Eva Falch

Lipids from residual fish raw materials

Quality assessment by advanced analytical methods

Doctoral thesis
for the degree of philosophiae doctor

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Norwegian University of
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PREFACE

Marine lipids have been my main scientific interest the last decade from working with quality improvement in a fish oil refining plant to quality assessment of fish feed and now research to improve the utilization of marine raw materials into high value lipids. I will thank SINTEF Fisheries and Aquaculture for giving me the opportunity to do a PhD. I was lucky to begin my career as SINTEF researcher partly by preparing the proposal for the Norwegian Research Council where my PhD was defined. It was thereby possible to make an influence on the activities and aims. The PhD period were set to three years from summer 02 till summer 05. In this period I have also been working part time on other research projects at SINTEF.

I have gained help and support from several people. It has been a joy to work with my supervisor, Dr. Turid Rustad, at Dep. of Biotechnology at NTNU. I am grateful for all the valuable scientific and practical advises she has given me and for her major contribution in improving my scientific writing. She has also contributed to encourage and supervise the ten students in biotechnology who has chosen our suggested topics for their thesis. Dr. Marit Aursand, my co-supervisor, and also my research manager at SINTEF, is an expert on NMR and lipids. She has been an outstanding source of inspiration both scientifically and through motivation, and I am very lucky to continue working with her.

I am also grateful to all my colleges at SINTEF Fisheries and aquaculture especially our experienced team working with marine lipids. Furthermore, I will thank the post graduate students for their contribution in finding relevant answers to bring us forward in this comprehensive field.

During my PhD work I was lucky to gain an EU scholarship for being a guest scientist at Dep. of Food Chemistry at The Royal Agricultural University of Denmark. A special thank to Acc. Prof. Mogens Andersen at KVL for teaching me ESR theory and practice. Prof. Thor Bernt Melø at Dep. of Physics at NTNU should also be thanked for helping me understanding the theory of ESR.

I am grateful to Dr. David Axelson for his help in making me see large multivariate data sets less complicated and also for valuable help in reading through my manuscripts. Much of my work has been in front of the computer managing the high field NMR magnets. In this situation I have received valuable help from PhD candidate Trond Størseth, Eng. Trond Singstad, Dr. Henrik Anthonsen, Dr. Beate Sitter, PhD candidate Inger Beate Standal and others working with

the HR-NMR instruments. A short working period visiting University of Århus and Prof. Hans J. Jacobsen introduced me to the interesting slow spinning NMR technique. Dr. Hanne Bertram, at Danish Institute of Agricultural Sciences, should also be thanked for initiating this stay and introducing me both to this research group and to the source of financial support (Nordic Network in Meat Science/NNMS).

My PhD work is part of a national project "Bærekraftig verdiskaping av marine biprodukter og bifangst" funded by the Norwegian Research Council. In the planning stages of this project, my supervisors and myself initiated an European project in the same research field. Luckily we got financial support from EU on the project "Characterisation and stabilisation of Gadidae species" with partners from 6 nations. This collaboration has also gained motivations on this area of research and resulted in strong international cooperation.

Finally, I will thank my nearest family Kato, Frida and Karoline. You will now hopefully see me in more action at home. I love you!

Yes!

SUMMARY

Fisheries and aquaculture generate considerable volumes of biomass that are wasted or applied for low value products. These biomasses may have potential as high value products due to their content of health beneficial nutrients, in particular the marine lipids that have been investigated in this current thesis. To upgrade these raw materials into high value lipids, more knowledge about availability, chemical composition and deterioration of the lipids is required. The work was divided into the following activities: (1) Provide data on the composition and quality of lipids in by-products from gadiform species (2) Study the applicability of different Electron Spin Resonance (ESR) spectroscopy techniques for assessment of the early stages of lipid oxidation in residual raw materials from fish (3) Study the applicability of ^{1}H Nuclear Magnetic Resonance (NMR) to evaluate lipid oxidation of marine oils and (4) Generate more knowledge of lipid composition and deterioration of cod lipids by adapting High resolution (HR) NMR applications for analysis of heterogeneous lipid extracts.

From a major sampling of gadiform species, current calculations showed that the residual raw materials (visceral fractions, heads, backbone and trimmings) made up ⅔ of the total catch. Furthermore, these calculations showed that an average daily production of cod fillet (10 000 kg) generated 1 000 kg of marine lipids with more than 30% of health beneficial n-3 fatty acids. Some significant variations in lipid composition were found among organs, species, and seasons. Liver contained higher levels of total lipids, consisting primarily of neutral lipids compared to viscera and trimmings that contained lower levels of total lipids comprising more polar lipids. The polar lipid fractions contained higher levels of polyunsaturated fatty acids. The lipids in the haddock (*Melanogrammus aeglefinus*) liver contained higher levels of polyunsaturated fatty acids (PUFAs) compared to cod (*Gadus morhua*), saithe (*Pollachius virens*) and tusk (*Brosme brosme*). The PUFA levels were lower in the viscera at the winter catch while no significant seasonal differences were found in the PUFA levels of trimmings or liver. Independent of the variations found, these raw materials contained considerable levels of the n-3 fatty acids.

