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Use of NMR spectroscopy in combination with pattern recognition techniques for elucidation of origin and adulteration of foodstuffs

Thesis for the degree of Philosophiae Doctor

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Summary

Consumers and food authorities are, to an increasing extent, concerned about factors such as the origin of food, how it is produced, and if it is healthy and safe. There are methods for general quality control to map the safety and nutritional value; however there is a need for suitable analytical methods to verify information such as the production method (wild/farmed), geographical origin, species, and process history of foods.

This thesis evaluates the applicability of using nuclear magnetic resonance (NMR) spectroscopy combined with pattern recognition techniques for authentication of foodstuffs. Fish and marine oils were chosen as materials. ^{13}C NMR was applied to authenticate marine oils and muscle lipids of both fatty and lean fish, according to production method (wild/farmed), geographical origin, species, and process history. ^{1}H NMR was applied on low molecular weight compounds extracted from cod muscle to authenticate fish according to species and processing conditions.

^{13}C NMR combined with pattern recognition techniques enabled the differentiation of marine oils according to wild/farmed and geographical origin of the raw material. It is suggested that this was mainly due to the different diets of the fish from which the oil was produced. It was also possible to authenticate marine oils according to species, and to say something about the level of mixtures detectable. The sn-2 position specificity of fatty acids in triacylglycerols was shown to be an important characteristic to separate oils of different species. Esterified fish oil (concentrates) could easily be differentiated from natural fish oil by their ^{13}C NMR profile.

^{13}C NMR on muscle lipids, combined with pattern recognition techniques enabled the classification of wild and farmed salmon. The classification according to geographical origin was somewhat more complicated. A combination of analytical methods may be the best approach to obtain reliable results on geographical origin of fish. The analysis of lean fish showed that it was possible to classify lean gadoids according to species, and two stocks of Atlantic cod could be differentiated. There were also minor differences in the sn-2 position specificity of 22:6n-3 in phosphatidylcholine (PC) and phosphatidylethanolamine (PE) among the species investigated.

^{1}H NMR on water soluble extracts of fish muscle provided information about a wide range of compounds, and the two species investigated (cod and haddock) displayed different ^{1}H NMR profiles. Dimethylamine (DMA) was used as a marker for frozen, non processed fish. When applying the ^{1}H NMR data in pattern recognition techniques, frozen fish could be differentiated from non-frozen fish, and in the classification of the cod of the different processing methods 80% of the samples were correctly classified.

Common for the methods presented in this thesis, NMR spectroscopy combined with pattern recognition for authentication of traceability data on foodstuffs, is the need for databases with analytical data on reference samples, covering the natural variation among the samples to be classified. Databases for marine oils need not be as extensive as for fish, since marine oils are generally produced from fish batches. Oils can also be analyzed directly without extensive sample preparation, and the greatest potential for official application may lie in the analysis of oils.

Table of Contents

Abbreviations

HR	High resolution
NMR	Nuclear magnetic resonance
NOE	Nuclear Overhauser effect
COSY	Correlated spectroscopy
LC	Liquid chromatography
MS	Mass spectrometry
GC	Gas chromatography
TMAO	Trimethylamine-N-oxide
TAG	Triacylglycerol
FA	Fatty acids
LC- PUFAs	Long chain polyunsaturated fatty acids
DUFAs	Diunsaturated fatty acids
MUFA	Monounsaturated fatty acids
SFA	Saturated fatty acids
DHA	Docosahexaenoic acid
EPA	Eicosapentaenoic
PL	Phospholipids
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PCA	Principal component analysis
LDA	Linear discriminant analysis
PLS-GA	Partial least squares-genetic algorithm
NN	Neural networks
PNN	Probabilistic neural networks
KNN	Kohonen neural networks
GTM	Generative topographic mapping
GRNN	General regression neural networks
SVM	Support vector machines
BBN	Bayesian belief networks
LOO CV	Leave one out cross-validation
TMS	Tetramethylsilane
TSP	Trimethylsilyl propionate

List of papers

^{13}C NMR studies on marine oils

Paper I Standal IB, Prael A, McEvoy L, Axelson DE, Aursand M. (2008) Discrimination of cod liver oil according to wild/farmed and geographical origins by GC and C-13 NMR. *Journal of the American Oil Chemists´ Society* 85:105-112.

Paper II Aursand M, Standal IB, Axelson DE. (2007) High-resolution C-13 nuclear magnetic resonance spectroscopy pattern recognition of fish oil capsules. *Journal of Agricultural and Food Chemistry* 55:38-47.

Paper III Standal IB, Axelson DE, Aursand M. (2009) Differentiation of fish oils according to species by ^{13}C NMR regiospecific analyses of triacylglycerols. *Journal of the American Oil Chemists´ Society* 86:401-407.

^{13}C NMR studies on fish muscle lipids

Paper IV Aursand M, Standal IB, Praël A, McEvoy L, Axelson DE. (2009) ^{13}C-NMR pattern recognition techniques for the classification of Atlantic salmon (*Salmo salar* L.) according to their wild, farmed and geographical origin. *Journal of Agricultural and Food Chemistry* 57: 3444-3451.

Paper V Standal IB, Axelson DE, Aursand M. ^{13}C NMR as a tool for origin testing of different cod species with emphasis on phospholipid profiles. *Revised version resubmitted Food Chemistry March 2009*.

^{1}H NMR studies on low molecular weight metabolites in fish muscle

Paper VI Standal IB, Gribbestad IS, Bathen TF, Aursand M, Martinez I. (2007) Low molecular weight metabolites in white muscle from cod (*Gadus morhua*) and haddock (*Melanogrammus aeglefinus*) analyzed by high resolution H-1 NMR spectroscopy. Magnetic Resonance in Food Science: from Molecules to Man 55-62.

Paper VII Martinez I, Bathen T, Standal IB, Halvorsen J, Aursand M, Gribbestad IS, Axelson DE. (2005) Bioactive compounds in cod (*Gadus morhua*) products and suitability of H-1 NMR metabolite profiling for classification of the products using multivariate data analyses. *Journal of Agricultural and Food Chemistry* 53:6889-6895.

Outline of the thesis

The first chapter of this thesis gives a short background to the need for methods to authenticate food, and gives an introduction to relevant methods to identify traceability information such as species, production method, geographical origin and process history of fish and marine oils. The emphasis in this chapter is on nuclear magnetic resonance (NMR) spectroscopy and its potential for authentication of fish and marine oils.

Chapter 2 presents the main objective, which was to evaluate NMR spectroscopy in combination with pattern recognition techniques as a method for elucidation of origin and adulteration of foodstuffs. The work was divided into three different studies, namely studies on 1) marine oils, 2) fish muscle lipids and 3) on low molecular weight metabolites in fish muscle. Chapter 3 gives a description of the materials and methods chosen in each study, while the results are presented and discussed in Chapter 4. Chapter 5 contains the conclusions drawn from this work, while some suggestions for further studies are given in Chapter 6.

1 Background

1.1 Traceability and authentication

Product declarations have mainly been focused on nutritional value, and authority control mainly limited to food safety. However, consumers are in increasing numbers concerned about factors such as the origin of food, how it is produced, and if it is healthy and safe. Food scandals have seriously undermined consumer confidence, and in recent years there has been an increasing focus on traceability in the food chain, both among authorities and consumers [www.trace.org].

Traceability is by definition "the ability to trace and follow a food, feed, food-producing animal or substance intended to be, or expected to be incorporated into a food or feed, through all stages of production, processing and distribution" [EC regulation 178/2002]. For fish and aquaculture products, recent EU directives require that fishery and aquaculture products should be labeled with information such as: 1) species, 2) geographical origin, and 3) production method of fish (i.e. wild/farmed) [EC regulation 2065/2001].

The large price differentials between farmed and wild fish and between different fish species (and stocks) make mislabeling and fraud profitable, and labeling information is not always correct. An example is the result of a survey by The Food Standards Agency (FSA, UK) in 2007 that investigated the extent to which fish described as "wild" were actually wild. 128 fish purchased from supermarkets, fishmongers, fish auctions, and special food shops were analyzed. The results showed that several samples were mislabeled; approximately 10% of the wild sea bass were found to be farmed, 11% for sea bream and 15% of the salmon [FSA, 2007].

To confirm the traceability data of products and to detect frauds, methods are necessary to determine such factors. There are several methods suitable for species identification in seafood, but traditional methods, 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 [Martinez et al., 2003]. At present, no official methods exist for unequivocal determination of the wild/farmed- and the geographical origin of fish, or to determine the process history of fish products (e.g. if the fish have been frozen/thawed).

Also when it comes to marine oils, there is a need for methods to verify composition and authenticity. The demand for fish oil, rich in the health beneficial (and valuable) long-chain n-3 polyunsaturated fatty acids (LC-PUFAs) docosahexaenoic acid (DHA, 22:6n-3) and eicosapentaenoic acid (EPA, 20:5n-3), is increasing, while the production of such oils has been rather constant the last years [Tacon et al., 2006; 2008]. Differences in price between oils of different origins and qualities, may lead to mislabelling and adulteration [Fantoni et al., 1996]. The demand for product specification and label requirements on marine oils varies depending upon whether the product is meant for human consumption or feed, if it is

a health food, a food ingredient, a remedy or an ingredient in a remedy. In general, precise directives for fish oil products on the health food market are lacking, but focus on traceability and authentication is increasing.

Reliable methods able to determine traceability information are important to secure consumers´ rights for correct information, to document information for the industry, to discourage commercial fraud and to prevent illegal captures of endangered/protected stocks.

1.2 Authentication of fish and marine oils

1.2.1 Authenticity issues

Relevant authenticity issues of fish are 1) species, 2) geographical origin and 3) production method (i.e. wild/farmed) [EC regulation 2065/2001]. However, other factors such as the quality, processing conditions, and whether the fish have been frozen and thawed are relevant when it comes to the history of fish [Dahle et al., 1998]. For marine oils, relevant traceability information is; the species used, production method (wild/farmed) and geographical origin of raw material, and the process history (natural fish oil vs. concentrates).

1.2.2 Overview of relevant authentication methods

The most relevant techniques to identify the production method of fish (wild/farmed) and geographical origins of fish are: genetic analysis, analysis of protein/enzyme profiles in some organs/tissues, analysis of fats and oils, stable isotope analysis or trace element signature analyses [Martinez, 2005, Martinez et al., 2008]. For discrimination between wild and farmed fish morphological analyses, individual tagging and carotenoid content (natural vs synthetic) are also relevant methods [Martinez, 2005].

Traditional methods for species authentication of fish are DNA or protein analysis, which may not work for marine oils which contain little or no DNA and proteins. Alternative methods for authentication of fish and fish oils are analysis of metabolite profiles, both of lipids (Chapter 1.2.3) and water soluble low molecular weight metabolites (Chapter 1.2.4). The most relevant “counterpart” to the methods used in this work (metabolite profiles) is isotopic analysis (see Chapter 1.2.5).

1.2.3 Lipid profiles for authentication

Fish lipids consist of both storage lipids (triacylglycerols) and membrane lipids (phospholipids).

The fatty acid composition of fish is the net result of a wide range of factors, including; diet, season, age, size, stage of sexual maturity, lipid metabolism and environmental factors [Sargent, et al., 1999; Dahslgaard et al., 2003] although the mechanisms are not fully understood. There is a potential of using fatty acid composition to study authenticity, as shown by a large number of studies [e.g. Aursand et al., 2000; Tritt et al., 2005; Busetto et al., 2008]. Lipid profiles have been shown to differ both between wild and farmed fish, geographical origins and among species/stocks;

Wild/Farmed

Since the commercial feed is generally very different from natural diet, the fat content and lipid composition of fish muscle have been used as criteria to differentiate farmed from wild fish [Aursand et al., 2000; Busetto et al., 2008]. Farmed fish usually have a higher fat content than wild fish [Martinez, 2008]. In addition, since the fatty acid profile of triglycerides reflects the diet [dos Santos et al., 1993; Sargent et al., 2002], the fatty acid composition (from Gas Chromatography, GC) or lipid profile (from ^{1}H- and ^{13}C- Nuclear magnetic resonance, NMR) may be used to discriminate wild from farmed fish [Aursand and Axelson, 2001; Tritt et al., 2005; Masoum et al. 2007; Mannina et al., 2008].

Geographical origin

Lipid composition of farmed fish can vary greatly among farms according to farming environment, feed quality, feed management and the culture methods implemented (e.g. feed rate) [Shearer et al, 2001; Sargent et al., 2002; Turchini et al., 2009]. Several studies have used lipid profiles to distinguish among fish of different farms [Molkentin et al., 2007; Martinez et al., 2009; Turchini et al., 2009]. It has also been shown that fatty acid composition differs among wild fish of different origins according to the available feed [Budge et al., 2002], salinity and temperature in addition to other environmental factors [Armstrong et al., 1994].

Species

Although the fatty acid composition of fish tissues varies according to factors such as season, age, diet, and environmental factors, there are genetically determined differences among species. This was evidenced by a recent study where analyses of several fish species showed that within-species variation was substantially less than among-species variation [Budge et al., 2002]. Both the fatty acid composition in triacylglycerols [Sigurgisladòttir et al., 1993; Budge at al., 2002; Recks et al., 2008,] and in phospholipids [Medina et al., 1995a; 1997; Joensen and Grahl Nielsen, 2001;], and the stereospecific structure of triacylglycerols vary among fish species [Gunstone et al., 1994; Aursand et al., 1995a; Nwosu and Boyd, 1997] and may therefore be used for authentication of species.

