Values (percentage of total fatty acids) of the 15 main fatty acids (level >1% of total fatty acids) observed in the cod liver oils are given in Table 1. As expected, the fatty acid composition varied both between and within the different groups of fish. The fatty acid composition of fish is influenced by a wide range of factors including: diet, season, size, age, stage of sexual maturity and according to genetic differences among species/stocks [6, 7, 23]. Cod are omnivorous, feeding on a variety of fishes (e.g. capelin, sprat, herring, other cod), crustaceans, crabs and clams. Feed used in cod farming, may contain raw material both from marine and vegetable origin. Typically fish meal and fish oil made from off-cuts of fish used for human consumption are used in the feed pellets, but also vegetable meal and oil (e.g. from soybean or rapeseed) can be used, in addition to cereal binders.

The higher level of 18:2n-6 in farmed cod in this study is in accordance with previous studies, showing a higher level of this fatty acid in farmed fish [24, 25]. Generally, cereal binders and vegetable oil used in fish feed has a higher level of 18:2n-6 and 18:1n-9 than fish oil, which makes these fatty acids promising candidates for biomarkers of farmed fish [26].

To ensure the quality standards requirements for medicinal cod liver oil, the European pharmacopoeia has specified monographs on cod liver oil [3] and there is a new monograph being introduced for farmed cod (*Gadus morhua*) [27]. The monograph on cod liver oils includes specification of composition of fatty acids. For cod liver oil of farmed cod, 18:2n-6 should be in the range from 3 to 11%, and for traditional cod liver oil, the range is 0.5–3%. The values of 18:2n-6 in this study are within the range specified by the European pharmacopoeia for farmed cod liver. Also when it comes to the health promoting PUFAS, all groups have levels of 22:6n-3 and 20:5n-3 which are in accordance with the levels specified in the European monograph (6–18 and 7–16%, respectively). However, the content of 22:6n-3 is slightly higher in wild than in farmed cod. Cod liver oil of wild origin in this study has thereby a higher n-3/n-6 ratio than the oil from farmed cod. A higher n-3/n-6 ratio in human diet is beneficial in reducing the risk of many chronic diseases, such as cardiovascular disease, cancer, and inflammatory and autoimmune diseases [28].

Classification Results

The GC and NMR data were analyzed by the multivariate methods PCA and LDA to observe groupings, to test if it was possible to differentiate wild from cultured cod, and to further elucidate differences between fish from the different geographical origins (farms/catch area).

GC: Unsupervised Classification Method PCA

Principal component analysis was performed as an unsupervised classification technique. Samples with similar lipid profiles will be closely located in the score plot. Principal component analysis, using the content of 15 fatty acids as variables, resulted in complete separation between

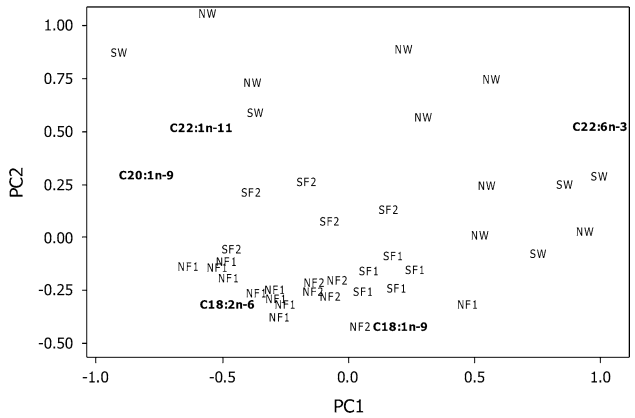

Fig. 1 PCA plot from GC data on the six groups of fish: wild fish from Scotland (SW) and Norway (NW), and farmed fish from farms in Scotland (SF1 and SF2) and Norway (NF1 and NF2). The GC values on fatty acids with a level over 1% of total fatty acid content were used in the analysis (mean-centered data), the position of the five most important variables is shown in the figure. The two first principal components explained 47 and 27% of the variance in the dataset

cod liver oils of wild and farmed origin (Fig. 1). Samples from wild fish are mainly positioned in the two right quadrants, while cultured fish appear primarily in the two left quadrants of the PCA plot. The two first principal components explained 47 and 27% of the variance in the dataset.