Advanced spectroscopic methods, such as ESR and NMR, which have not previously been used for these specific applications were adapted to provide reliable data on lipid composition and deterioration. Free radical assessments by the ESR spin trapping technique detected the very early stages of the lipid oxidation and only a few minutes of oxidation of

docosahexaenoate (DHA) gave significant changes in the ESR spectra. The levels of free radicals trapped in cod liver oil and salmon oil during the first hours of oxidation were in accordance with the oxidative stability measured by conventional methods. This work highlights some of the precautions that are important to take in order to gain successful and trustworthy results by using ESR. ^{1}H NMR was found to provide valuable information about the oxidative changes occurring in reference standards of DHA. It was possible to study specific lipid oxidation products such as different hydroperoxides, aldehydes and also cyclic compounds. This information is not usually obtained by single conventional analytical methods. The spectra also demonstrated the effectiveness of α-tocopherols as antioxidant. Multivariate data analysis helped elucidate the changes in spectra during storage and showed a reduction of resonances originating from n-3 fatty acids during oxidation. However, the sensitivity was low (detection levels ~0.01 mM) but was shown to be improved by using the new Cryoprobe technology.

The changes in lipid class composition were significant during the short time of storage of cod gonads. Important changes due to lipase activity, such as formation of free fatty acids, hydrolysed phospholipids and also esterification of cholesterol were found. These changes were observed by NMR and from thorough interpretation of ^{13}C NMR spectra data on specific phospholipids, triacylglycerols, positional distribution of PUFAs in phospholipids, free fatty acids and hydrolysed acylglycerols and phospholipids obtained non-destructively from the total lipid extracts. NMR is unique due to its non-selective properties providing multi component determination in a single step. This technique is highly recommended for qualitative analysis of heterogeneous marine lipid extracts.

Additional analysis by using the magic angle spinning NMR technique showed that valuable compositional data may be obtained from analysis of intact gonads.

CONTENT

ABBREVIATIONS

FA	Fatty acids
FFA	Free fatty acids
PUFA	Polyunsaturated fatty acids
HUFA	Highly unsaturated fatty acids (≥20 carbon atoms and ≥ 3 double bonds)
SFA	Saturated fatty acids
MUFA	Monounsaturated fatty acids
GC	Gas chromatography
LC	Liquid chromatography
MS	Mass spectroscopy
TLC	Thin layer chromatography
NMR	Nuclear magnetic resonance spectroscopy
HR-NMR	High resolution nuclear magnetic resonance spectroscopy
MG	Mono acylglycerols
DG	Di acylglycerols
ESR	Electron spin resonance spectroscopy
TMS	Trimethyl silane $(CH_3)_4Si$
TSP	Trimethyl Sodium Propionate
PL	Phospholipids
PC	Phosphatidylcholine
PE	Phosphatidyletanolamine
TAG	Triacylglycerols
DHA	Docosahexaenoic acid (C22:6n-3)
EPA	Eicosapentaenoic acid (C20:5n-3)
TRI DHA	Tri-docosahexaenoate
DHA EE	Ethyl docosahexaenoate
PBN	α-Phenyl-N-tert-butylnitrone
TEMPO	2,2,6,6-tertamethylpiperidine-1-oxyl
GADIFORM	fish species of the gadiform order; cod, saithe, haddock, tusk and ling
CLO	Cod liver oil
PCB	Polychlorinated biphenyls
Cod	*Gadus morhua*
Saithe	*Pollachius virens*
Haddock	*Melanogrammus aeglefinus*
Tusk	*Brosme brosme*
Ling	*Molva molva*

1. LIST OF PAPERS

BY-PRODUCTS FROM FISH

Paper I E. Falch, T. Rustad, and M. Aursand, By-products from gadiform species as raw material for production of marine lipids as ingredients in food or feed. Process Biochemistry. 2006, 41, 666-674.

Paper II E. Falch, M. Sandbakk, and M. Aursand, On-board handling of by-products to prevent microbial spoilage, enzymatic reactions and lipid oxidation, In book: Maximizing the value of marine by-products (Prof. F. Shahidi, Ed) – accepted manus - invited authors

LIPID OXIDATION

Paper III E. Falch, H. Anthonsen, D. Axelson, and M. Aursand, Correlation between ^{1}H NMR and traditional analytical methods for determining lipid oxidation in ethylesters of Docosahexaenoic acid. Journal of the American Oil Chemist Society. 2004, 81(12) 1105-1109

Paper IV E. Falch, J. Velasco, M. Aursand, M. Andersen, Detection of radical development by ESR spectroscopy techniques for assessment of oxidative susceptibility of fish oils. 2005, Eur. J. Food Res. 221(5) 667-674.

Paper V E. Falch, and M. Aursand, Resonance spectroscopy to study lipid oxidation in fish and fish products, Handbook of modern magnetic resonance, Edited by Graham Webb, Kluwer Academic/Plenum Publisher, New York, USA (2006). In Press

COMPOSITION OF LIPIDS BEFORE AND AFTER HYDROLYSIS

Paper VI E. Falch, T. R. Størseth, and M. Aursand, HR NMR to study quality changes in marine by-products, Magnetic Resonance in Food Science. The Multivariate Challenge. SB Engelsen, PS Belton and HJ Jakobsen (eds.), The Royal Society of Chemistry, Cambridge, 2005, 11-19

Paper VII E. Falch, T. R Størseth, and M. Aursand, High resolution NMR for assessment of lipid classes and acyl stereospecific position of fatty acids in marine phospholipids, Submitted to Chemistry and Physics of Lipids, November 4th

Paper VIII E. Falch, T. R. Størseth, and M. Aursand, High resolution NMR for studying lipid deterioration in cod (*Gadus morhua*) gonads.