Species: stereospecific triacylglycerol structure

Fish oils and lipids of fatty fish are mainly composed of triacylglycerols, which consist of fatty acids esterified to three different stereospecific positions on a glycerol molecule, namely sn-1, sn-2 or sn-3. The stereospecific distribution of fatty acids in triacylglycerols is characteristic, and more or less unique, for different types of natural fat [Aursand et al., 1995a]. Analyses of triacylglycerol structure may therefore be used to study authenticity and adulteration, as has previously been shown for fish oils [Aursand et al., 1995a], milk fat [Andreotti et al., 2002], animal fat [Szabò et al., 2007] and olive oils [Wollenberg, 1990; Sacchi et al., 1992; Vlahov et al., 2006; Vichi et al., 2007]. Traditional methods to determine the distribution of fatty acids in acylglycerols are usually based on laborious and time-consuming chromatographic/enzymatic methods [Buchgraber, 2004]. Recently, various mass spectroscopic- (MS) (such as MALDI-TOF MS, RP-HPLC-MS-MS) [Schiller et al., 2004; Leskinen et al., 2008; Zehethofer and Pinto, 2008] and ^{13}C NMR methods [Sacchi et al., 1993a; Gunstone and Seth, 1994; Aursand et al., 1995a; Vlahov et al., 2006] have been developed that are less labor intensive, and do not require enzymatic treatment of the sample.

The stereospecific distribution describes the composition of fatty acids in sn-1, sn-2 and sn-3 position, while the regiospecific distribution gives the composition in sn-1,3 and sn-2. The European pharmacopoeia on salmon oil (and cod liver oil from farmed cod) includes this regiospecific distribution (sn-2 position specificity) of fatty acids in triacylglycerols by ^{13}C NMR spectroscopy to minimize the risks of blends and adulteration. Regiospecific analyses by ^{13}C NMR is treated more exhaustively in Chapter 1.4.2.

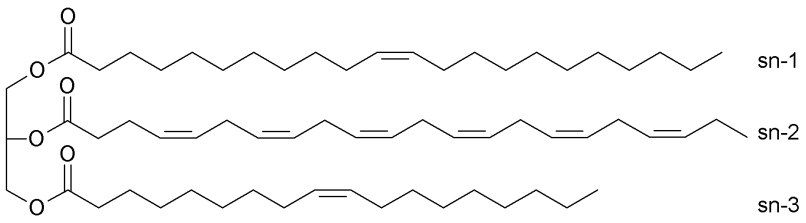

Figure 1. Structure of a triacylglycerol (TAG) molecule; three fatty acids esterified to the stereospecific positions sn1, sn-2 and sn-3.

Species/stocks; phospholipid profiles

Muscle lipids of lean marine fish species consists primarily of phospholipids (PLs), and even though polar lipids are affected by diet to some degree [Morkore et al., 2007], they are

less influenced by diet than triacylglycerols [Lie et al., 1992; Jobling et al., 2008]. Therefore, when studying species or stock differences, phospholipid analysis is recommended [Medina et al., 1997; Joensen et al., 2000].

Fatty acid composition of heart tissue phospholipids has allowed differentiation between species and stocks of the redfish sebastes [Joensen and Grahl-Nielsen, 2001; 2004], and two stocks of cod reared under identical conditions [Joensen et al., 2000]. Analyses on eggs from various cod stocks, suggested that fatty acid compositions of phospholipids were more dependent on stocks then on diet [Pickova et al., 1997]. Species of Atlantic tuna have been distinguished by phospholipid-fatty acid profiles of white muscle [Medina et al., 1997].

Analyses of polar lipids may also be applicable to study the production method of fish. A preliminary study by Mannina et al. (2008) found that the content of cholesterol and phosphatidylethanolamine (PE) differed between wild and farmed fish.

Process history

Marine oils enriched in the health beneficial n-3 fatty acids can be tailored by winterization and molecular distillation, but more commonly chemical- or enzymatic esterification/trans-esterification is applied [Breivik, 2007]. The resulting products often consist of mono- and diacylglycerols, and/or ethyl esters in addition to the intact triacylglycerols. Most of the concentrated products on the market are blends of natural fish oil and their derivatives. By using suitable techniques (e.g. high performance liquid chromatography (HPLC) or ^{13}C NMR) such compositional differences between natural and modified fish oils, may be detected [Siddiquie et al., 2003].

1.2.4 Low-molecular weight metabolite profiles for authentication

In addition to analysis of lipids, the content low molecular weight metabolites in fish tissue are of relevance to study authenticity (and nutritional value) of fish and fish products [Martinez, 2005]. Low molecular weight metabolites in fish tissues include free amino acids, nucleotides and related compounds, peptides, organic bases, sugars and inorganic constituents [Konosu and Yamaguchi, 1982]. In general, the level of these metabolites will vary according to species, season, environmental conditions, diets, parts and tissues of body, freshness, stress and postmortem handling and processing [Konosu and Yamaguchi, 1982]. Classical methods to analyze these compounds are amino acid analyses or by HPLC [Reid et al, 2006], but also NMR [Martinez et al, 2005] is a potential technique as shown in Chapter 1.4.3.

The study of the global profile of low molecular weight metabolites has been extensively used in many research areas, such as toxicology, environmental sciences and clinical disease diagnostics [Lin et al., 2007; Viant et al., 2008]. However, few studies have used this approach for authentication purposes of fish, a short summary of relevant papers are given in the next sections.

Species

Each species and tissue has a particular osmolyte profile that contributes to the species-specific taste [Konosu and Yamaguchi, 1982]. However, to our knowledge, none had used this information for species authentication of fish.

Wild/farmed

Recently, Mannina et al. (2008) used the ^{1}H NMR metabolic profiles of organic and aqueous sea bass extracts to discriminate wild and cultured seabass. The preliminary results in this study show that in addition to fatty acid composition, cholesterol- and phosphatidyl-ethanolamine content, the content of some water-soluble metabolites, such as choline, trimethylamine oxide, glutamine, fumaric and malic acid may be useful to discriminate between wild and farmed fish. The authors emphasize that the NMR technique gives a more complete picture of the metabolic profile than techniques only focusing on specific compounds.

Freezing/thawing

Customers are willing to pay higher prices for fresh fish, and there is an increasing number of incidents of thawed fish mislabeled as fresh. Relevant methods to determine if fish have been frozen are reviewed by Martinez (2005), and include: physical determination (Torrymeter), physiological determination (of fish lenses), enzymatic methods, near-infrared (NIR) spectroscopy, dry extract spectroscopy by infrared refleaction (DESRIR) and Magnetic resonance imaging (MRI). In species of gadoids dimethylamine (DMA) is formed enzymatically upon freezing (fastest between -5 and -10ºC) from the osmolyte trimethylamine-N- oxide (TMAO), and DMA may in this concern be a marker for products that have been frozen. However, it has also been shown that DMA can be formed from TMAO non-enzymatically, e.g. in heat-processed dry fish [Spinelli and Koury, 1979; 1981].

1.2.5 Isotopic analyses for authentication

Isotopic techniques are based on the quantitative determination of hydrogen, carbon, nitrogen and oxygen isotopes at natural abundance levels. The isotopic composition of the constituent of fish and marine oils (proteins, carbohydrates, fats, minerals) depend on various factors. Some of these factors depend on geographical origin, while others are related to production methods (such as composition of the feed, seasonal and geological factors). The two main techniques used to determine the isotope ratios of natural products are isotope ratio mass spectrometry (IRMS) and site-specific natural isotope fractionation studied by nuclear magnetic resonance (SNIF-NMR). By SNIF-NMR the natural abundance of ^{2}H isotopomers may be accurately quantified [Martin and Martin, 1991], whereas IRMS only gives a mean value of the deuterium content of a given chemical

species. SNIF-NMR is the official method adopted by EU for the authentication of wines [EC regulation 2348/91].

Production method and geographical origin

Several studies have used stable isotope analyses to study production method and geographical origin of fish and marine oils. Stable isotope analysis, often in combination with fatty acid analysis, has recently been applied to discriminate wild from farmed salmon [Aursand et al., 2000; Thomas et al., 2008] and turbot [Bell et al., 2007; Busetto et al., 2008], to identify organic farmed salmon [Molkentin, 2007] and to discriminate rainbow trout of different diets [Moreno-Rojas, 2008]. Using this technique, it has also been possible to distinguish amon Atlantic salmon from different sources [Aursand, 2000; Thomas et al., 2008] and to discriminate among intensively farmed freshwater Murray cod from farms in different geographical areas [Turchini et al., 2009]. In the latter study, the combination of three isotopic ratios ($\delta^{13}C$, and $\delta^{15}N$ clearly linked fish to a specific commercial diet, while $\delta^{18}O$ linked fish to a specific water source) was used to discriminate among fish from different farms.

1.3 Nuclear magnetic resonance in general

The following sections will provide an overview of NMR spectroscopy, and is based on Further information can be found in textbooks on the subject [Claridge, 1999; Abraham et al., 1988].

1.3.1 History of NMR

Nuclear magnetic resonance (NMR) was for the first time observed in 1946 by two independent groups [Bloch et al., 1946; Purcell et al., 1946], and for this work they shared the Nobel Prize of physics in 1952. Since then NMR has grown as an analytical method in numerous disciplines (chemistry, biology, medicine, materials science and geology). In 1950 it was discovered that the resonance frequency of a nucleus is influenced by its chemical environment (chemical shift) and that a nucleus could further influence the resonance of another through the phenomenon of chemical bonds. Later it was discovered that the chemical shift (and spin-spin couplings) could be directly related to chemical structure. In the 1970s the signal to noise ratio in NMR was greatly increased through the introduction of Fourier transformation (FT) of the time-domain response resulting in a frequency domain spectrum. This work was pioneered by Richard R. Ernst, who in 1991 received the Nobel Prize of chemistry for his contributions in the development of high resolution nuclear magnetic resonance spectroscopy.

Superconducting magnets and computer controlled instruments appeared in the 1970s and further increased the potential of the method. And with the higher fields provided by the superconducting magnets, the observation of e.g. carbon-13 nuclei were possible. In the

1970s also the multipulse experiments were introduced (2D). As a result of further instrumental development (computing technology advances allowed data manipulation in reasonable amounts of time), an enormous number of pulse techniques has been introduced.

1.3.2 The principles of NMR

The basis of NMR is that nuclei exhibit magnetic properties such as nuclear spin (I) and magnetic moment (μ). Magnetic resonance arises from a nucleus with I≠0 placed in a magnetic field (B_0) if radio frequent energy (rf) is applied to the nucleus (**Figure 2A**). The radio frequency pulse applied in NMR spectroscopy induces transmissions between different energy states. The excited nucleus undergoes relaxation by emission of radiation and this time dependent decay (**Figure 2B)** is acquired and Fourier transformed into a frequency-domain spectrum (**Figure 2C)**.

Various nuclei in the sample (e.g. ^{1}H, ^{13}C and ^{31}P) can be excited depending on the radiofrequency applied. Chemically distinct atoms due to differences in molecular environments will give rise to peaks at different frequencies (chemical shifts). The intensities of peaks in NMR spectra are directly related to the nuclei responsible for them, and make quantification of constituents possible (with suitable experimental parameters).

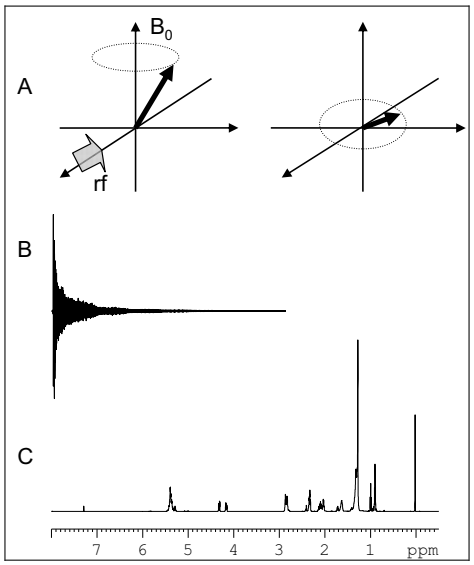

Figure 2. Vector model of population of spinning nuclei in a magnetic field (B_0) with a radio frequency pulse (rf) applied causing a flip of the vector (in this example a 90º flip). The time dependent decay signal (B) is acquired and Fourier transformed into a frequency-domain spectrum (C). The FID and the resulting ^{1}H NMR spectrum is from salmon oil.

1.3.3 Instrumental developments

The first NMR experiments were performed in a magnetic field of 8-30 MHz for protons. Nowadays instruments machines operating at 950 MHz for protons are available. In addition magic angle spinning (MAS) NMR spectroscopy has made the analysis of intact tissue possible. Spinning of the sample at the magic angle, reduces the chemical shift anisotropy contributions to line broadening normally seen because of lack of mobility in tissues. However, large molecules like proteins and lipids appear as broad signals in the MAS NMR spectrum. NMR automation utilizing sample changers and/or flow-injection systems have become valuable. Cryoprobes, with higher sensitivity have been developed, which has allowed for analysis of sample amounts previously considered too small a few years ago [www.bruker.de].

1.4 NMR spectroscopy in the analysis of food

NMR spectroscopy has been shown to be particularly well suited in the profiling of biological material [Nicholson et al., 1995; Fan, 1996]. Since NMR is a non-specific high-throughput analytical method, NMR spectra can act as "fingerprints", which can be used to compare, discriminate or classify samples [Le Gall and Colquhoun, 2003].