There are no clear groupings of the fish of different origins (i.e. Norwegian and Scottish), but samples of the same farm are positioned close to each other (i.e. SF1). The distribution of samples is dominated by the level of five fatty acids, namely: 18:2n-6, 22:6n-3, 22:1n-11, 20:1n-9 and 18:1n-9.

The PCA score plot shows the same result as found in Table 1, the level of 22:6n-3 is higher in wild fish, while the levels of 18:2n-6 is lower in wild fish compared to farmed fish. The wild fish show relative high variations in the content of fatty acids, as can be seen from the wide spread of samples of wild fish (SW and NW). The cultured fish, on the other hand, have a more uniform fatty acid composition within each group, which is not surprising, due to the formulated feed.

NMR: Unsupervised Classification Method PCA

A typical ^{13}C-NMR spectrum of cod liver oil is shown in Fig. 2 (farmed cod). ^{13}C NMR gives in addition to fatty acid composition, information on lipid classes and positional distribution of triacylglycerols. Cod liver oil consist primarily of triacylglycerols, and the different carbon atoms (methyl, methylene, glyceryl, olefinic and carbonyl-) give signals in different regions of the spectrum the olefinic and methyl region are enlarged in Fig. 2.

The PCA was performed on the ^{13}C-NMR data (123 chemical shift intensities). The score plot of PC1 versus PC2 is shown in Fig. 3. The first two principal components account for 42% of the total variance in the dataset. Clear separation both between wild and farmed fish and between the different farms/catch area can be observed. Each group forms a separate cluster in the score plot, even though the groups SF1 and SF2 are closely distributed. The clustering result was obtained from the autoscaled data matrix, and many peaks contributed to the classification. To find the most discriminating chemical shifts, variable selection was performed using the Partial Least Squares-Genetic Algorithm (PLS-GA). The ten most important variables (chemical shifts) were determined to be (in decreasing order of importance) 31.48, 22.53, 127.86, 29.14, 129.66, 27.13, 29.08, 128.11, 29.672 and 14.05 ppm. These peaks, highlighted in Fig. 4, are all in the olefinic and methyl/

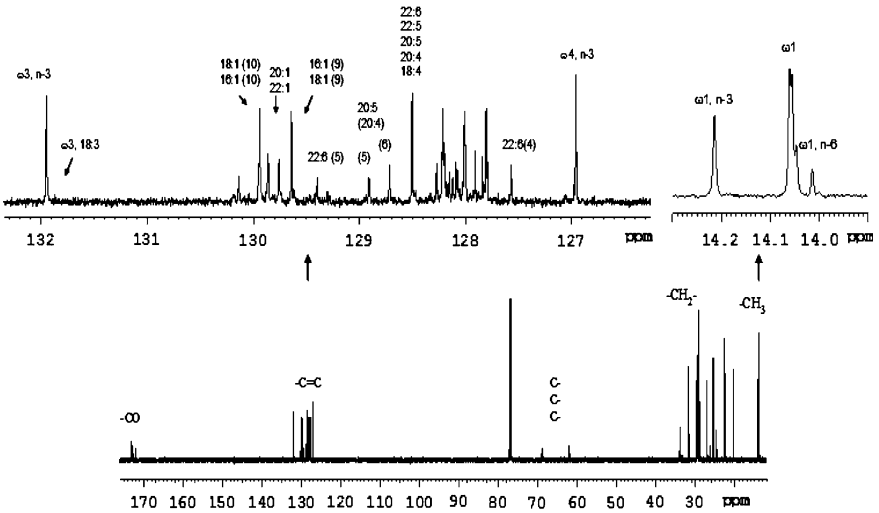

Fig. 2 Typical ^{13}C-NMR spectrum of cod liver oil (farmed cod) illustrating the different regions. The olefinic- and methyl region are enlarged and the main resonances assigned according to Aursand and Grasdalen [8]. ω carbon number from the methyl end