The following papers and proceedings present studies carried out during this period and may be used as a background reading on utilization of cod by-products but they are not regarded as part of the thesis:

Falch, E.; Jonsdottir, R.; Rustad, T.; Shaw, N.B.; Arason, S.; Kerry, J.P., Dumay, J.; Berge, J.P.; Sandbakk, M.; Aursand, M. Geographical variation in lipid composition of different by-products from species of the Gadidae family. *Journal of Food Composition and Analysis* **2005(a).** In press

Falch, E.; Øverby, A.; Rustad, T. Natural antioxidants in cod liver oil: Pit falls during oxidative stability assessment, In *WEFTA 2005 book*, Luten, J.; Jacobsen, C., Bekaert, K., Oehlenschlager, J.; Sæbø. A. Eds. Accepted manus (after minor changes) **2005 (b**)

Falch, E.; Størseth, T.; Aursand, M. Quality changes in fish by-products evaluated by high resolution nuclear magnetic resonance spectroscopy. Proceedings in the Novel Analytical Method part of the 34th WEFTA Meeting, September 2004, Lübeck, Germany, 2004; pp 216-219.

Rustad, T.; Falch, E. Making the most of fish catches. *Food Science & Technology,* **2002,** pp 36-39.

Slizyte, R.; Dauksas, E.; Falch, E.; Storrø, I.; Rustad, T. Yield and composition of different fractions obtained after enzymatic hydrolysis of cod (*Gadus morhua*) by-products. *Process Biochemistry* **2004,** *40* (3-4), 1415-1424.

Slizyte, R.; Dauksas, E.; Falch, E.; Storrø, I.; Rustad, T. Characterisation of protein fractions generated from hydrolysed cod (*Gadus morhua*) by-products, *Process Biochemistry*, **2005**, *40*(6), 2021-2033

Dauksas, E.; Falch, E.; Slizyte, R.; Rustad, T. Composition of fatty acids and lipid classes in bulk products generated during enzymic hydrolysis of cod (*Gadus morhua*) by-products, *Process Biochemistry*, **2005,** *40*(8): 2659-2670

Slizyte, R, Alves-Filho, O.; Falch, E.; Rustad, T. The influence of drying processes on functional properties of fish protein hydrolysates from cod (*Gadus morhua*) by-products, Proceedings from 2nd Nordic Drying Conference, 25-27th June 2003.

Michelsen, H, E. Falch and T. Rustad, (2004) Utilisation of by-products from farmed Atlantic salmon (*Salmo salar*) Proceedings at the 34th WEFTA Annual Meeting, 12-15 September, Lubeck Germany

2. INTRODUCTION

Globally, more than 134 million tonnes of fish and shellfish are caught each year [FAO, 2005], and of this about ¼ is discarded. The amounts discarded include species that are under-utilized, by-catch, unconventional or unexploited and also ***residual*** raw material from utilized fish also known as ***by-products***. These fractions might be sources of the health beneficial marine lipids with applications in health promoting products for human and animal nutrition [Rustad and Falch, 2003,]. The use of fish oil for human consumption has been increasing gradually for the last decades [Aidos et al, 2002] and dietetic research has shown that most people do not have enough n-3 fatty acids in their diet [Horrock and Yeo, 1999; Simopolous 2002]. The levels of the characteristic long chain polyunsaturated n-3 fatty acids (PUFA) such as eicosapentaenoic acid (EPA) and docosahexaenioic acid (DHA) make marine lipids unique compared to other lipid sources. These fatty acids are believed to play a preventive role in cardiovascular disease and in the alleviation of other health problems [Uauy and Valenzuela, 2000; Vanschoonbeek et al. 2003; Stillwell and Wassall, 2003; Calder, 2004]. In recent years, the industry has shown a growing interest in new product development including incorporation of different lipid components from fish in pharmaceuticals (eg. capsules), and in functional food as microencapsulated lipids [Johnsen et al, 2002]. New fish oil products based on refined oil or its derivatives with higher concentrations of specific fatty acids are constantly being introduced into the international market. Production of structured lipids by enzyme technology is also industrially established and new processes are on the way to being industrialised [Borch, 2005; Holm; 2005; Sjold-Jørgensen, 2005; Haraldsson, 2005; Guo et al, 2005; Scrimgeour, 2005] where marine n-3 PUFAs might be important ingredients [Halldorson and Haraldsson, 2005; Haraldsson, 2005]. The health properties and the biochemical stability of the products are expected to be dependent on the molecular nature of the lipids (e.g., as ethyl ester or triacylglycerols and their positional distribution in the triacylglycerol molecule) and the relative concentration of the n-3 fatty acids.