Both ^{1}H NMR and ^{13}C NMR are able to detect a range of metabolites in a non-targeted way. The major advantage of ^{1}H NMR spectroscopy compared to ^{13}C NMR is the higher sensitivity and thereby shorter acquisition times per experiment– preferred in metabolic profiling. On the other hand, ^{13}C NMR has a greater range of chemical shifts, which leads to less overlapping of signals, and is the preferred tool in lipid analysis when interpretation of spectra is the goal.

1.4.1 NMR spectroscopy for authentication purposes

Combined with chemometric techniques NMR spectroscopy can be a useful and rapid method to assess food authenticity and it has been applied to a wide range of foodstuffs, including fruit juices [Cuny et al., 2008], olive oil [Sacchi et al., 1998; Brescia et al., 2003a], wines [Brescia, et al., 2003b], and honey [Consonni and Cagliani, 2008; Donarski et al., 2008]

The studies on NMR for authentication of olive oils are numerous. Virgin and extra virgin olive oils are high price products. Some olive oils are entitled to Protected Designation of Origin (PDO) or Protected Geographical Indication (PGI) labels under EC Regulation No 2081/92. However, these oils can easily be adulterated with other high oleic oils, such as refined olive oil, hazelnut and rapeseed oils. High resolution (HR) ^{13}C NMR has been used to discriminate virgin olive oils from several countries from other oils, and of mixtures of other types of oil [Sacchi et al., 1992; Zamora et al., 2001; Brescia et al. 2003a, Rezzi et al., 2005]. Also ^{1}H NMR has been applied to study olive oil authenticity [Sacchi et al., 1998;

Mannina et al., 2001]. Levels of minor compounds related to quality and genuineness have been detected by ^{1}H NMR [Sacchi et al, 1996; 1997]. In addition methods with separation/concentration of marker compounds by HPLC, and subsequent detection by NMR have been applied to differentiate oils of different origins [Zamora, 2002].

Despite the complexity of ^{1}H and ^{13}C NMR spectra of biofluids and tissue extracts, many resonances can be assigned directly, based on chemical shift values (from literature or by spiking of pure compounds) and/or signal multiplicities, or by applying various multipulse techniques (i. e 2D). Although assignment of resonances is not necessary for classification of spectra, spectral assignment may lead to insight into the biochemistry and nature of the samples analyzed.

1.4.2 NMR spectroscopy of fish lipids

NMR spectroscopy has been particularly valuable in the study of lipids, since it provides multi-component information and can be applied non-destructively. NMR gives a fingerprint of the sample analyzed and may be used as a rapid profiling technique.

^{1}H NMR

^{1}H NMR on lipids gives information about lipid classes (triacylglycerols, diacylglycerols, ethyl esters, phospholipids) [Sacchi et al., 1997; Siddiquie et al., 2003; Falch et al., 2005a], and minor compounds such as free glycerol and cholesterol, added vitamin A and E, and trans fatty acids [Siddiqui et al., 2003], sterols and phenols [Sacchi et al., 1996] and different oxidation products, such as aldehydes [Falch et al., 2005b; Sacchi et al., 2006] and peroxides [Silwood and Grootveld, 1999]. The molar percentages of n-3, DHA and EPA acyl groups, and also some unsaturated and saturated acyl groups can be determined [Aursand et al., 1993; Sacchi et al., 1993b; 2006; Igarashi et al., 2002; Guillen et al., 2003; 2008]. ^{1}H NMR analysis can be carried out with a high degree of automation and gives a rapid fingerprint (2-5minutes) of the lipid profile. The DHA and n-3 fatty acid content (mole%) are also possible to quantify directly from intact material such as fish tissue by using the magic angle spinning technique [Aursand et al, 2006]. The ^{1}H NMR technique can be used to follow changes in the fatty acids of fish oils during oxidation [Tyl et al., 2008, Guillen et al., 2008] and heating [Sacchi et al., 2006]

^{13}C NMR

Traditionally the analysis of fatty acid composition is done by gas chromatography (GC), but this technique requires pre-treatment of the lipid sample. An alternative to GC is ^{13}C NMR spectroscopy. In addition to fatty acid composition of fish [Aursand et al., 1992], the ^{13}C NMR spectra give information about lipid classes [Gunstone, 1991; Aursand et al., 1992; Falch et al., 2006], the positional distribution of PUFAs in triacylglycerols and phospholipids [Gunstone and Seth, 1994; Medina and Sacchi, 1994a; Aursand et al., 1995a;

Falch et al., 2006], and content of other compounds such as cholesterol and wax esters [Gunstone, 1993]. The technique has also been shown to give information about the process history of marine oils, e.g., extraction, processing and storage [Siddiquie et al., 2003], and to study changes in lipid composition following thermal processing such as canning [Medina et al, 1994b; 1995b; 1998; 2000].

Authentication studies on fish/marine oils using NMR lipid profiles

Recently, ^{13}C and ^{1}H NMR spectroscopy has been used to detect and identify a wide range of compounds in encapsulated cod liver oil [Siddiqui et al 2003]. Natural sources of cod liver oils could be distinguished from those subjected to chemical modifications [Siddiqui et al. 2003]. ^{1}H NMR of muscle lipids has also been applied to differentiate between wild and farmed salmon and sea bream of different origins [Masoum et al., 2007; Rezzi et al., 2007]. Both HR ^{1}H and ^{13}C NMR, in conjunction with chemometrics, allowed the differentiation between a limited number of wild and farmed salmon and other species [Aursand et al., 2001].

Regiospecific analyses

As introduced in Chapter 1.2.3, both the fatty acid composition and the stereospecific structure of triacylglycerols vary among fish and may therefore be used for authentication of species.

Fatty acid composition may easily be manipulated to fit the requirements. As previously mentioned European pharmacopoeia on salmon oil (and cod liver oil from farmed cod, includes the regiospecific distribution (sn-2 position specificity) of fatty acids in triacylglycerols by ^{13}C NMR spectroscopy. Quantitative analysis of the distribution of fatty acids in triacylglycerols by ^{13}C NMR is possible due to the different chemical shifts of fatty acids in sn-1,3 and sn-2 position. Both the carbonyl region (172-174) ppm, the olefinic signals (126-134ppm), the glycerol region (74-60 ppm), and the aliphatic region (19-35ppm) of ^{13}C NMR spectra have been used in regiospecific analysis [Ng, 1985; Wollenberg, 1990; Sacchi et al., 1993a; Aursand et al., 1995a; Vlahov et al., 2006; Miller et al., 2006,].

Previous regiospecific ^{13}C NMR studies reported the composition of lipids in triacylglycerols of tuna oil [Sacchi et al., 1993a], and differences between cod liver-, harp seal- and Atlantic salmon oil [Aursand et al., 1995a], and between menhaden-, Chilean-, anchovy- and cod liver oil [Gunston and Seth, 1994]. The positional distribution of fatty acids in lipids from skin, white and dark muscle of mackerel [Ackman et al., 1991], and differences between fish oils [Ando et al., 1992], menhaden and salmon oil [Nwosu and Boyd, 1997], and in Albacore tuna [Aubourg et al., 1996] have been studied by chromatographic methods. Knowledge of triacylglycerol structure has also become

increasingly important since the stereospecific structure influences the lipid metabolism [Lien et al., 1994; 1997; Karupaiha and Sundram, 2007] and bioavailability of fatty acids.

1.4.3 NMR spectroscopy of low molecular weight compounds.

As discussed in Chapter 1.2.4, the type and amount of low molecular weight metabolites in fish may be used for product authentication. Several authors have used ^{1}H NMR to study low molecular weight metabolites in fish muscle. The ^{1}H NMR technique has been used to study changes in fish muscle during freezing [Howell et al., 1996; Sitter et al., 1999], and to quantify betaine in mussels [de Vooys and Geenevasen, 2002]. Recent studies used the technique to determine the freshness of sea bass submitted to ionizing radiation and modified atmosphere packaging [Reale et al., 2008]. Assignments of the ^{1}H NMR spectra from perchloric extracts of Atlantic salmon have been performed [Gribbestad et al. 2005].

Intact fish tissue can be analyzed by high field NMR. Aursand et al. (1995b) analyzed minced muscle from Atlantic salmon, without any additional pretreatment, by ^{13}C NMR spectroscopy, while Gribbestad et al. (2005) demonstrated that high resolution magic angle spinning (HR-MAS) spectroscopy can be employed to identify single chemical components (such as hypoxanthine, anserine etc.) in salmon muscle non-destructively.

1.5 Pattern recognition techniques applied to NMR spectroscopic data

Foodstuffs often contain a high number of NMR detectable compounds, and it is not always possible to detect variations between samples by visual inspection. The difficulties in sorting out important signals in a complex spectrum and to classify samples may be overcome by using suitable pattern recognition techniques. Pattern recognition techniques have been frequently and successfully applied to NMR data in a variety of applications related to food composition and authentication [Lindon et al., 2001; Alam and Alam, 2005].

Generally pattern recognition techniques are applied for three purposes; exploration, classification and prediction. Both the area/intensities of peaks and full spectra can be used as input for multivariate analysis. The assignment of spectral resonances gives information about the chemical composition of the samples, but it is not necessary for classification purposes. In spectra where peaks overlap, peak areas can be obtained by deconvolution (curve resolution) [Sacchi et al., 1995; Gribbestad et al., 1999]. When full spectra are used, they are normally converted to ASCII or JCAMP file formats. Regions without signals or unwanted signals are removed prior to multivariate analysis [Lindon et al., 2001]. Potential problems about inconsistencies in ppm values between samples in the data analyses should be solved by manual alignment or data pre-treatment methods [Forshed, 2003].

Both unsupervised and supervised pattern recognition techniques were used in this thesis The term “unsupervised” means that the model is created without any knowledge about class membership. Other methods are termed “supervised” and are used for sample

classification. A description of the methods used in this thesis is given in the materials and methods section (Chapter 3.3).

It is important to test the reliability of a chemometric classification. In principal component analysis (PCA), the approach of finding the optimal model dimensionality (number of PCs) is called cross-validation [Wold, 1987]. In supervised classification, generally, two methods can be applied to evaluate the quality of the prediction, namely cross-validation or dividing the samples into training- and test-sets. By cross-validation "the predictive ability of a model formed on a part of the dataset can be tested by how well it predicts the remainder of the data" [Brereton, 2003]. Cross-validation often gives over-optimistic results, in example when there are correlations in the variables. Similarly, training sets give generally fairly good predictions, because the model is created from samples in this dataset. Still, this does not mean that the model itself is good for "real" samples [Brereton, 2003]. Therefore, often a "test" dataset is created to test the model, consisting of new samples in an independent dataset.

2 Objectives

The main objective was to evaluate NMR spectroscopy in combination with pattern recognition techniques as a method for elucidation of origin and adulteration of foodstuffs. More specifically, the objects were;

1. Study the applicability of ^{13}C NMR spectroscopy as a method to authenticate marine oils according to; production method (wild/farmed) and geographical origin of raw material, process history and species.

2. Study the applicability of ^{13}C NMR spectroscopy of muscle lipids as a method to authenticate lean and fatty fish according to: production method (wild/farmed), geographical origin and species/stocks.

3. Study the applicability of ^{1}H NMR spectroscopy of low molecular weight metabolites in fish muscle for authentication according to; species, processing methods and freezing/thawing.

3 Materials and Methods

The materials and methods used in the studies are given in the papers, this section will provide a short background of the chosen materials and methods.

3.1 Materials

3.1.1 Marine oils

The prices of marine oils have generally been determined by the content of the health-beneficial omega-3 fatty acids DHA and EPA. However, there is an increasing focus on traceability of marine oils, and in the future, prices may to a greater extent be determined by factors such as: species, geographical origin and production method of raw material, and the process history (natural vs. concentrates). At the time when this investigation began, there had been few studies using the fingerprint obtained from ^{13}C NMR spectroscopy of marine oils for authentication purposes [Aursand et al, 2001].

In this study (Paper I, II, III) the usefulness of using ^{13}C NMR spectroscopy for authentication of marine oils according to wild/farmed and geographical origin of raw material, species and according to changes in lipid composition following certain processes was evaluated.

To study differences between fish oil from wild and farmed raw material, cod liver oils were chosen as materials (Paper I). The contributions of farmed species (Atlantic salmon, Rainbow trout, and Atlantic cod) are increasingly important sources of the valuable fish oils. Since this raw material (with generally high fat content) allows the production of oil to take place directly after slaughtering, these products may have better freshness than oils from wild fish. Multivariate analysis of ^{13}C NMR spectra was applied to authenticate cod liver oils according to wild/farmed and geographical origin, and the results were compared with the results from GC (Paper I).

Materials analyzed in Paper II included commercial health oil capsules of various species and origins, oil of different manufacturing steps from commercial producers, in addition to lipids extracted from cod livers, and from salmon/trout; blends of real salmon oil and diluent oil (commercial South American fish oil) were also analyzed.

In the latter work (Paper II), the full ^{13}C NMR spectra were used in the statistical analyses. Previous studies of marine oils have shown that ^{13}C NMR provided valuable information on sn-2 position specificity of fatty acids in triacylglycerols, which were characteristic for certain species (see Chapter 1.4.2). The possibility of using ^{13}C NMR regiospecific information for authentication of species of marine oils is evaluated in Paper III. Atlantic salmon, in addition to the pelagic fish species mackerel and herring were chosen as raw material. These pelagic fish species are used in fish oil production today, but there is a

potential for increasing the omega-3 oil production by utilising by-products from fillet-production of these species to a greater extent.