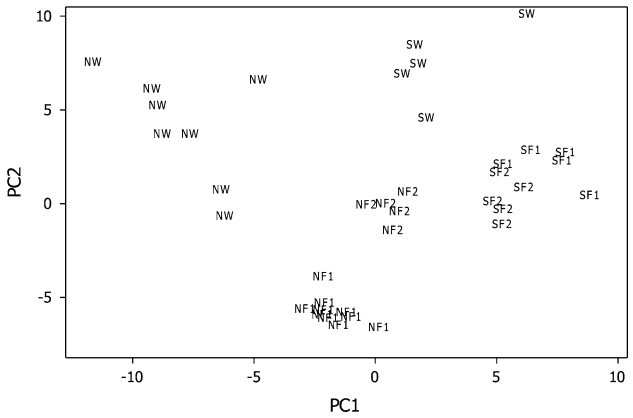

Fig. 3 PCA plot from ^{13}C-NMR data on the six groups of fish: wild fish from Scotland (SW) and Norway (NW), and farmed fish from farms in Scotland (SF1 and SF2) and Norway (NF1 and NF2). 123 chemical shift intensities were used as variables in the analysis (autoscaled data). The two first principal components explained 23 and 19% of the variance in the dataset

methylene region of the spectra. The top 10 variables were chosen since they give the best compromise between classification accuracy and minimization of overfitting of the data. Some of these peaks could be assigned according to literature values on ^{13}C-chemical shifts of fatty acids in fish lipids [8]. The peak at 31.48 ppm was assigned to the ω3 carbon atom of n-6 FA, while the peak at 22.53 ppm was assigned to the ω2 carbon atom of n-6 FA. Figure 5 illustrates the differences in signal intensity of the most important peak in the wild/farmed classification, the level of this peak at 31.48 ppm, arising from n-6 fatty acids, are higher for farmed than for wild fish, consistent with the GC results.

GC and NMR data: Supervised Classification LDA

For a more quantitative result on the discrimination power of the two techniques GC and ^{13}C NMR, LDA was performed. Both the ability to separate cod liver oil of wild from farmed fish, and to discriminate the six different groups of fish (SW, NW, SF1, SF2, NF1, NF2) were investigated. Results from LDA with cross validation as validation method are given in Table 2. Wild versus farmed classifications resulted in 100% correct classification (38/38) for NMR data (two first principal components as variables). Results from GC data gave one misclassified sample and a correct classification rate of 97% (37/38) for wild/farmed classifications (three first principal components, explaining 47, 27, and 10 % of the variance respectively, used as variables). In the classification of cod liver oils according to the origin (38 samples from six different farms/catch areas), the correct classification rate was 63% for GC data and 95% for NMR data.

These results demonstrate the potential of lipid profiling by GC and ^{13}C NMR as authentication methods of cod liver oils according to wild/farmed origin. When it comes to the geographical origin (farm/catch area), ^{13}C NMR seems better able to separate oils of different groups in this study. Compared to GC, ^{13}C NMR is a less sensitive technique, however ^{13}C NMR can be applied nondestructively and without extensive sample manipulation. In addition, ^{13}C NMR provides a more complete picture of the lipid profile than GC, since the positional distribution of fatty acids in triacylglycerols and information about lipid classes can be obtained. The technique is suited to detect if chemical modifications have been made to the natural oil (i.e addition of diacylglycerols or ethyl-esters) [14, 15] and to study deterioration and oxidation [29]. The future goal is to

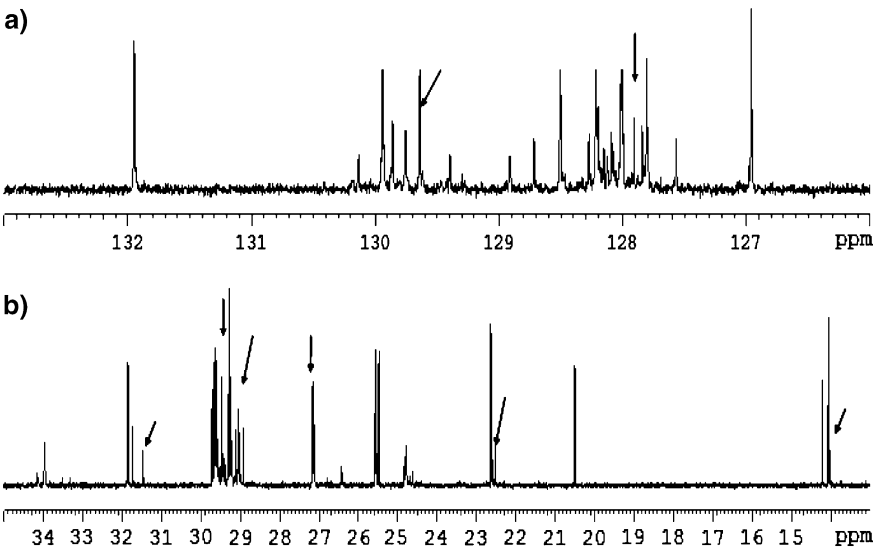

Fig. 4 **a–b** The ^{13}C-NMR spectrum of cod liver oil (farmed cod) with the most important variables in the wild/farmed classification highlighted, as determined by PLS-GA variable selection method