Marine raw materials are highly susceptible to lipid oxidation and to enzymatic hydrolysis leading to unpleasant flavour and reduction of nutritional value. These processes are among the main reasons for these large volumes being discarded or produced for low value applications in a market demanding high quality marine lipid as food ingredients [Kolanowski and Laufenberg, 2005]. Marine lipids are complex classes of compounds containing various different

fatty acids free or esterified mainly to phospholipids or triacylglycerols. Other lipid components found in fish are sterols, waxes, hydrocarbons, vitamins, antioxidants and degradation products from lipids. These components affect the biochemical processes at different levels. The spoilage processes affecting lipid composition might be due to endogenous enzymes (particularly lipases and phospholipases), microbial processes or to oxidative reactions. These spoilage processes proceed simultaneously leading to production of a wide variety of reaction products. Analytical methods to elucidate compositional changes that occur in this material are valuable (1) to determine the shelf life, (2) to reveal unknown processes, (3) for evaluating methods of conservation and (4) to secure the food safety by controlling chemical compounds. Due to intensive research and development of new and more powerful analytical techniques, it is now possible to study the chemical composition and the biochemical changes that occur in marine lipids. To date, analytical methods to study compounds affecting the organoleptic and nutritional quality of marine lipids are diverse and selective to specific lipid compounds. High resolution NMR spectroscopy to study mixtures of compounds provides information on a broad range of chemical compounds that could otherwise only be obtained by the use of numerous conventional analyses, such as GC, HPLC and TLC methods. Furthermore, HR NMR is non-destructive and pre- treatment steps that might influence the composition are not needed [Gunstone, 1993; 1994; Diehl, 2001; Siddiqui et al., 2003].

Lipid oxidation is observable by a trained sensory panel at low detection levels [Kulås et al, 2003] but more efficient and objective methods for assessment of early changes are needed. Resonance spectroscopy techniques may be applied to study changes in lipids. In this PhD work Nuclear Magnetic Resonance (NMR) spectroscopy and Electron Spin Resonance (ESR) spectroscopy are the two main techniques used to study changes in marine lipids, but also traditional methods such as peroxide value, conjugated dienes, thiobarbituric acid reactive substances, anisidine value and oil stability index are presently applied. While the ESR method is limited to study free radicals, NMR can show a detailed composition of the lipids.

The future demand for the nutritious marine lipids should, to a certain extent, be met by maximizing the value of the total catch of fish by utilization of the residual raw materials. Such action will prevent excessive harvesting and help secure a sustainable development of our fish stocks, that today are fully exploited [FAO, 2004, 2005]. Conversion of by-products into higher value products will increase the profitability of the utilisation and will motivate the industry to increase the utilisation of these fractions. In order to increase the total utilisation of fish, the fishermen should be able to predict and calculate the amounts and composition of the by-product. In the

recently developed *Processing table* in *FishBase* (www.fishbase.org) [Froese and Pauly, 2000], data on the weight fraction of by-products are about to be included. For the gadiform species, which are the targeted species in this PhD work, such data are not yet established. The industry should have data on the available amount of residual raw material, amount and composition of the lipids and to what extent the content and composition vary with factors such as fraction, species, sizes and fishing grounds. High value applications of these lipids generally require a specified chemical composition and a minimum and stable delivery quantum.

Gadiform species (cod, saithe, haddock and tusk), which are the targeted species in this PhD work, are among the main commercial fish species caught in the Northeast Atlantic Ocean. The amount of available lipids from gadiform species is calculated in the first part of this thesis [Paper I] along with a presentation of the technology for the on-board handling of fish by-products to prevent microbial spoilage, enzymatic reactions and lipid oxidation [Paper II]. The second part deals with the lipid oxidation, which is the most important factor reducing the shelf life of edible oils and should be conquered to meet the demand for food grade quality lipids. In this chapter the advanced techniques ESR and NMR are evaluated for their effectiveness [Paper III – V]. The last part of this thesis deals with the development of NMR as a tool for compositional analysis of cod lipids, in particular phospholipids and triacylglycerols [Paper VII]. The effectiveness of different NMR techniques for analysis of biochemical changes in lipids is investigated [Paper VI and VIII].

3. BACKGROUND

3.1 Fish by-products or residual raw material

Residual raw material from fish includes head, bones, trimmings and visceral fractions such as liver, stomach and gonads. At present, there is a limited utilization of these residual raw materials, and the utilised fractions are mainly of low value and not intended for human consumption.

Fisheries and aquaculture are important global industries due to its supply of healthy food. Norway, which is one of the leading exporters of fish, is managing some of the most important productive biomasses in the world. Fisheries and aquaculture are important for their contribution to export income and also for employment, especially in the coastal regions. It is regarded as a very important future industry due to the production of renewable resources [Almås, 1999; RMW, 2004, Pedersen, 2005]. The potential by-products from the Norwegian fishing industry are estimated to be approximately 605 thousand tonnes of which 75% are currently utilised [Rubin, 2005]. Only 50 thousand tonnes are processed into food or other high value products [Rubin, 2005]. It is therefore of great potential interest for the fishing industry to land and utilize a greater part of the total catch for higher value products.

The current practice of dumping potential by-products to waste results in both a loss of valuable food, as well as undesirable environmental impact if the biomass are dumped close to the coast. FAO has recently reported that Atlantic cod, along with other fish species in the Northeast Atlantic, are overexploited or depleted [FAO, 2004, 2005]. Fishing quotas and licences controlling the catches and the global catch has not been increasing during the last two decades, and the total catch cannot be expected to increase above current levels. Maximizing the utilization of the total catch of fish is therefore securing the sustainability of our marine resources and makes it possible to partly meet the increasing demand for marine food and feed.