3.1.2 Muscle lipids

Atlantic salmon is by far the most important species in aquaculture. European producers of salmon include Scotland, Norway and Ireland, but Atlantic salmon is also produced in countries such as Tasmania, Chile and Canada [Eurostat, 2007]. At the time these studies began, there was no official method to verify if salmon was wild or farmed, or geographical origin.

The study in Paper IV is part of a larger EU project, where salmon from the major European producing areas were analyzed by a wide range of methods to investigate possible methods to differentiate wild and farmed salmon, and to provide some information on the geographical catch or farmed area. In addition to the ^{13}C NMR technique (as reported here in Paper IV), the ^{1}H NMR [Masoum et al., 2007], ^{2}H NMR (SNIF-NMR), different isotopic techniques [Thomas et al., 2008] and gas chromatography were evaluated [Axelson et al., submitted]. In this study, market samples were also classified and a peer test was performed (see discussion).

Studies using lipid profiles for authentication of fish, have mainly focused on fatty fish species, such as salmon, which are rich in triacylglycerols (and reflect dietary fatty acid composition). In this work we also wanted to study the applicability of muscle lipid profiles of lean fish, such as gadoid fish species, for authentication purposes. As mentioned in a previous chapter, muscle lipids of lean fish consist mainly of phospholipids, and since phospholipids are known to be less influenced by diet than triacylglycerols [Lie et al., 1992], analysis of phospholipids is preferred to study species- and stocks differences [Medina et al., 1997; Joensen et al., 2000].

Paper V is an evaluation of the applicability of using the ^{13}C NMR fingerprint obtained from muscle lipids to differentiate the lean gadoid fish species cod, saithe, haddock and pollack. The Atlantic cod was represented by two stocks, namely Norwegian coastal cod and north-east arctic cod. North-east arctic cod command superior prices, and Norwegian coastal cod was listed as an endangered species in 2006 (www.artsdatabanken.no). As previous studies had shown that one could distinguish among oils of different fish species by the sn-2 position specificity of fatty acids in triacylglycerols (Paper III), in paper V it was evaluated whether similar differences could be found in the sn-2 position specificity of fatty acid in phospholipids.

3.1.3 Low molecular weight metabolites in fish muscle

In addition to the health beneficial omega-3 fatty acids, fish are considered healthy food due to the content of small bioactive molecules (such as taurine, betaine anserine, etc.). The

potential of using the content of small (low-molecular weight) metabolites for authentication purposes was introduced in previous chapters. We wanted to study the applicability of using ^{1}H NMR spectroscopy of low molecular weight metabolites in fish muscle to authenticate fish according to species, processing methods and freezing/thawing.

Cod is an important species in Norwegian fishery. Approximately ¼ of the total Norwegian catch (tons) is from cod and other cod fishes. In Norway, cod is the most important white-fish for export, with a value of 6.2 billion NOK in 2007. Cod is mainly exported in the form of clipfish (33%), salted (20%) or dried (10%), but also as frozen, fresh, round fish or as fillets [SSB, 2008].

To be able to study differences and changes of the raw material, it was necessary to interpret the ^{1}H NMR spectra of extracted low molecular weight compounds of cod muscle (Paper VI). In addition, prospective differences between two cod species (cod and haddock) were investigated (Paper VI). Next it was evaluated if the metabolic profile obtained was characteristic enough to differentiate fish according to common industrial processing and preservation methods, such as freezing, drying, salting and cooking methods, boiling and frying of cod (Paper VII).

Low molecular weight metabolites in fish muscle were extracted by perchloric acid extraction [Glonek et al. 1982] in such a way that unstable compounds should be preserved during the catching and extraction procedure of the fish [Erikson et al., 1997; Gribbestad et al., 2005]. ^{1}H NMR spectra were run, and assignments of the peaks were achieved by comparison with literature values, 2D experiments, and spiking with reference compounds.

3.2 NMR techniques applied

3.2.1 ^{1}H and ^{13}C NMR

Both ^{1}H and ^{13}C NMR lipid profiles have been applied for authentication of lipids. In this thesis, focus was on the ^{13}C NMR technique in the analysis of marine lipids. The ^{13}C NMR spectra are less crowded than ^{1}H NMR spectra, and the interpretation of the spectra (to find important constituents/variables to separate different oils) are easier in ^{13}C NMR due to the wider chemical shift range. Interpretation of ^{1}H and ^{13}C NMR spectra of fish lipids have been made in previous studies [Gunstone 1991; 1993; Aursand and Grasdalen, 1992; Aursand et al., 1993; Sacchi et al., 1993a]

For the analysis of muscle lipids extracted from lean fish muscle, which mainly consist of phospholipids, ^{13}C NMR was the chosen technique. The alternative, ^{31}P NMR, would provide information on phospholipid species present, but in this study the focus was on ^{13}C NMR since this technique in addition gives valuable information about sn-2 position specificity of fatty acids in phospholipids molecules (Paper V). Assignments of resonances were done according to previous ^{13}C NMR studies of marine phospholipids [Medina et al., 1994a; Falch et al., 2006; 2007].

In the analyses of low molecular metabolites in fish muscle, ^{1}H NMR was the chosen technique. ^{1}H NMR is the most commonly applied NMR technique in analysis of osmolytes, due to its higher sensitivity compared to ^{13}C NMR spectroscopy. The spectra of the perchloric acid extract of fish muscle are also readily interpretable, and resonances were identified from spiking with pure compounds, 2D techniques and comparison with previous ^{1}H NMR studies on biological material [Nicholson et al., 1995; Fan et al., 1996; Sitter et al., 1999; Gribbestad et al., 2005].

An automatic sample changer was applied in the experiments performed on the 500MHz instrument. The use of an automatic sample changer with automatic shimming minimizes the time between subsequent samples and makes it possible to analyze up to 120 samples without the need for an operator during the experiment (e.g. over night). In the study of regiospecific distribution, a 600MHz instrument was used to increase the resolution of closely positioned resonances in the carbonyl region.

3.2.2 Semi-quantitative ^{13}C NMR

The natural abundance of ^{13}C is low, about 1.1 % compared to ^{1}H 100% abundance. In addition, the ^{13}C nucleus is inherently less sensitive than ^{1}H. In order to increase the S/N ratio, decoupling of ^{1}H resonances, responsible for splitting of the ^{13}C -resonances (1H-^{13}C coupling), is normally applied in routine ^{13}C NMR [Claridge, 1999]. Broadband decoupling and power gated decoupling (decoupling in both acquisition period and relaxation delay, and only relaxation delay, respectively) give rise to a phenomenon called the nuclear Overhauser effect (NOE), leading to enhancement of ^{13}C signals to varying extent. By applying inverse-gated decoupling, with decoupling only in the acquisition period, a decoupled spectrum without NOE can be obtained [Claridge, 1999]. Another prerequisite for a quantitative spectrum in this regard, is that the nuclei are fully relaxed before subsequent pulses. A recycle time $\geq 6.5T_1$ (T_1 ;longitudinal relaxation time) is needed with a pulse angle of 90º [Gillet and Delpuech, 1980; Wollenberg, 1990; Aursand et al., 1993;]. Since T_1 values of (Jeol EX-400) are up to 6.15 seconds (for ω2 carbon atom of n-3 fatty acids) [Aursand et al., 1993] and because the limited sensitivity of the ^{13}C NMR nucleus requires approximately 1000 scans, this leads to long experimental times for quantitative measurements (10 h) [Aursand et al., 1993]. As shown by Aursand et al., 1993 paramagnetic compounds such as $Cr(acac)_3$ can be used to decrease the relaxation time. Such paramagnetic compounds result in line-broadening; but a concentration of 0.025M resulted in minimal line broadening [Aursand et al., 1993].

A semi-quantitative approach (with recycle time less than $6.5T_1$ was chosen in the ^{13}C NMR studies (Paper I-V), because quantitative measurements require considerable longer experimental time. Even though the signal intensities within each spectrum are not quantitative, the relative intensities for corresponding signals across different spectra are comparable. It is the overall relationship among resonances that determine class assignments when applied in pattern-recognition techniques. The key factor is that the

spectra are acquired under the same conditions as much as possible. An evaluation of the reproducibility among labs was done in the EU Cofaws project (see Chapter 4.4).

3.2.3 Regiospecific analyses of triacylglycerols and phospholipids by ^{13}C NMR

In the regiospecific analyses of triacylglycerols (Paper III) and phospholipids (Paper V), a semi- quantitative approach was chosen (see Chapter 3.4). According to literature, T_1 values of carbonyl carbons are not particularly influenced by the position of its acyl chain (sn-1,3 or sn-2) [Ng, 1985; Wollenberg, 1990; Aursand et al., 1993]. Because the carbonyl carbons have equal NOE factors [Wollenberg, 1990] and similar T_1 values quantitative carbonyl spectra can be obtained from semi-quantitative spectra (with relaxation delay times (much) shorter than the traditional $\geq 6.5\ T_1$. To enhance spectral resolution, long acquisition times (10-20s) can be applied [Wollenberg, 1990; Sacchi et al., 1993a].

The regiospecific analyses can be optimized, by using the DEPT (distortionless enhancement by polarization transfer) pulse sequence [Vlahov et al, 2001; 2006] and 2D techniques, such as HSQC-TOCSY [Simova et al., 2003]. However, the technique used in this thesis is straightforward, -and also gives the total ^{13}C NMR fingerprint which may be valuable, for instance when other oils are added as adulterants.

3.2.4 Metabolite-oriented approaches in NMR spectroscopy

Generally, four metabolite-oriented approaches can be used as defined by Fiehn (2001) and specified for HR NMR by Mannina et al. (2008); 1) metabolite target analysis; analysis of a specific compound (concentrated and isolated from other compounds); 2) metabolite/metabolic profiling; is used when one is interested in the specific role of a selected metabolic pathway and requires identification and quantification of a selected number of metabolites; 3) metabolomics; which involves a comprehensive quantification of metabolites in the sample; and 4) metabolic fingerprinting (often called metabonomics in medicine and nutrition); where one use the metabolite fingerprint (e.g. NMR-fingerprint) with all signals without necessarily any identification/quantification prior to sample classification; however this approach may detect individual signals that can be related to sample classification [Fiehn, 2001]. Lipidomics can be defined as a subset of metabolomics.

In this work, focus was on using the metabolic fingerprint obtained by NMR spectroscopy to classify samples, without necessarily identification and quantification of the metabolites analyzed. Still, to elucidate differences between groups, some kind of identification of resonances is important, and we also applied target analysis of specific compounds (e.g. level of dimethylamine as indicator of frozen fish and regiospecific distribution of triacylglycerols and phospholipids).

3.3 Multivariate analyses

Generally, principal component analysis (PCA) was the first multivariate technique applied to each dataset. PCA analysis is easy to interpret (visually), and was used to get an overview of the data and to observe prospective differences among groups.

Even though unsupervised, exploratory techniques, such as PCA are helpful to observe differences among classes, this technique is not necessarily suited to deal with more complex tasks. When the number of groups to be separated becomes larger more sophisticated supervised chemometric techniques are required. Furthermore, advanced multivariate methods, such as neural networks, have the advantage that they give quantitative measurements of the classifications.

A short description of the multivariate methods used in this thesis is given below.

Principal component analysis (PCA) [Jolliffe, 1986; Wold et al., 1987] 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 the original data (in decreasing order). 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, but PCA is not a quantitative, statistical method.

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. LDA 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 [Kim et al., 2003].

Partial least squares-genetic algorithm (PLS-GA) [Leardi and Gonzalez, 1998] have been extensively used for variable selection. Variable selection attempts to identify and remove the variables that are not relevant for the classification. Usually, spectroscopic measurements consist of a very large number of variables, and this dimensionality is circumvented using e.g. PLS regression. The PLS prediction can be improved by using search algorithms, and the genetic algorithm is one such optimization tool.

Neural Networks (NN). PCA and LDA are linear methods, while the neural networks are non-linear methods. The way a neural network operates is compared to the way in which the brain works [Lindon et al., 2001]. The descriptors (e.g. chemical shift intensities) are entered at the input neurons for each sample, and the network is trained by performing an

optimization of weights in the interconnections between neurons until (after input of all of the data of the training set) the values output at the output layer neurons are as close as possible to the values required. In a case with 5 classes of samples, there will be 5 output neurons (and a sample which falls into class 2 would be 0-1-0-0-0). The input to a neuron is the sum of the outputs of all the neurons in the previous layer multiplied by the weight factor for each connection (which are optimized at each stage of training the network). The output of a neuron is related to the summed inputs by a non-linear transfer function. Different neural network employ different learning rules, but all in some way determine pattern statistics from a set of training samples and then classify new patterns on the basis of these statistics.

Probabilistic neural networks (PNN) [Specht, 1990] are organized into three layers: input, pattern and summation. PNNs are particularly powerful in analysis of highly non-linear classification problems. In PNN the relationship between the data and the resulting classification are determined by the data itself, without any assumption about detailed mathematical relationship in this concern [Lindon et al., 2001]. The PNN operates by defining a probability density function (pdf) for each class based on the training set data and an optimized kernel width parameter, also optimized by a genetic analysis. PNN have the advantage that it uses all the information in the dataset, while avoiding the problem of over-fitting to achieve classifications [Patterson et al., 1996].

Generalized regression neural networks (GRNN) [Specht, 1991] perform regression rather than classification tasks, but work in a similar way as the probabilistic neural network.