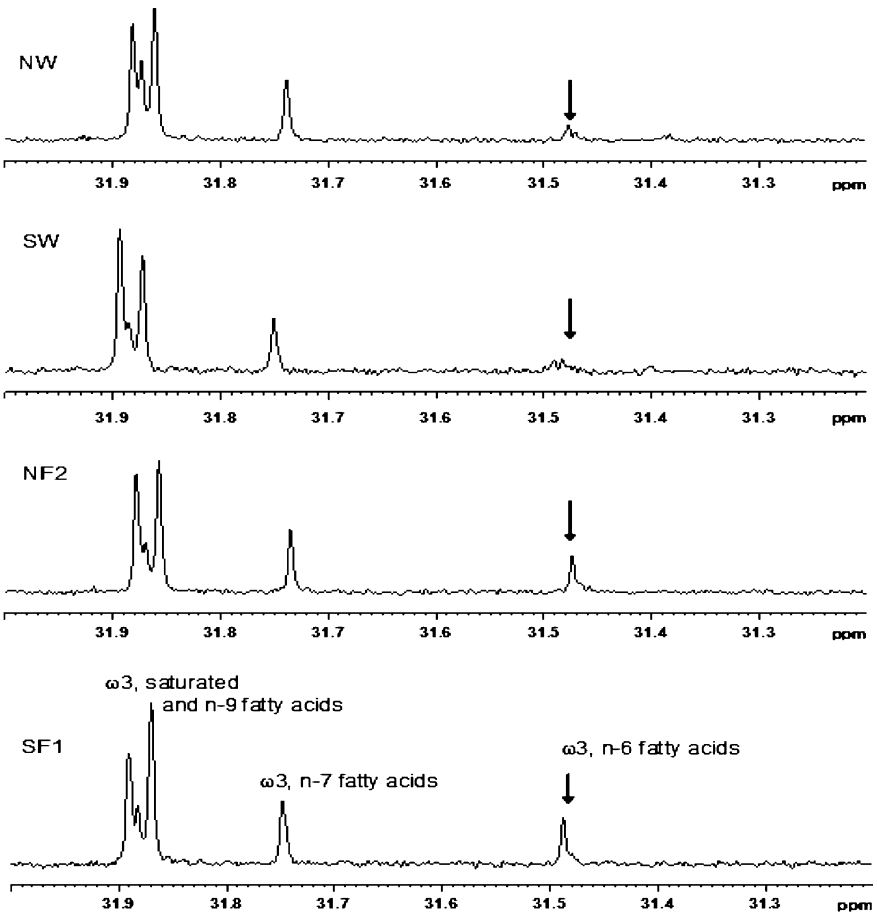

Fig. 5 The ^{13}C-NMR spectra of cod liver oils extracted from wild fish from Scotland (SW) and Norway (NW), and farmed fish from farms in Norway (NF2) and Scotland (SF1). The peak at 31.48 ppm, assigned to ω3 carbon atoms of n-6 fatty acids, was determined to be the most important variable in wild/farmed classification of cod liver oils. ω carbon number from the methyl end

Table 2 LDA classification results according to wild/farmed and geographical origin of the six groups of cod liver oils (wild fish from Scotland (SW) and Norway (NW), and farmed fish from farms in Scotland (SF1 and SF2) and Norway (NF1 and NF2))

Dataset used in the classification	W/F classification	Geogr. origin (group)
GC	97% (37/38)	63% (24/38)
NMR	100% (38/38)	95% (36/38)

establish robust databases with authentic material, so that unknown samples can be statistically treated and grouped according to origin (species, production method, geographical origin). The result, a reliable authentication method of marine oils, would be important to secure correct labeling, to discourage commercial fraud and to prevent illegal capture.

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