There are several definitions of the term ‘by-products’ and when fish is the target product the definitions are mainly excluding these resources as food ingredients. Fish by-products have been previously defined as ‘products made from raw materials which are for reasons of species, size, quality, availability or processing capacity, unsuitable for direct human consumption’ [Windsor and Barlow, 1981] and ‘any carcase or part of any animal or fish or any product of animal

origin not intended for direct human consumption with the exception of animal excreta and catering waste' [Walsh, 1994]. The European Commission [2003a] has used the following definition of fish by-products: 'Secondary or incidental product of an industrial or manufacturing process - including wild caught fish or part thereof that are not used for human consumption, and materials (e.g. head, frames and trimmings) generated from processing of wild and farmed fish for human consumption as well as mortalities from fish farms'. Fish are included in the recent 'animal by-product regulations' from the European Commission [2003b]. These definitions are delimiting the by-products into low value products and a new terminology is needed for the fractions that are produced into food. The Norwegian meat industry has implemented the term Plus Products ('PLUSSprodukter') [Sollerud, 2005], meaning that this production will add value to the total production. The term co-products are also used. Internationally, the term by-product is still used for secondary products or products that are not regarded as the main product [Bechtel, 2003; Shahidi, 2005]

Residual raw material from gadiform species (cod species) comprise visceral fractions and cut-offs from filleting (Fig. 1). Today, these raw materials are processed into consumer products in some parts of the world. In Iceland, the main volumes of cod head are dried and exported to Africa [Syversen et al, 2000; Arason, 2003]. Trimmings are de-boned and converted to minced fish product. Liver is either canned or, more commonly, used in the production of cod liver oil. Stomachs are considered as a culinary delicacy in Iceland and some Eastern countries [Archer, 2001]. There is also a limited production of hydrolysed proteins from the protein rich residual raw materials. Cod roe is used as ingredients in spreads [Bledsoe et al, 2003], while the milt is less utilised, but minor volumes are used for extraction of DNA or phospholipids [Eximo A.S. (www.eximo.no)]. Phospholipids, in general, are widely used in food, pharmaceuticals and cosmetic products due to their emulsification, stabilisation, antioxidants and nutritional properties [Schneider, 2001; Sampalis, 2003; Guo et al, 2005; Vikbjerg, 2005]. Marine phospholipids are also used in speciality feed particularly for marine larvae nutrition [Coutteau et al, 1997; Leigh et al, 2004].

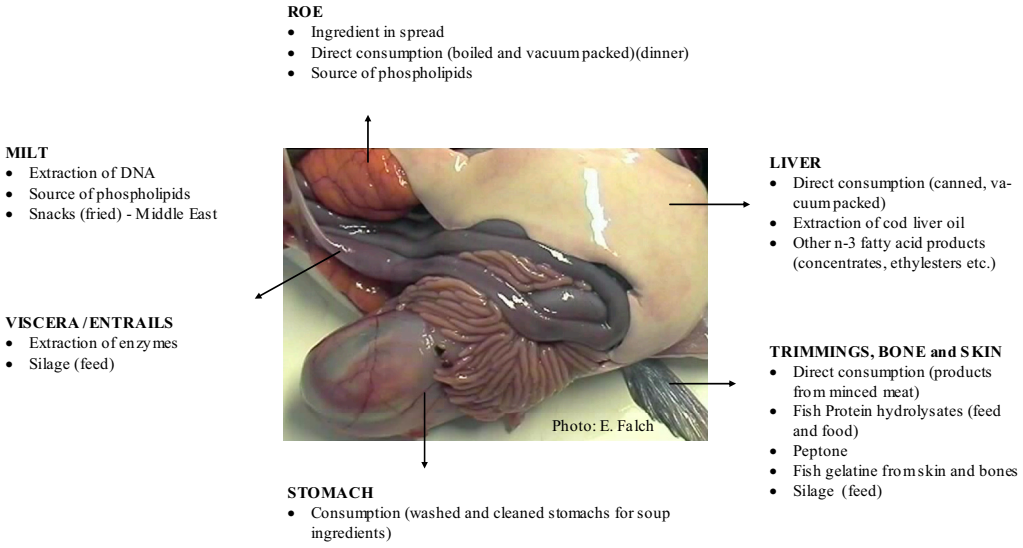

Fig. 1. Overview of possible applications of different residual raw material from processing of cod.

3.2 Marine lipids and health aspects

Fish lipids are natural sources of polyunsaturated n-3 fatty acids (PUFA) such as docosahexaenoic acid (DHA; 22:6n-3) and eicosapentaenoic acid (EPA; 20:5n-3). The n-3 PUFAs in fish are reported to prevent coronary heart diseases [Dyerberg et al. 1978 Vanschoonbeek et al. 2003], have a positive effect on brain and nervous system [Nettleton, 1993] and stimulating the immune system [Khalfoun et al, 1997; Field et al, 2001]. In recent years these fatty acids are used in antipsychotic treatment [Peet, 2004] and new health effects are still being discovered [Uauy and Valenzuela, 2000; Vanschoonbeek et al. 2003; Stillwell and Wassall, 2003; Calder, 2004].