Kohonen neural networks (KNN) [Kohonen, 1997; de Boishebert et al., 2006] are designed for unsupervised learning, in contrast to other networks. During the training process the input patterns (e.g. selected chemical shifts) are presented without specifying what the output of the network should be. Samples are grouped together based on their similarity. It is primarily used for the examination of data sets for which no or only a little a priori knowledge concerning the internal relationships is available. Once the network has been trained, each unit in the Kohonen map might be associated with an object class and then the map may be used for classification and visualization purposes.

Generative Topographic Mapping (GTM) [Bishop, 1998] provides an alternative to the widely used KNN maps, and this approach reportedly may avoid some of the theoretical deficiencies of the Kohonen approach, although the basic results are typically similar in practice. The GTM map provides a useful method for visualization of class relationships.

Support Vector Machines (SVM) [Cortes and Vapnik, 1995; Anguita et al., 2005] perform classification by constructing an N-dimensional hyperplane that optimally separates the data into two categories. SVM models are closely related to neural networks. A SVM model using a sigmoid kernel function is equivalent to a two-layer, perceptron neural network. In the parlance of SVM literature, a predictor variable is called an attribute, and a transformed attribute that is used to define the hyperplane is called a feature. The task of

choosing the most suitable representation is known as feature selection. A set of features that describes one case (e.g., a row of predictor values) is called a vector. So the goal of SVM modeling is to find the optimal hyperplane that separates clusters of vector in such a way that cases with one category of the target variable are on one side of the plane and cases with the other category are on the other size of the plane. The vectors near the hyperplane are the support vectors.

Bayesian Belief Networks (BBN) [Pearl, 1998; Heckerman, 1995] consists of: 1) nodes which represent the random variables, where each node has states (a set of probable values for each variable); 2) directed edges (arrows) which connect the nodes (represent dependencies; absence of arrows indicate independence); 3) a conditional probability table which is associated with each node (prior probability); and 4) a directed acyclic graph where the graph represents independence relationships between variables. The most important advantages for this work are that BBN´s calculate explicit probabilities for a hypothesis, can be used as a classifier and can find the variables with the most impact on the classification.

4 Results and Discussion

The research activities performed in this works are divided into three studies as presented in Chapter 2.

4.1 ^{13}C NMR studies on marine oils for authentication purposes (PAPER I, II and III).

The goal of this study was to evaluate ^{13}C NMR spectroscopy as a method to authenticate marine oils according to; production method (wild/farmed) and geographical origin of raw material, process history and species.

4.1.1 Wild/farmed and geographical origin of raw material

By using ^{13}C NMR in combination with various multivariate analyses, differentiation between marine oils according to whether the material was from wild or farmed fish was achieved, both in the analyses of cod liver oils (Paper I, Paper II), and for the salmon oils (Paper II). Fish oils are generally made from storage lipids of fish (triacylglycerols), and the fatty acid compositions of triacylglycerols are to a great extent influenced by dietary fatty acid input [Sargent et al., 2002]. Since farmed fish have a diet that differs from the available food for wild fish, this is reflected in the fatty acid composition [Sargent et al., 2002].

The farmed cod liver oil displayed a higher level of 18:2n-6 and 18:1n-9 than the wild cod liver oil, which may be attributed to cereal binders and/or vegetable oil used in fish feed. Principal component analysis (PCA) of both the GC and the ^{13}C NMR showed clear separation between cod liver oils of wild and farmed origin. A quantitative result of the discrimination power was obtained by linear discriminant analysis (LDA), which gave correct classification rates of 97% for GC data and 100% for NMR data in the wild/farmed classification. The most important variables in the GC classification were found to be the values for 18:2n-6, 18:1n-9, 22:6n-3, 22:1n-11 and 20:1n-9. Similarly, the most important peaks in the wild/farmed classification from NMR data included peaks at 31.48 ppm, and 22.53ppm, which could be assigned to ω3 and ω2 carbon atoms of n-6 fatty acids respectively. The fact that farmed fish has a higher level of fatty acids normally found in vegetable oils has been shown in several studies [Aursand et al., 2001; Busetto et al., 2008]. Livers from farmed cod are a promising source of valuable omega-3 fatty acids, and the values on the health beneficial fatty acids 22:6n-3 and 20:5n-3 are in the range specified by the European Pharmacopoeia for cod liver oil both from farmed and wild cod in this study.

For the analyses of cod liver oils, there were clear groupings according to different geographical origins (Paper I and II). This was particularly evident in the analysis of the cod liver oils in Paper I, where the classification rates were 63% for GC data and 95% for NMR data of cod liver oils from Scotland and Norway. In paper II, cod liver oils from

different geographical origins (Icelandic Sea and Barents Sea) could be differentiated. It is suggested that this separation between cod liver oils from different geographical origins, is mainly caused by differences in the available feed (diet). Fatty acid composition of cod livers reflect dietary input [dos Santos et al., 1993], however also environmental, metabolic and genetic factors influence lipid composition [Sargent, 1999]. In paper I, clear grouping of cod liver oils from the different farms was observed. This separation is most likely the result of different feeds used, even though (as mentioned in the background), it is known that lipid composition varies according to farming environment, feed management and the culture methods implemented (e.g. feed rate) [Turchini et al., 2008].

4.1.2 Process history

As observed in the analysis of various fish oil capsules in Paper II, there were clear differences between ^{13}C NMR of natural and chemically modified fish oils. Products that had gone through chemical esterification/transesterification processes (Omega-3 concentrates) were recognized by the content of diacylglycerols and monoacylglycerols in addition to the triacylglycerols found in natural fish oil (see Paper II, Figure 6), and these samples formed clear clusters in the statistical analyses (e.g. in the Kohonen neural network (KNN) analysis). Such compositional differences have been observed by several authors [Gunstone 1993; Sacchi et al., 1993a; Siddiquie et al., 2003;], but classification of marine oils due to these characteristics has previously not been made. Generative topographic mapping (GTM) analysis illustrated further that there were subgroups within the group of cod liver oils (e.g. cod liver oils of various processing steps).

4.1.3 Species and market samples

PCA of the full ^{13}C NMR spectra of the different commercial fish oils (Paper II) showed substantial separation of most classes according to species. Similarly, probabilistic neural network (PNN) predictions gave a validation result of 96.2% correctly classified, and trout, salmon and cod liver oils were completely classified. Interestingly, samples described as "natural salmon oil" clustered with South American fish oils (which are generally produced from anchovies) in the PCA plot. Also some of the commercial cod liver oils were grouped within this group. The suspicion that these samples were mislabeled was further strengthened by the results from the KNN analysis.

Fatty acid stereospecific/regiospecific composition is characteristic for fish species. In paper III, a more thorough evaluation of species differences in the regiospecific distribution (sn-2 position specificity) of fatty acids in triacylglycerols were done for Atlantic salmon (*Salmo salar L.*), mackerel (*Scomber scombrus*), and herring (*Clupea harengus*.). The species Atlantic salmon, mackerel and herring, could readily be differentiated from their ^{13}C NMR carbonyl profile, which gives information about regiospecific distributions of fatty acids (Paper III). In particular, the sn-2 position specificity of 22:6n-3 was markedly higher in herring, compared to Atlantic salmon and mackerel. Compared to previous ^{13}C

NMR studies, the values on sn-2 position specificity of 22:6n-3 and 20:5n-3 reported for salmon and herring in the present study differ from marine oils from other species, such as seal and cod liver oil [Aursand et al., 1995a], tuna [Sacchi et al., 1993a] and anchovy, menhaden and chilenian oil [Gunstone et al, 1994], as illustrated in **Figure 3**. Particularly, for anchovies the sn-2 position specificity of 20:5n-3 is low compared to other fish species [Gunstone et al., 1994]. Recent results by Mannina et al. (2008) show that the regiospecific distribution of fatty acids in muscle of sea-bass differ from the above mentioned studies on fish oils, in that 20:5n-3 and 22:6n-3 was preferentially distributed in the sn-1,3 position.

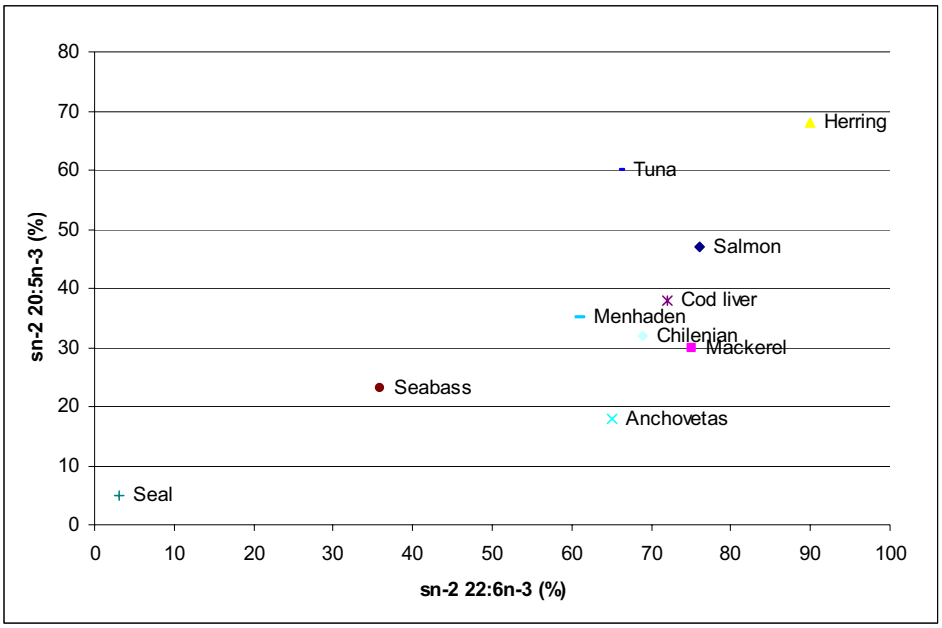

Figure 3. sn-2 position specificity among different marine oils. According to; the present study, Aursand et al., 1995a, Gunstone et al., 1994 and Sacchi et al., 1993a.

Furthermore, PCA of the data points in the carbonyl region revealed that the distribution of saturated/monounsaturated) fatty acids (SFAs/MUFAs) differed among the species. More recent results show that the distribution of SFAs and MUFAs also differs between salmon- and anchovy oil; two commercially important fish oils (**Figure 4**). While the regiospecific distributions of 22:6n-3, 20:5n-3 and 18:4n-3 are easily obtained from semi-quantitative ^{13}C NMR spectra, the resolution of closely spaced resonances in the region where saturated/monounsaturated (and other fatty acids, such as 18:2n-6 and 18:3n-3) (173.3-

173.2 ppm and 172.8-172.9 ppm) requires high field (typically 600MHz and more) and optimized spectra (good digital resolution- and thereby long experimental times). The spectra in **Figure 4** were obtained in approximately 3 hours on a 600MHz instrument fitted with a cryoprobe.

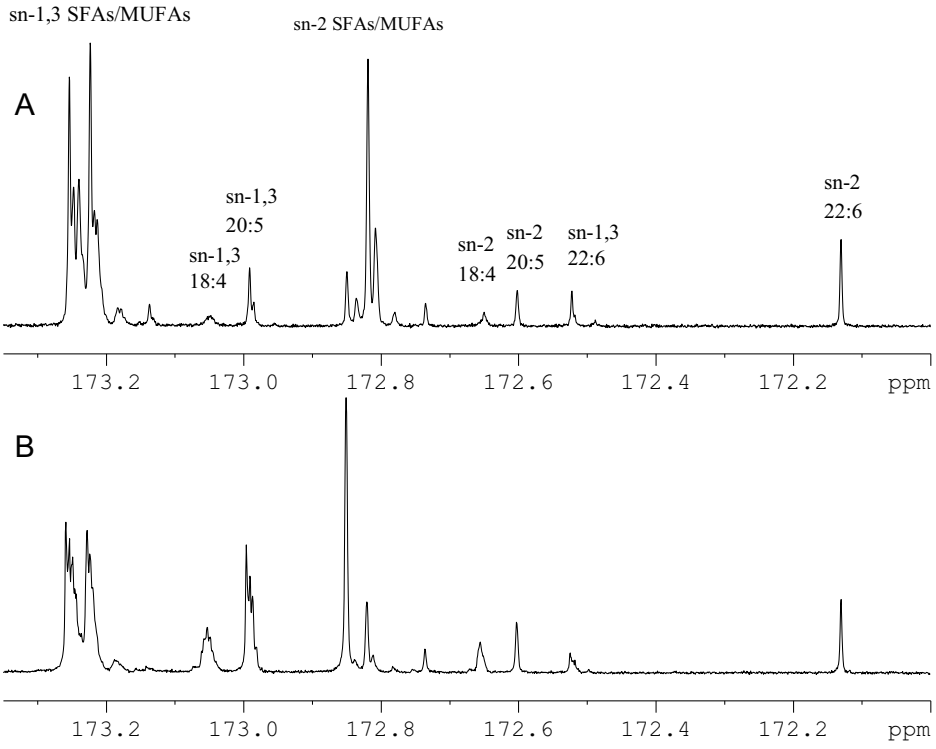

Figure 4. 600MHz ^{13}C NMR carbonyl region for A) salmon- B) anchovy oil illustrating the different profiles of the two species. The spectra were obtained on a 600MHz instrument fitted with a cryoprobe at Bruker BioSpin GmbH (DE).

