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Analytical Methods

^{13}C NMR as a tool for authentication of different gadoid fish species with emphasis on phospholipid profiles

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ABSTRACT

The aim of this study was to evaluate if phospholipid profiles obtained by ^{13}C nuclear magnetic resonance (NMR) spectroscopy is characteristic enough to separate species of lean gadoid fish. ^{13}C NMR data were obtained from muscle lipids of five categories of lean gadoid fish, namely, north-east arctic cod and Norwegian coastal cod (*Gadus morhua*), haddock (*Melanogrammus aeglifinus*), saithe (*Pollachius virens*), and pollack (*P. pollachius*). A total of 27 fish caught at the same location on the Norwegian coast in the traditional fishing season (March/April) in 2006 were analysed. The sn-2 position specificity of 22:6n-3 (docosahexaenoic acid, DHA) in phosphatidyl choline (PC) and phosphatidyl ethanolamine (PE) for the different species/stocks were investigated, and the full ^{13}C NMR spectra applied in multivariate analysis. Stereospecific distribution calculations showed significant differences among species in the distribution of 22:6n-3 in PC and PE, and the pollack group displayed the lowest values for 22:6n-3 in sn-2 position, both in PC and PE. This first screening showed that by using the ^{13}C NMR fingerprint of muscle lipids, linear discriminant analysis gave a correct classification rate of 78% according to the five categories of lean gadoid fish, while successful classification (100%) was achieved with Bayesian belief networks (BBN) predictions.

1. Introduction

Gadoid species, such as Atlantic cod, saithe, haddock and pollack, constitute the most important species in Norwegian fisheries. In 2007 ~550,000 tonnes of these fish species were landed from Norwegian wessels. The catches of Atlantic cod and saithe are approximately similar (220,000 tonnes), but the catch values of Atlantic cod (north-east arctic cod) and saithe is 31% and 10% respectively (Fiskeridirektoratet, 2007). EU directives have introduced labelling regulations which requires that fishery and aquaculture products should be labelled with information such as: species, geographical origin, and production method of fish (i.e. wild/farmed) (EC, 2001). However, differences in quality and price between fish of different species and origin, may lead to falsification and mislabelling, and there is a need for methods able to verify that the traceability information is correct, to protect consumer rights and to prevent illegal capture. Traditional methods for species identification of fish are DNA or protein analyses (Martinez et al., 2003), however it would be convenient to use additional and complementary techniques for processed products.

Analysis of lipids is a potential tool for authentication of fish and marine oils (Aursand, Standal, & Axelson, 2007; Aursand, Standal, Praël, McEvoy, & Axelson, 2009; Hidalgo & Zamora, 2003). Triacylglycerol (TAG) fatty acid composition in muscle of fatty fish, such as salmon, and in livers of gadoid fish, such as cod, reflects the diet (dos Santos, Burkow, & Jobling, 1993; Lie, 1991), which makes it possible to discriminate between wild and farmed fish from lipid analysis (Aursand, Mabon, & Martin, 2000; Aursand et al., 2009; Standal, Praël, McEvoy, Axelson, & Aursand 2008). However, fatty acid composition of fish is a net result of a wide range of factors, including, lipid metabolism, season, age, size and stage of sexual maturity and environmental factors (Sargent, Bell, McEvoy, Tocher, & Estevez, 1999). It has also been shown that certain fatty acids seem to be maintained within limits which depend on the species (Aursand, Jørgensen, & Grasdalen, 1995). Mus-

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cle of the lean marine fish species consists primarily of phospholipids (PLs), and even though polar lipids are affected by diet to some degree (Mørkøre, Netteberg, Johnson, & Pickova, 2007), they are less influenced by diet than TAGs (Lie, Hemre, & Lambertsen 1992; Nanton, Lall, & McNiven, 2001). Therefore, when studying species or stock differences, PL analysis is recommended (Joensen, Steingrund, Fjallstein, & Grahl-Nielsen, 2000). Analyses on eggs from various cod stocks, suggested that fatty acid compositions of PLs were more dependent on stocks then on diet (Pickova, Dutta, Larsson, & Kiessling, 1997). Species of Atlantic tuna have been distinguished from PL–fatty acid profiles of white muscle (Medina, Aubourg, & Pérez Martín, 1997).

The standard analysis of fatty acids is by gas chromatography (GC), but the use of high resolution (HR) ^{13}C nuclear magnetic resonance (NMR) spectroscopy in the analysis of lipids is increasing. ^{13}C NMR is a multicomponent technique, which gives, in addition to fatty acid composition as GC, information on lipid classes and sn-2 position specificity of fatty acids in TAGs, which are characteristic for certain species (Aursand et al., 1995), and PLs (Falch, Størset, & Aursand, 2006). Previous analyses of marine oils showed that one could easily distinguish oils of different species according to their TAG profile obtained by ^{13}C NMR (Aursand et al., 1995; Standal, Axelson, & Aursand, 2009).

^{13}C NMR studies on marine PLs include analysis on cod gonads (Falch, Størseth, & Aursand, 2007; Falch et al., 2006), and tuna muscle PLs (Medina & Sacchi, 1994; Sacchi et al., 1993). However, to the authors' knowledge, ^{13}C NMR studies of muscle lipids extracted from different species and stocks of lean gadoid fish, have not been published previously.