The interest in using phospholipids as carriers of n-3 PUFAs or drugs (in pharmaceuticals) is increasing and studies of lipid metabolism in infants have shown a higher absorption with DHA in phospholipids than DHA in breast milk [Makrides et al, 2002]. The acyl regiospecific distribution of the fatty acids in the triacylglycerols and phospholipids play a key role in digestion and absorption of lipids. Previous studies have shown that EPA and DHA in the *sn-2* positions are more readily absorbed compared to structured lipids where these fatty acids are esterified to one of the other positions of the triacylglyserol [Christensen et al, 1995]. The positional distribution of the natural forms of these lipids is therefore important, also when

producing structured lipids and concentrated marine n-3 PUFAs for health improvement. ^{13}C-NMR is one of the valuable methods used to study the positional distribution, both in triacylglycerols and phospholipids [Paper IV]. Additional components in marine lipids with recognised positive nutritional effect are the fat soluble vitamins which previously (before 1970) were understood as the bioactive compounds in marine lipids.

Reaction products from lipid oxidation [Kulås et al, 2003] and lipolysis (free fatty acids) [Refsnegaard et al, 1998, 2000] are known to affect the organoleptic properties of fish products. There is also reason to be aware of possible negative health effects of reaction products generated during lipid oxidation. Primary reaction products from lipid oxidation are unstable and break down to form a wide range of reaction products with varying toxicological significance [Kubow, 1990]. Some of the compounds are toxic at high concentrations and studies have shown that hydroperoxides and aldehydes might cause damage of DNA [Yang and Schaich, 1996]. Furthermore, animal studies have shown that a high fish diet increased oxidative stress potential in the mammary glands of spontaneously hypertensive rats [Metha et al, 1994] and increased oxidation in different organs of experimental animals fed PUFA rich diets [Diniz et al, 2004]. Feeding of oxidised cod liver oil to different experimental animals was reported to provide a wide spectrum of injurious effects [Sanders, 1994]. However, a concrete relevance in development of diseases is still unclear. A high intake of PUFAs is known to increase the physiological requirements for antioxidants that are also effective in-vivo [Muggli, 1994; Sanders, 1994] and a balance in the oxidants and antioxidants in a PUFA rich diet is believed to be important. Marine lipids also contain cholesterol, particularly the organs that contain high levels of phospholipids, such as fish gonads [Paper II, VI-VIII]. There is considerable evidence that the biological activities of some of the cholesterol oxidation products (oxysterols) are associated with human diseases [Addis, 1986; Linseisen and Wolfram, 1998a,b; Kubow, 1990; Hwang, 1991; Sanders, 1994; Valenzuela et al, 2004]. The cholesterol oxidation products are linked to atherogenesis, cytotoxicity, mutagenesis, and carcinogenesis and health implications of oxysterols have been recently reviewed [Linseisen and Wolfram, 1998a,b; Hwang, 1991]. The development of new analytical techniques for assessment of reaction products from lipid oxidation and other biochemical reaction products in lipids might give new information on the specific products formed.

One of the important issues in the discussion of the utilisation of marine lipids in healthcare products has been the levels of persistent organic pollutants and heavy metals accumulating in the lipid fractions of fish. Particularly, the levels of PCB and dioxins are reported to be high in some fish species [Smith and Gongolli, 2002] and purification of oils is therefore required before

consumption. While there are technologies available for removal of these pollutants in the neutral oil fractions [Hjaltason, 2002; Maes et al, 2005; Breivik and Thorstad, 2005], it is reported that no cost effective processing alternatives for removing dioxins in viscous lipids (incl. phospholipids) or fish meal are published [Baron et al, 2005].

In spite of the potential risk of consuming toxic compounds from fish, the health benefits are concluded to be of principal importance [Sidhu, 2003; Wang et al, 2005]. These potential toxic substances, particularly in oils, may be minimized by gaining more knowledge about the chemical composition and use of the most suitable handling and purification processes.

3.3 Lipid resources from cod residuals

Gadiform species such as cod, saithe, haddock and tusk are important species for the North European Fisheries. Of the total landings of approx. 2.9 mill tonnes of raw material to Norwegian fisheries, gadiform fish species comprise more than 500 thousand tonnes [Fiskeridirektoratet, 2004] with a value of more than 40% of the total value of the Norwegian catch of fish and shellfish. Fundamental for high value utilisation of these by-products is that the processors are able to deliver first class and standardized qualities with a known chemical composition.

It is well known that the chemical composition of fish may vary with factors such as age, body size, stage of sexual maturity and diet [Damberg, 1963; Jangaard et al, 1967a,b; Love et al, 1972; Tocher and Sargent, 1984; dos Santos et al, 1993; Ingolfsdottir et al, 1998]. Most of the published data on amount and composition of by-product fractions have a biological rather than a processing point of view and data on the compositional variation are of great value for processors of marine lipids.