4.1.4 Mixtures

A prospective method to reveal fraud and mislabeling of fish oils should be able to detect if the natural oil have been diluted with other (cheaper) oils. An evaluation of the levels of diluent oil that can be detected and quantified was done by generalized regression neural networks (GRNN) in Paper II. The results indicate that the degree of mixture that could be quantified for real salmon oil diluted with South American oil was 5%.

As shown in a previous chapter, regiospecific data are valuable to identify which species a fish oil is made from. For mixtures, it will be possible to identify dilutions by regiospecific analyses when the content of the oils are significant. Previous ^{13}C NMR regiospecific studies on olive oil showed that an addition of 10% esterified oil (corresponding to a level of 2.5-3 mole% saturated in the sn-2 position) to pure olive oils could easily be detected [Sacchi et al., 1992]. Smaller amounts of diluent fish oils may not be easily observed in this region due to low signal intensities of carbonyl carbons (which require long experimental times), and closely spaced resonances. Additions of vegetable oils will be easier to detect, since added vegetable oil will lead to elevated levels of minor fatty acids in fish oils. As an example; in Paper II it can be seen that fish oil with added palmitic acid has a characteristic carbonyl profile compared to natural fish oil due to higher level of the fatty acid 18:2n-6. The just mentioned addition of palmitic acid to salmon oil was even more evident from the olefinic region (Paper II).

4.2 ^{13}C NMR studies on muscle lipids for authentication purposes (Paper IV, Paper V)

The goal of this study was to evaluate ^{13}C NMR spectroscopy of muscle lipids as a method to authenticate fish according to: production method (wild/farmed), geographical origin and species/stocks. Both fatty fish (Atlantic salmon) and lean fish (Atlantic cod, saithe, haddock, pollack) were used as materials.

4.2.1 Wild/farmed and geographical origin of salmon

Several multivariate methods were used in the classification of wild vs. farmed salmon from ^{13}C NMR data (Paper IV), and the predictions were tested both using cross-validation and by using training/validation datasets. Wild and farmed predictions were excellent both using Probabilistic Neural Networks (PNN) and Support Vector Machines (SVM). By using validation test sets, PNN gave a correct classification rate of 98.5%. For the SVM approach, 100% of the samples were correctly predicted according to wild/farmed. These results are in accordance with the preliminary study on Atlantic salmon by Aursand and Axelson (2001). Differences between wild and farmed fish could be observed in several samples from the ^{13}C NMR spectra directly in that the farmed fish had a higher content of 18:2n-6 (also confirmed by GC analyses from the same study [Thomas et al., 2008]. As discussed previously, discrimination between wild and farmed fish is generally straightforward, since the fatty acid composition reflects the diet and farmed fish has a different diet than wild fish.

When it comes to the market samples, five samples labeled as “wild” appear to have been farmed. These results are in accordance with the other methods tested on the same fish [Masoum et al., 2007; Thomas et al., 2008].

The geographical origin predictions were somewhat more difficult. Correct classification rates ranged from 82.2% to 99.3% by PNN and SVM respectively. However, some classes

consisted of relatively few samples in this classification. As for the marine oils in previous chapter, the discrimination among fish of the different farms is most likely the results of different feeds used [Turchini et al., 2009]. However, there are some factors that complicate the use of lipid profiling when it comes to differentiation among fish of different farms.

Lipid composition of farmed fish can fluctuate depending on the oil and meal used in the feed, it is also common practice to shift from one diet to another according to feed cost, availability and growth stage of the fish [Turchini et al., 2009]. The changes in the FA composition of the triacylglycerol fraction following changes in the composition of the diet have been explained using a dilution model [Jobling, 2004]. Fatty acid composition of fish has been shown to be affected by diets fed more than three months before a change in diet [Martinez et al., 2009, Turchini et al., 2009]. A recent study on the identification of the farm origin of salmon showed that the fatty acid composition of the feed was not reflected in the muscle lipids [Martinez et al., 2009], and a possible explanation was that the fish had only been given the analyzed feed for the last period of time, so that the washing period of previous feed had not been completed. In addition, the fact that lipid metabolism is influenced by factors such as growth stage, culture system and general environmental conditions confounds the influence of dietary fatty acid composition on fillet compositions [Turchini et al., 2009].

A recent study [Turchini et al., 2009] used a combination of morphological, chemical, and isotopic analyses (IRMS) to discriminate among intensively farmed freshwater Murray cod from different farms in different geographical areas. The authors deduce that fatty acid composition is not a particularly good discriminator of the origin of farmed Murray cod, since it is more reflective of diet than the farm system [Turchini et al., 2009]. However, the authors emphasize that one should consider alternative statistical methods based on dissimilarities between fish rather than on correlations between variables when discrimination between fish of different farms is the goal [Turchini et al., 2009]. This was demonstrated by a recent study, which showed the great potential of lipid profiling (by GC and ^{13}C NMR) combined with advanced chemometrics to identify the farm origin of farmed fish and also the farm origin of escaped fish from the same farm [Martinez et al., 2009]. As pointed out by the authors, since both the genetic “background” of the fish and the feed may vary over time, to be able to trace a fish back to its farm; one needs to establish and continuously update databases with representative reference material [Martinez et al., 2009].

As several authors have concluded; a combined use of several techniques may be the optimal for reliable results to determine the geographical origin of fish [Luykx et al., 2008; Thomas et al., 2008; Martinez et al., 2009].

4.2.2 Species and stocks of lean gadoid fish

In paper V ^{13}C NMR muscle lipid profiles were obtained from four different species of lean gadoid fish, (including two stocks of Atlantic cod), namely north-east arctic cod and Norwegian coastal cod (*Gadus morhua*), haddock (*Melanogrammus aeglifinus*), saithe (*Pollachius virens*), and pollack (*Pollachius pollachius*).

Results from the ^{13}C regiospecific analysis, showed that 22:6n-3 was preferentially positioned in the sn-2 position in both phosphatidylcholine (PC) and phosphatidylethanolamine (PE), in accordance with previous results on cod milt and roe [Falch et al., 2006]. Average values on the sn-2 position specificity of 22:6n-3 for the different fish categories varied between 77-85% for PC and 65-76% in PE. The pollack group displayed the lowest values for 22:6n-3 in sn-2 position, both in PC and PE, and there were significant differences among species both in PC and PE. However, the species specific differences in sn-2 position specificity observed here were not as obvious as the differences in triacylglycerols of the fatty fish species in paper III. This is not surprising, as the lean species investigated are closely related species (all are gadoid fish). By using the full ^{13}C NMR fingerprint of muscle lipids, successful classification (100%) according to the five categories of lean gadoid fish was achieved with Bayesian belief networks (BBN) predictions. These results are in accordance with previous studies on Atlantic tuna, which showed that three species could be differentiated from their fatty acid composition of the phospholipid fraction [Medina et al., 1997]. Interestingly, the north-east arctic cod samples were easily differentiated from the coastal cod samples, and this may be attributed both dietary and metabolic differences. The results are in accordance with previous results, which showed that two stocks of cod reared under identical conditions with the same diet had different fatty acid composition in their heart tissue [Joensen and Grahl-Nielsen, 2001]. The authors concluded that these differences were expected to be genetic.

Even though this study shows that it was possible to discriminate amongst species and stocks of lean gadoid fish based on ^{13}C NMR lipid profiling of muscle lipids (which mainly consist of phospholipids), further studies are needed to assess the range of variation according to dietary, environmental and metabolic differences within species/stocks. As examples from metabolomic toxicology have shown, influences from the environment may obscure genetic variations in the metabolome (and conversely) [Bundy et al., 2009].

4.3 ^{1}H NMR studies on low molecular weight metabolites in fish muscle for authentication purposes (Paper VI and Paper VII)

The goal of this study was to evaluate ^{1}H NMR spectroscopy of low molecular weight metabolites in fish muscle for authentication according to species, processing methods and freezing/thawing.

4.3.1 Species

A wide range of low molecular weight metabolites was assigned in cod and haddock white muscle (Paper VI). The results show that labile compounds (e.g. adenosine diphosphate, ADP) were preserved during the sample handling and extraction. A total of 35 compounds were identified in the NMR spectra, many of which give information on the metabolic condition of the sample analyzed. Cod and haddock displayed, to a large degree, the same compounds, but both intra- and interspecific differences in relative peak intensities were registered. Since this study was on a limited number of samples, it was not possible to attribute the differences to species specific factors or to others, such as environmental factors, development stage or physical condition. The results of Paper VI were used to identify compounds reflecting the physiological state and nutritional value of fish of different processing conditions in paper VII.

4.3.2 Processing conditions and freezing/thawing

In the different cod samples analyzed in paper VII (fresh, fried, boiled, frozen, thawed, drip loss, clipfish), bioactive constituents identified included; taurine, betaine, anserine, creatine and trimethylamine N-oxide (TMAO). Highest loss of bioactive compounds from the fillet took place during freezing and thawing, mostly in what is known as drip loss. The results showed less significant changes in bioactive compounds during cooking and frying, in contrast to recent studies on cod fillets [Larsen et al., 2007], which reported substantial loss of bioactive compounds during similar preparation methods. Frozen fish could be distinguished from not frozen unprocessed cod by the presence of dimethylamine (DMA), which is formed enzymatically from TMAO during freezing. However, DMA was also observed in the clipfish samples, which do not necessarily imply that this fish had been frozen, since DMA may also be formed non-enzymatically by e.g. heating processes and drying [Spinelli and Koulry, 1981]. It has previously been shown for non-processed fish that DMA may be used as a marker for freezing in gadoid fish species, unless the fish is badly spoiled prior to freezing [Huss, 1995]. Upon death and during ice storage, the TMAO is metabolized to trimethylamine (TMA) by certain bacteria.

Relatively large intra-group variability in peak intensities was observed in this study, which may be originating from the large individual variation known to be found in fish; however, the extraction procedure (with freeze-drying of the muscle prior to extraction), might have impaired the metabolic composition. In the multivariate analysis, 80% of the samples were correctly classified according to processing method (7 groups) by probabilistic neural network (PNN) analysis. However, analysis of fresh, unprocessed and cooked samples permitted a total correct classification of these samples. One important conclusion from this work is that when clear, unambiguous diagnostic resonances exist for certain processing conditions, no multivariate analysis is required. Small, although characteristic differences among samples may be obscured by the main bulk of compounds found in closely related samples when multivariate analysis is applied. When it comes to the applicability of

metabolic fingerprint as a method for product classification, one needs representative databases covering the natural variation found in the samples.

4.4 General discussion

4.4.1 Reproducibility issues

When using HR NMR fingerprinting as an authentication method, it is important to try to run the samples as "similar" as possible. The whole procedure from sample preparation to analysis by pattern recognition techniques can be affected by factors unrelated to the sample characteristic of interest [Defernez and Colquhoun, 2003].

The repeatability and reproducibility of the NMR analysis itself are generally good. The NMR results are not influenced by age of columns, as for chromatographic methods. However, small differences in experimental conditions, such as instabilities in apparatus, temperature variations, operator experience/performance, inhomogenities in the applied magnetic field, or differences in relative concentrations of the samples analyzed, may lead to erroneous classification [Defernez and Colquhoun, 2003].

In these studies it was determined that all spectra had acceptable linewidth and lineshape after the NMR analysis. An evaluation of the reproducibility of the method (semi-quantitative ^{13}C NMR in combination with pattern recognition techniques) was done in context with the studies on wild and farmed salmon (Paper IV). A peer test was performed in order to assess how well the method could be applied in another laboratory and whether the results were comparable. Ten samples of oil (5 blind duplicates) were run at three different laboratories according to an established protocol. The FIDs from the different labs were collected, and sent to one of the partners, which performed the data treatment and manual alignment of peaks in each of the ^{13}C NMR spectra, and exported for principal component analysis. Even though the ^{13}C NMR experiments were not run quantitatively, the results showed good reproducibility. This is in accordance with results from metabolomic toxicology, which showed good reproducibility among laboratories given reasonably well defined protocols [Viant et al., 2009].

Furthermore, it has been shown that it is important to choose the most appropriate extraction method [Lin et al., 2007]. Especially in analysis of labile compounds, small differences in the sampling handling and extraction procedure may lead to large differences in the level of metabolites among individuals of the same group. As observed in paper VII, the extraction procedure (with freeze-drying of the fish muscle prior to extraction) may have contributed (in addition to the large individual variations known to be found in fish) to the large sample to sample variance. Also in the analysis of lipids, the extraction procedure is important, and the yield of lipid components depends on the tissue investigated [Cabrini et al., 1992]. The Bligh and Dyer procedure was the chosen method both in the analysis of fatty fish (in a modified form as evaluated by Thomas et al., (2008)) and of lean fish. The

Bligh and Dyer procedure (1959) have been shown to give the highest yield of phospholipids in lean fish compared to other methods [Cabrini et al., 1992; Jensen, 2003].

pH influences the chemical shift in perchloric acid extracts [Lin et al., 2007]. In the ^{1}H NMR study of low molecular metabolites in processed cod (Paper VII), pH was adjusted to 7.5 ± 0.1, and small chemical shift differences between samples were aligned automatically. In the analysis of lipids, concentration was the factor that influenced chemical shifts most [Mannina et al., 2000]. It was observed in these studies that automatic alignment of peaks worked well, since the chemical shift differences in each region were similar.