The aim of this study was to evaluate if ^{13}C NMR phospholipid profiles are characteristic enough to discriminate fish species and stocks. For the study five categories of lean gadoid fish were selected, namely: two stocks of Atlantic cod (*Gadus morhua* L.) (north-east arctic cod and Norwegian coastal cod), and the species haddock (*Melanogrammus aeglifinus*), saithe (*Pollachius virens*), and pollack (*P. pollachius*). The ^{13}C NMR spectra were investigated to observe any differences in stereospecific distribution of the fatty acid 22:6n-3 in phosphatidyl choline (PC) and phosphatidyl ethanolamine (PE) among the different groups analysed. The full ^{13}C NMR fingerprints were applied in multivariate analysis to observe groupings, to classify samples according to species/stocks, and to determine important variables in the classification.

2. Materials and methods

2.1. Materials

The following species and stocks of lean fish were caught outside the coast of Vikna, Nord-Trondelag, Norway during March/April 2006 (in the traditional fishing season): north-east arctic cod (AC, $n = 6$) and Norwegian coastal cod (CC, $n = 6$) (*G. morhua L.*), haddock (H, $n = 6$) (*M. aeglefinus*), saithe (S, $n = 5$) (*P. virens*), pollack (P, $n = 4$) (*P. pollachius*). The fish was frozen within 24 h by the fisherman and transported to the laboratory. In total, 27 fish were analysed. The average round weight of the different fish categories (with standard deviation) are north-east arctic cod: 2.9 ± 0.6 kg, coastal cod: 2.2 ± 0.3 kg, haddock: 0.7 ± 0.2 kg, saithe: 2.7 ± 1.2 kg and pollack: 4.3 ± 1.1 kg.

2.2. Lipid extraction

Lipid was extracted from white fish muscle under the back dorsal fin according the Bligh and Dyer method (1959). Before analysing the lipid extract by NMR, parts of the chloroform phase were removed by evaporation.

2.3. ^{13}C NMR

Approximately 90 mg of the oil sample was transferred to 5 mm NMR tubes and diluted with 0.6 mL deuteriated chloroform ($CDCl_3$, 99.8% purity, Isotec Inc., Matheson). The ^{13}C NMR spectra were run semi-quantitatively with a high number of scans, to achieve sufficient signal to noise (S/N) ratio to evaluate the stereospecific distribution of fatty acids in PLs. Comparisons between intensities of resonances within the same spectra should be performed with care in semi-quantitative ^{13}C NMR spectra, however, comparison of signals among the different spectra is possible. Previous studies have shown that a semi-quantitative approach can be applied in positional distribution measurements, since the nuclear overhauser effect (NOE) effect is similar for carbonyl-carbons and that the T1 values of these carbons does not vary much according to the position of the fatty acids in the glycerol molecule (Aursand et al., 1995; Wollenberg, 1990). ^{13}C NMR spectra were obtained on a Bruker Avance DMX 600 instrument (Bruker BioSpin GmbH, Rheinstetten, Germany) operating at 150.9 MHz for carbons, using a 5 mm BBO probe at 298 K. Power gated decoupling was applied. The following acquisition parameters were used: time domain 64k, pulse width 60°, sweep-width 200.8 ppm, acquisition time 1.08 s, relaxation delay 0.5 s, number of scans 16k. Zero filling and exponential line broadening (0.15 Hz) were applied before Fourier transform. The chemical shift scale is referred indirectly to TMS by the triplet of CDCl3 at 77.11 ppm.

2.4. Peak picking of full ^{13}C NMR spectra

Peak positions and intensities were obtained for resonances >0.5% of the maximum peak intensity within each spectrum. The resulting peak list was exported for manual alignment due to small variations in chemical shifts among samples. The solvent resonances were removed, before the data matrix (254 variables for the 27 samples investigated) were exported for multivariate analysis.

2.5. Calculation of the sn-2 position specificity of 22:6n-3 in PC and PE

Peakfitting was applied to the carbonyl region of the ^{13}C NMR spectra, to facilitate integration of peaks arising from 22:6n-3 in sn-1,3 and sn-2 position of PC and PE and to reveal hidden peaks (in particular 22:6n-3 in sn-2 position of PE consisted of more than one peak). The ^{13}C NMR data were processed in xwinnmr as previously described, and the full spectra (1r file) were converted into ASCII files. Data in the carbonyl region (174–172 ppm) were selected (681 data points) and imported to PeakFit 4.12 (SeaSolve Software Inc, San Jose, CA). The AutoFit Residuals method was chosen as peak-fitting method. This procedure initially places peaks by finding local maxima in a smoothed data stream. Hidden peaks are than optionally added where peaks in the residuals occur (Peakfit 4.12). Peaks were fitted, assuming Lorentzian lineshape, with a linear baseline substracted prior to fitting. The area of the peaks arising from sn-1 and sn-2 22:6n-3 in PC and PE were registered, and the relative distribution of 22:6n-3 in sn-2 position calculated. Comparisons between means for the five fish categories were performed by one-way analysis of variance (ANOVA) with a significance level of 0.01 (Excel, Microsoft XP).

2.6. Multivariate analysis

The ^{13}C NMR data were analysed by the multivariate methods principal component analysis (PCA) (Jolliffe, 1986; Wold, Esbensen, & Geladi, 1987), linear discriminant analysis (LDA) and Bayesian belief network (BBN) (Heckerman, Geiger, & Chickering, 1995; Pearl, 1988) to observe groupings and to test if it is possible to