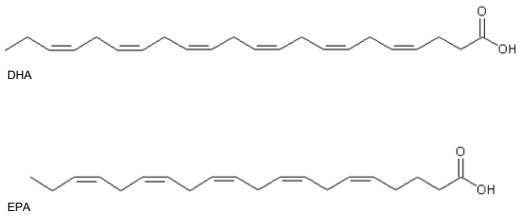

Fig. 2. The chemical structure of EPA and DHA which are the most characteristic n-3 fatty acids of marine origin

3.4 Biochemical changes in marine lipids

Lipolytic activity post mortem

Marine raw materials are highly perishable and conservation immediately post mortem is fundamental for producing food grade products [Paper II]. Deterioration is due to microbial spoilage and biochemical processes; however, microbial spoilage is not within the scope of this thesis work and will therefore not be discussed here. Biochemical processes, such as hydrolysis catalysed by endogenous enzymes, are important factors reducing the nutritional value and shelf life of marine raw material and are thereby limiting their utilization [Paper II]. The lipolytic enzymes can be divided in two main groups: (1) lipases which hydrolyse triacylglycerols into acylglycerols and free fatty acids and (2) phospholipases which hydrolyse phospholipids into lysophospholipids, free fatty acids, acylglycerols and phosphatide derivatives. Basic descriptions of these reactions are shown in Fig. 3. Levels of free fatty acids are among the main parameters used to classify different quality levels of fish oils. The guideline specifications of FFA in crude fish oils are 2% [Codex, 1999] and 2-5% [Young, 1985] while the levels should generally be below 2% (as acid value) in medicinal cod liver oil [European Pharmacopoeia, 2003]. Levels of free fatty acids in crude oil may be reduced by alkali refinement to levels less than 0.05% [Gunstone, 2004] but if the levels are too high, it may be reduced by traditional refinement. The raw material handling is therefore crucial.

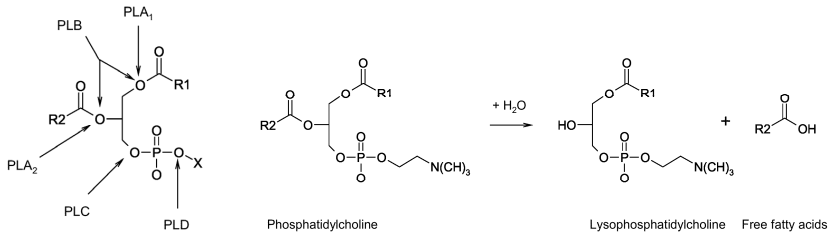

Fig. 3 Various enzymes catalysing phospholipid hydrolysis. PLA_1, PLA_2 and PLB are phospholipases belonging to the group acyl hydrolases together with lysophospholipases while PLC and PLD are phosphodiesterases. R_1 and R_2 denotes fatty acids and X represents the polar head group. The hydrolysis reaction of phosphatidylcholine is shown on the right hand side.

The lipolytic activity in residual raw materials from gadiform species and inactivation conditions has recently been determined [Søvik, 2005; Søvik and Rustad, 2005]. These studies demonstrated

seasonal variations in lipolytic activity in liver, viscera and cut-offs from Atlantic cod showing higher activity in these raw materials during the summer and spring compared to the winter catch. The activity of lipases was also reported to be influenced by fishing ground. Heating at 85^{o}C for 10 minutes has been used for complete inactivation of lipases [Rustad, personal comm.]. In addition to prevent taste deterioration of lipids [Refsgaard et al, 2000], inactivation of lipases is also believed to reduce the lipid oxidation since the free fatty acids are reported to oxidize more rapidly than the esterified fatty acids [Shewfelt, 1981]. Triglyceride hydrolysis is suggested to lead to increased oxidation while phospholipid hydrolysis is reported to produce the opposite effect [Shewfelt, 1981].

Lipid oxidation

Marine oils are highly susceptible to oxidation, mainly due to the high content of long chain PUFAs. Since photo-oxidation is easily prevented by eliminating light, only the more challenging auto-oxidation will be discussed in this thesis. Auto-oxidation (Fig. 4) of fatty acids proceeds through a chain reaction where a loss of hydrogen ions initially leads to formation of free radicals. The radicals react further into peroxyl radical (ROO·) and produce hydroperoxides. The hydroperoxides are labile compounds that easily cleave or produce alcoxyl radicals (RO·) leading to a variety of reaction products. Non-radical species such as aldehydes, ketones, acids, alcohols and also more complex reaction products such as epoxy- and polymeric compounds (Fig. 4) are formed during the propagation and termination steps.

In fatty acids, the bisallyllic CH bonds (-CH=CH-$\underline{CH_2}$-CH=CH-) have the lowest bond energy (75 kcal/mol) and this is therefore the most reactive site for auto-oxidation [Simic et al, 1992]. In bulk oils, the rate constant for producing hydroperoxides is proportional to the number of these bisallyllic sites, which make the long chain PUFAs found in marine lipids very prone to auto-oxidation. The oxidizability of DHA is reported to be five times greater than in 18:2 [Cosgrove et al, 1987]. In addition, the composition of primary oxidation products (hydroperoxides) is more complex with higher levels of bisallyllic sites in the fatty acids. Frankel [1998] has reported more than 10 hydroperoxides from oxidation of DHA (4-, 7-, 8-. 10-, 11-, 13-, 14-, 16-, 17-, and 20 hydroperoxide) and 8 from oxidation of EPA (5-, 8-, 9-, 11-, 12-, 14-, 15-, and 18 hydroperoxide), while Paper III, in this thesis, demonstrates 10 resonances (-OO$\underline{H}$) developed during oxidation of ethyl docosahexaenoate and approximately 14 developed during oxidation of tridocosahexaenoate by the use of ^{1}H NMR. Kobayashi et al [2003], who identified each of the monohydroperoxides in EPA and DHA by GC-MS, also reported an inverse oxidisability of