4.4.2 Need for database with reference materials

The method of using HR NMR fingerprints for authentication requires that analytical data on suitable reference samples are collected in a database, which makes it possible to classify an unknown sample. This database should cover all kinds of individual variations, to prevent erroneous accusations of mislabeling/fraud. For fish, this is a challenge, since the composition of fish tissues are influenced by a wide range of factors (diet, season, age, size and stage of sexual maturity and according to lipid metabolism and environmental factors) [Sargent et al., 1999], and this calls for extensive databases. For marine oils, the reference material need not be so extensive, since fish oils are normally produced from batches, implying that individual variations are less relevant. However, also marine oils show great variation within the same species according to seasons, year of production and geographical origin [Stansby, 1979; Nichols, 2009]. Analogous official databases have already been established for wines. The European wine databank on authentic European wines consists of isotopic and nuclear magnetic resonance determinations (SNIF-NMR) and is used to discover product enrichment and addition of water [EC regulation 2348/91], and it may also be used to verify the variety and geographical origin of wines [EC regulation 2729/2000].

4.4.3 NMR spectroscopy as an authentication method (advantages/disadvantages)

There are several advantages of NMR spectroscopy; it is a non-targeted multicomponent technique, and can be used for screening a wide range of foods (oils, liquids, extracts, or intact food by HR MAS). The technique can also be used for structural elucidation and to detect and quantify marker compounds.

The method presented in this thesis, NMR spectroscopy in combination with pattern recognition techniques for authentication, can be applied to a wide range foodstuffs, and this has already been demonstrated for olive oils and other vegetable oils [Sacchi et al., 1998; Brescia et al., 2003; Rezzi et al., 2005], fruit juices [Cuny et al., 2008], soft drinks [Charlton et al., 2008], dairy products [Brescia et al., 2005], wine [Brescia et al., 2003b], tea [Le Gall et al., 2004], honey [Consonni and Cagliani, 2008] and meat [Sacco, 2005]. The approach can also be applied to study quality of raw material [Aubourg et al., 1998],

and changes in foods during processing. As examples from the feed industry show; HR NMR instruments, situated at the factory, can be used for such purposes [www.ewos.com]. HR NMR, combined with chemometrics, is also well suited to study changes in metabolite profiles according to stress, pollutants, farming and genetic modifications (an example of the latter is a recent study of peas by Charlton et al. (2004)). Also in the study of phospholipids, which are of significant functional importance, there is a potential to use HR NMR (^{13}C or ^{31}P) as a fingerprint for various classifications [Schiller et al., 2007].

The main disadvantages of NMR spectroscopy as an authentication method are the price of the instruments, and the limited sensitivity (compared to GC and MALDITOF-MS). Since the datasets may consist of typically 64k variables, there is a need for special software able to deal with such large datasets, and automated procedures. On the other hand, the development in this field is fast, and the whole data-preprocessing procedure can be automated [Viant et al., 2009] which reduce data pre-processing times considerably. In addition, software for identifying and quantifying metabolites are now commercially available (e.g. www.chenomix.com), which reduces time for analysis to a minimum.

To date, the technique has not been adopted for official authentication of food, but the largest potential for official application may lie in the analysis of oils [Le Gall and Colquhoun, 2003]. A prospective official application of the technique will most likely take place in central laboratories, and not in fishing vessels, factories or by the local food authorities. Instrumental developments will increase the information obtainable, and reduce experimental times. 500MHz and 600MHz instruments are becoming more common in research laboratories, and instruments at higher fields are commercially available (e.g. 950 MHz). Cryoprobe ^{1}H NMR spectroscopy has detected limits of contaminants in water of approximately 1 μM [Charlton et al., 2006]. Also flow injection systems have been used to study authenticity of olive oils [Rezzi, 2005].

Even though ^{13}C NMR is a less sensitive technique compared to GC, ^{13}C NMR has the advantage that intact lipids can be analyzed non-destructively. Since pure fatty acid compositions of marine oils are easy to manipulate, another important advantage of ^{13}C NMR is that it provides information about lipid classes and regiospecific distributions. Particularly, the ^{13}C NMR information about sn-2 position specificities of fatty acids in triacylglycerols and phospholipids are important characteristics to distinguish oils and muscle lipids of different species. The ^{13}C NMR technique is also able to detect minor components [Siddiquie et al., 2003], and to observe lipid changes (e.g. oxidation) during food processing [Medina et al., 1998] and storage [Falch et al., 2007]. In complex spectra, interpretation and quantification can be facilitated by NMR on chromatographically obtained fractions [Zamora et al., 2002].

The major advantage of ^{1}H NMR in analysis of low molecular compounds for authentication is that NMR is a non-targeted technique, which provides quantitative signals from every compound containing protons, in contrast to targeted approaches, such as liquid

chromatography and GC-MS. Information about a wide range of compounds, including compounds relevant to the nutritional value, can be derived from ^{1}H NMR spectra. The challenge in the use of ^{1}H NMR fingerprinting for authentication purposes, is to extract the important information out of the wealth of information present (need for suitable chemometric treatment) and as previously described, there is a need for representative databases to map the natural variation within groups.

5 Concluding remarks

Marine oils

The results presented show that ^{13}C NMR is a valuable tool for differentiation of marine oils according to origin. When applying the ^{13}C NMR spectra in pattern recognition techniques it was possible to authenticate marine oils according to;

- wild/farmed origin of raw material, both of cod liver oils and salmon oils.
- geographical origin, although this may be due to the different feed used, which may vary
- species (and mixtures of two species down to a dilution of 5%). Mislabeled market samples could be recognized.
- process history; natural fish oil that had gone through esterification processes (omega-3 concentrates) could easily be differentiated from natural fish oil.

The sn-2 position specificity (obtained from the ^{13}C NMR carbonyl region) allowed discrimination among the species mackerel, salmon and herring, and this seems to be an important characteristic when differentiating marine oils according to species.

Fish muscle lipids.

When applying the ^{13}C NMR spectra of muscle lipids in pattern recognition techniques it was possible to classify salmon according to;

- wild/farmed origin
- geographical origin, although somewhat more complicated. A combination of methods may be more useful here as stated by other authors [Luykx et al., 2008]

When applying ^{13}C NMR spectra of muscle lipids of lean fish in pattern recognition techniques it was possible to;

- classify different cod species
- discriminate between two stocks of Atlantic cod

It was shown that the ^{13}C NMR spectra provided information about the regiospecific distribution of 22:6n-3 in PC and PE, which showed minor differences among the cod species investigated.

Low molecular weight metabolites

^{1}H NMR on water soluble extracts of fish muscle provided information about a wide range of compounds, including several labile and bioactive compounds;

- cod and haddocks analyzed displayed different ^{1}H NMR profiles. Further studies are needed to conclude whether this is attributed to species-specific differences
- DMA was used as a marker for frozen, non processed fish

When applying ^{1}H NMR data of low molecular weight metabolites in fish muscle in pattern recognition techniques it was possible to classify fish according to

- frozen/ not frozen
- processing conditions with 80% correct classification.

Common: The large variations between individual fish represent a challenge when applying HR NMR fingerprinting as authentication method. This was evidenced both in the classification of salmon according to geographical origin by ^{13}C NMR analyses of lipids, and classification of cod submitted to several processing conditions from ^{1}H NMR on low molecular weight metabolites. Any use of metabolic fingerprinting for authentication demands a database of authentic samples covering natural variation among the groups investigated (-and this database needs to be continuously updated in some cases). Databases for marine oils need not be as extensive as for fish, since marine oils are generally produced from fish batches.

6 Suggestions for further work

The results in this thesis show that ^{13}C NMR spectroscopy combined with chemometrics is a powerful tool to elucidate origin (wild/farmed, species and geographical-) and process history of fish and marine oils, and to detect adulterations. The applicability of the method is being evaluated further in a project funded by the NFR and Norwegian Food Safety Authority (NFR: 178264). In this project, analytical data (GC, ^{13}C NMR, analysis of pollutants on a subset of the samples) on reference samples of marine crude oils of different species and origins are collected, and added to an extensive database. Classification of an unknown sample is achieved by various multivariate techniques. A next step in this work would be to establish databases of refined oils.

As shown in this work, ^{13}C NMR regiospecific analyses are of value for authentication purposes. However, it has been shown that there is a degree of variation within species (e.g. according to temperature [Miller et al., 2006]), and studies investigating and mapping this variation (according to e.g. diet, season, age, size, maturity, and environmental factors) will be of value. Mapping of other species would also be important. It would be interesting to use instruments at higher fields (e.g. 850MHz instrument) to achieve reliable values on sn-2 position specificity of SFAs and MUFAs.

In this work, we analyzed mixtures of oils (salmon oil diluted with South American oil), and the results showed that the dilution could be quantified down to a 5% dilution. Thorough studies on the detection limits of other oils as adulterant should be performed (both for ^{13}C NMR regiospecific analyses and ^{13}C NMR fingerprinting).

^{13}C NMR phospholipid profiles in muscle lipids of lean fish were characteristic enough to discriminate species/stocks of gadoid fish. It would be interesting to investigate to which extent these characteristics can be attributed to genetic, dietary and/or environmental factors. Furthermore, the sn-2 position specificity of 22:6n-3 in PC and PE was rather similar among the species/stocks investigated. It would be interesting to investigate more species, preferably with more distant genetic relationships. For fish with higher lipid content, it would be convenient to separate the phospholipids from the triacylglycerols prior to NMR analyses.

When it comes to ^{1}H NMR metabolite profiling of low molecular weight metabolites in fish, the individual variation is a challenge, and the applicability of this technique combined with chemometrics for authentication according to species or processing conditions requires extensive databases. Detection of the most relevant signals and marker compounds for specific groups or processes will improve the classifications. It would be interesting to study the applicability of HR MAS, which allows intact fish muscle to be analyzed, for authentication purposes.

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PAPER VI

LOW MOLECULAR WEIGHT METABOLITES IN WHITE MUSCLE FROM COD (*GADUS MORHUA*) AND HADDOCK (*MELANOGRAMMUS AEGLEFINUS*) ANALYZED BY HIGH RESOLUTION ^{1}H NMR SPECTROSCOPY

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1 INTRODUCTION

Fish is considered as healthy food because of its content of n-3 polyunsaturated fatty acids (PUFAs), proteins and small bioactive compounds such as taurine, anserine, betaine and trimethylamine N-oxide (TMAO). PUFAs play a preventive role in cardiovascular diseases and in the alleviation of other health problems. Regarding the function of the small molecules, some of them stabilize protein structure[1] or regulate osmotic pressure in cells[2], while others have additional functions; taurine is an essential growth factor, and necessary for regulating the function of eyes, heart, muscles, brain and central nervous system[3,4,5], anserine is an antioxidant[6] and betaine is important for a proper liver function[7].

Previous studies used ^{1}H NMR spectroscopy to study changes in cod (*Gadus morhua*) and haddock (*Melanogrammus aeglefinus*) muscle during frozen storage[8]. Sitter *et al*. applied ^{1}H NMR to monitor several metabolites simultaneously during frozen storage of Atlantic halibut (*Hippoglossus hippoglossus L.)*[9]. Gribbestad *et al*. gave a detailed assignment of the ^{1}H NMR spectra from Atlantic salmon[10], while Martinez *et al*. applied the technique to evaluate changes in bioactive components following freezing, thawing, cooking and salting of cod[11].

The present study reports for the first time a full interpretation of the ^{1}H NMR spectrum of perchloric extracts from cod (*Gadus morhua*) and haddock (*Melanogrammus aeglefinus*), in order to identify compounds reflecting the physiological state and nutritional value of fish.

2 MATERIALS AND METHODS

2.1 Fish

Cod (*Gadus morhua*) (n=5, 3.6+0.6 kg) and haddock (*Melanogrammus aeglefinus*) (n=5, 0.34+0.05 kg) were caught in the Trondheims fjord (March and April respectively) and held live in a big landing net for about 6 h. Fish were individually taken out from the net and killed by a blow to the head within 10-20s. Samples of white muscle (1-2 g) were excised right under the first dorsal fin, accurately weighted and frozen in liquid nitrogen within 30-60 s after slaughter.

Perchloric acid (PCA) extraction was performed in ice according to Glonek *et al.*[12]. Ice-cold PCA (2mL, 7% w/v) was added accurately weighted frozen fish sample (1-2 g) and homogenized to a paste consistency (10-20s) with an Ultra Turrax homogeniser. Ice-cold PCA (1 mL, 7% w/v) was added to remove the residue on the homogeniser blade. The homogenate was centrifuged (3500 × g, 10 min, 4 º C). The supernatant was transferred to a new tube, while the extraction of the pellet was repeated twice as described above. All supernatants were pooled and neutralized (pH=7) with 2 M KOH in ice before centrifugation (3500 × g, 10 min, 4 º C). The resulting supernatant was stored at -80ºC until lyophilization.

2.2 NMR spectroscopy

The lyophilized extracts were redissolved in 0.7 mL of D2O containing trimethylsilypropionate-2,2,3,3-d4 (TSP, 0.5mM) and transferred to 5-mm NMR sample tubes. High resolution ^{1}H NMR spectra were recorded at ambient temperature on a Bruker Avance DRX500 spectrometer. The ^{1}H spectra were obtained using water presaturation in the relaxation delay. A sweep width of 10 kHz was collected into 32K points. Number of scans was set to 512. The raw data were multiplied with a 0.3 Hz exponentional line-broadening factor before Fourier transformation into 64K data points.

Some extract samples were spiked with small amount of the pure compounds (taurine, creatine, β-alanine, choline and anserine) to ensure correct assignment of peaks. The number of scans in the analyses of the pure compounds in D2O was reduced to 16.

The ^{1}H homonuclear correlated spectra (COSY) were obtained by applying water presaturation during relaxation delay and using gradient pulses for selection. The COSY spectra were recorded by acquisition of 90 transients per increment for 512 increments collected into 4K data points. A spectral width of 12 kHz was used in both dimensions. The time domain was zero-filled and apodized with a squared sine window function in both dimensions before Fourier transformation.

3 RESULTS AND DISCUSSION

3.1 Assignment of spectra

The 1D ^{1}H NMR spectra of the perchloric acid extracts from cod are given in Fig. 1. (Table of the assigned metabolites is available on request). The assignments were made using two dimensional ^{1}H homonuclear correlated spectroscopy (COSY), by spiking with some pure compounds and by comparison with ^{1}H NMR spectra of pure compounds in D2O. Additionally, the resonances of the compounds identified in the present samples were compared to those of the same compounds from other biological materials including human samples[13,14,15], plants[16], Atlantic Halibut (*Hippoglossus hippoglossus L.*)[9] and Atlantic salmon (*Salmo salar*)[10] for further confirmation.

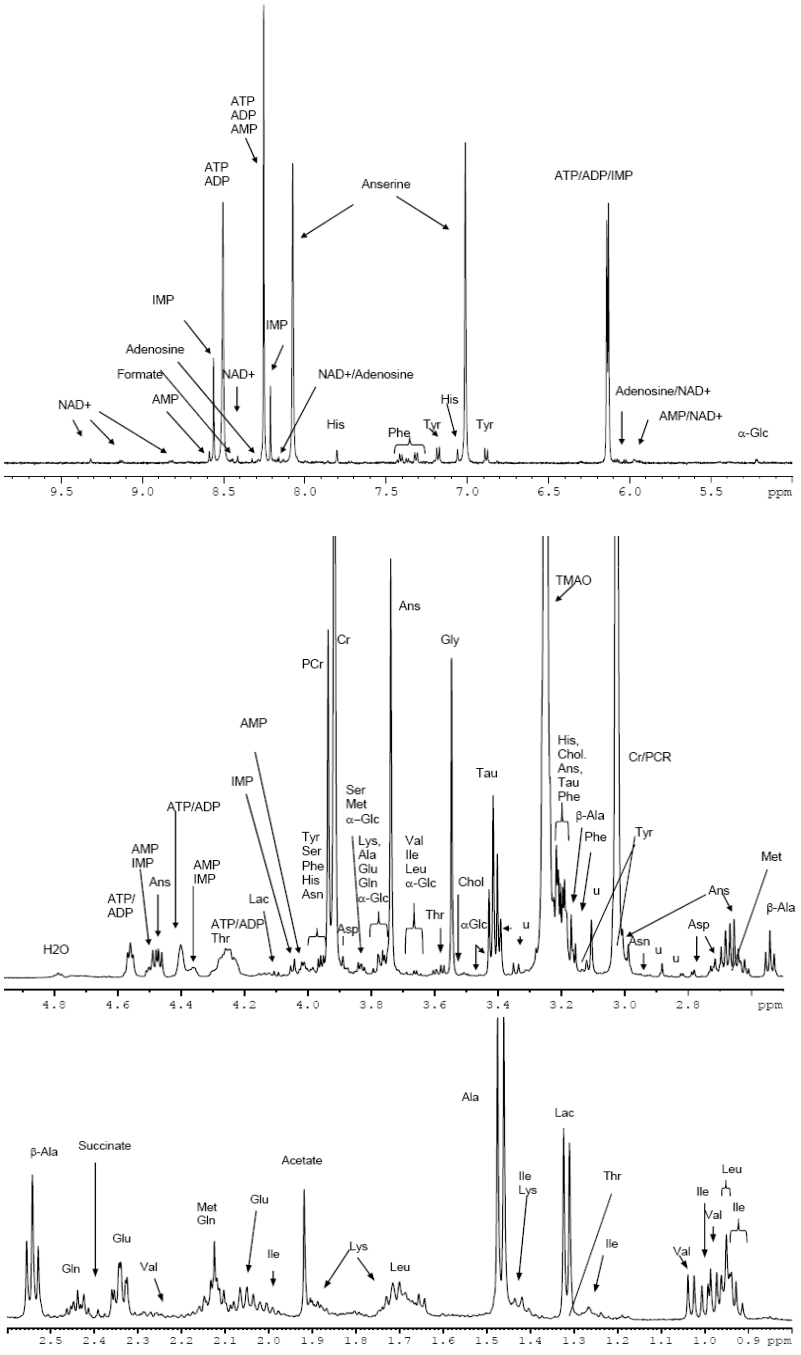

Figure 1 *Representative 500MHz ^{1}H NMR spectrum of PCA extracts (pH=7.2) from white muscle of cod.*

A wide range of metabolites was assigned, many of which give information on the metabolic condition of the sample analyzed. Differences both within and between the two fish species were observed. Relatively large differences between individuals of the same species were observed. This sample to sample variance may be due to the individual variation known to be found amongst fish samples[17] and differences in the handling of the fish (i.e. stress level)[18]. In addition, small inconsistencies in the extraction procedure might influence the level of labile compounds in the post-mortem fish muscle. Spectra of cod and haddock displayed to a large degree the same signals. This is consistent with earlier findings, which showed considerable overlap in chemical composition of the nitrogenous extractives of the two species[19,20].

3.2 Selected assigned metabolites

The peak of the absolute highest intensity in the spectra arises from the natural osmolyte trimethylamine N-oxide (**TMAO**). Many suggestions about the function of TMAO have been made. It is now generally believed that TMAO has an osmoregulatory role[21]. TMAO is reduced by bacteria to trimethylamine (TMA), which is responsible for the unpleasant "fishy" odor and taste of spoiled fish[22]. The signal arising from TMA (singlet at 2.91 ppm)[13] was not detected in the fish analyzed in our study, since the samples were taken immediately after death.

Creatine (Cr) and phosphocreatine (PCr) display singlets of high intensity in the spectra. Most of the Cr is phosphorylated (PCr) in resting muscle and supplies energy in the form of high energy phosphate for muscular contraction. Fish exposed to stress prior to death have lower values of PCr and ATP than unstressed fish[18]. The observed signal from PCr (3.92 ppm) in this study suggests that PCr has been preserved during the catching and the extraction procedure of the fish.

The signals from **amino acids** in the NMR spectra most likely stem from free amino acids since these are abundant in fish muscle; however, there might be contributions from peptides too. These compounds play important roles in physiological functions including osmoregulation and buffering capacity[21], in addition they contribute to the aroma and flavor of the fish[22] and increase its antioxidant capacity[23,24]. During storage and processing, proteins are degraded, and the level of free amino acids and peptides in muscle changes.

High content of **taurine** has been shown to be characteristic of white-fleshed fishes[22]. **Choline,** a precursor of acetylcholine, is important as a methyl donor in various metabolic processes including lipid metabolism[21]. Betaine, a derivative of choline, has been associated with osmoregulation[25] and acts as methyl donor in the synthesis of methionine from homocysteine. Previous studies showed that ^{1}H NMR could be used to quantify betaine in mussels[26] and cod[11]. In the present work betaine could not be detected because the two singlets arising from betaine (at 3.91 and 3.27 ppm), are presumably hidden under the higher intensity signals of creatine and TMAO.

Distinct peaks from **anserine** were visible both for cod and haddock samples. This compound is believed to function as a buffer during anaerobic metabolism, a fact reflected by the high levels in muscles used for burst activity[27]. In addition to an intracellular buffering function[28,29], anserine have been proposed to have additional roles controlling enzyme activity[30], inhibiting oxidative reactions[31] and as neurotransmitter[32]. Anserine decomposes to its constituents β-alanine and 1-methylhistidine by hydrolysis, a fact that permitted Ruiz-Capillas *et al*.[33] to estimate the loss of quality during ice storage by measuring the levels of 1-methylhistidine, β-alanine, anserine and tryptophan.

Adenosine triphosphate *(ATP)* predominates the **nucleotides** in muscle of live animals under normal conditions, but after death a series of enzymatic reactions leads to

decomposition of ATP to adenosine diphosphate (*ADP*), adenosine monophosphate (AMP), inosine monophosphate (*IMP*), inosine (Ino) and hypoxanthine (Hx). When the level of ATP has dropped under a critical level; the muscle enters *rigor mortis*, however both the extent of rigor and the nucleotide level varies along the fish body[34]. Another important factor in analyzing ATP in muscle is the sample handling and extraction procedure[35,11]. Hx is a contributor to the bitter off-flavour of spoiled fish while IMP is usually associated to the desirable taste of fresh fish. The degree of freshness is often expressed as the K-value[36], an indication of the content of ATP relative to its degradation products. Sitter *et al*. showed that ^{1}H NMR of perchloric extracts is a possible method for studying ATP degradation, and estimated the loss of freshness and K-value during ice storage of Atlantic halibut (*Hippoglossus hippoglossus L*)[9]. However, in some species the degradation of nucleotides is too fast, as in Pacific cod (*Gadus morhua macrocephalus*), or too slow, as in plaice (*Paralichthys olivaceus*), for the K-value to be of practical use in quality control[37].

In the present work, AMP and IMP were unambiguously identified in the spectra, while ATP and ADP could not be distinguished due to overlapping peaks, both in the 1D and the 2D spectrum. IMP content of unstressed fish at slaughter is found to be low[38,34]. The relatively high level of IMP in the spectra indicates that the fish were stressed at the time of slaughter, while the absence of detectable signals from Hx in the spectra is in accordance with the freshness of the fish. In comparison Gribbestad *et al*. assigned hypoxanthine in the spectra of salmon muscle which had been stored on ice for more than two days[10].

The doublet arising from **lactate** in the 1D spectrum is well resolved. Previous studies have shown that the level of lactate in fish muscle can be calculated both from ^{1}H NMR and ^{13}C NMR spectra[39,10]. The lactate concentration reflects the initial glycogen stores before death, the handling of the fish and the extraction procedure.

3.3 Species differences: cod vs haddock

Examples of differences between cod and haddock are given in Figure 2, where peaks arising from β-alanine, glutamate and glutamine are all of lower intensity in haddock than in cod. Conversely, the signals from α-glucose and β-glucose were higher in haddock than in cod (results not shown). More samples need to be analyzed to make conclusions about whether these differences are due to species-specific differences or differences in postmortem handling of the fish, fishing grounds, season, sex, species, size or biological conditions.

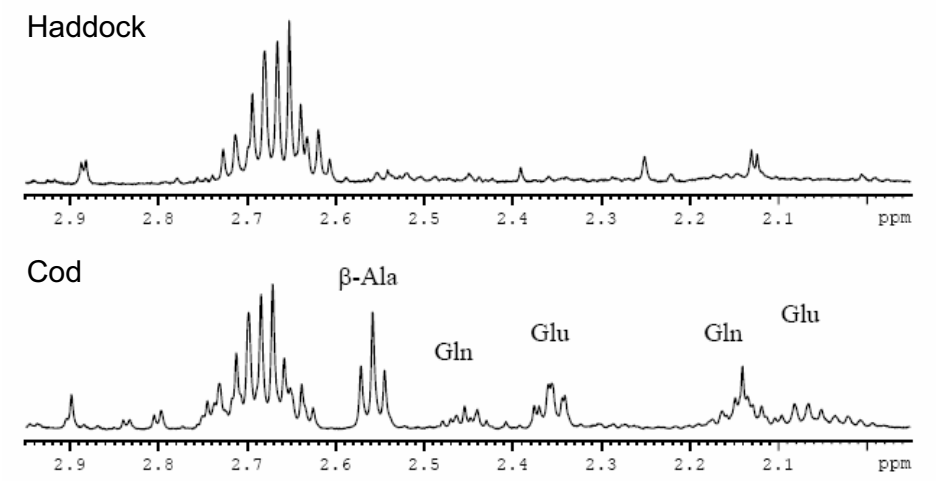

Figure 2 *Representative 500MHz ^{1}H NMR spectra of perchloric acid extracts of white muscle from haddock and cod. The lower level of β-Ala, Glu, and Gln in haddock compared to cod are examples of differences between the metabolic profiles obtained in this study.*

4 CONCLUSIONS

A wide range of metabolites was assigned, many of which give information about the metabolic condition of the sample analyzed. Cod and haddock displayed, to a large degree, the same compounds, however both intra- and interspecific differences in relative peak intensities were registered. Fish is known to display large individual differences in metabolite composition, and the present study does not permit one to attribute the differences to species specific factors or to others, such as environmental factors, development stage or physical condition. However, the results of this work may have practical applications in the use of high resolution NMR to further elucidate biochemistry and nutritional aspects of fish.

Abbreviations

Ile, isoleucine; Leu, leucine; Val, valine; Lac, lactate; Ala, alanine; Lys, lysine; Glu, glutamate; Met, methionine; Gln, glutamine; β-Ala, β-alanine; Ans, anserine; α-Glc, α-glucose; β-Glc, β-glucose; Asp, aspartate; Asn, asparagine; TMA, trimethylamine; Cr, creatine; PCr, phosphocreatine; Tyr, tyrosine; Phe; phenylalanine; Chol, choline; His, histidine; TMAO, trimethylamine N-oxide; Tau, taurine; Gly, glycine; Thr, threonine; Ser, serine; AMP, adenosine monophosphate; IMP, inosine monophosphate; ATP, adenosine triphosphate; ADP, adenosine diphosphate; NAD+, Nicotinamide adenine dinucleotide; u, unassigned peak.

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