these fatty acids in emulsions, showing that DHA was more stable than the fatty acids containing fewer bisallyllic positions. Lydberg et al [2005] detected 8 monohydroperoxides from oxidation of DHA. While the levels of monohydroperoxides decreased after a few days of storage, the levels of polyhydroperoxides increased throughout the oxidation period. Fatty acids containing more than two double bonds are previously reported to produce significant amounts of hydroperoxy epidioxides at early stages of lipid oxidation [Frankel, 1998] and polymerisation and cyclisation of unsaturated fatty acids in acylglycerols are common reactions.

The lipid composition has a significant influence on the auto-oxidation. Marine lipids comprise more than 25 different principal fatty acids as part of acylglycerols, phospholipids and hydrolysed derivatives (lysophospholipids, mono- and diacylglycerols and free fatty acids) with different oxidizability. Recent studies of oxidisability of DHA have shown that DHA in phospholipids are more resistant to lipid oxidation than DHA in triacylglycerols [Song et al, 1997; Lydberg et al, 2005]. Other lipid constituents in fish such as sterols (mainly cholesterol), steryl esters, fat soluble vitamins and antioxidants may also oxidize to produce new compounds. Pro-oxidative compounds such as haemoglobin from blood and pro-oxidative metals are also influencing the lipid oxidation progress and these compounds in fish have been recently thoroughly investigated [Lauridsen et al, 1999; Richards and Hultin, 2002; Kristinsson and Hultin, 2004; Undeland et al, 2004].

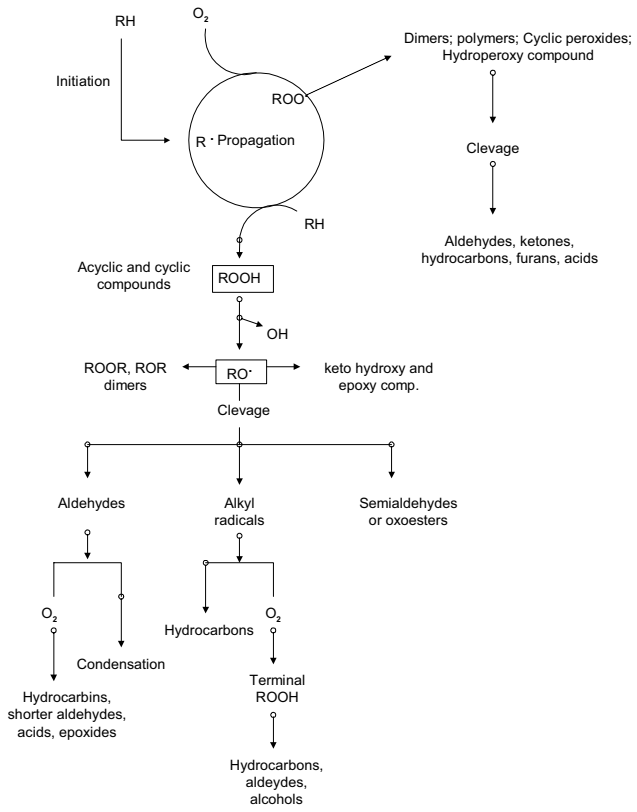

Fig. 4. Overview of the lipid oxidation processes [Nawar, 1996].

3.5 Analytical tools for quality assessment

Traditional methods

Some volatile lipid oxidation products in fish oil are detectable by sensory analysis at levels as low as 10^{-2} - 10^{-5} µg/g oil [Kulås et al, 2002], and methods to evaluate early stages of oxidation before the oil reaches the end of its shelf life are therefore required. The complex nature of marine lipids requires advanced analytical techniques for compositional analysis and for assessment of degradation products affecting the sensory attributes and nutritional value.

Since lipid oxidation is the most important factor limiting the shelf-life of marine oils, the industry has generally implemented methods to assess these changes in their quality control systems. There are a number of methods available to determine lipid oxidation in food systems, but there is not one single method that alone can give a complete and satisfactory description of

the oxidative status [Frankel, 1998]. Table 1 gives the basic principles along with some important advantages and disadvantages of relevant analytical methods for determining lipid oxidation.

The most common methods for analyzing primary lipid oxidation are the peroxide value or conjugated dienes. Since the peroxides are labile components that are rapidly transformed into secondary products [Frankel, 1998] these methods have to be combined with analysis of secondary oxidation products. Thiobarbituric acid reactive substances (TBARS) and anisidine value are both methods that determine the presence of aldehydes, which are secondary oxidation products. The Totox value is still one of the most commonly used oxidation parameter in plant laboratories and commercial laboratories in Norway. This value is a combination of the peroxide value and the anisidine value [Frankel, 2005]. Due to rapid polymerisation of EPA and DHA compared to the formation of stable peroxides of these fatty acids, peroxide value is reported to be an unreliable indicator of lipid peroxidation in fish [Choo et al, 1987].

Other techniques for evaluating oxidation are based on accelerated oxidation. The Oil stability index method [Jebe et al, 1993], the Rancimat test [Mendez et al, 1997] and oxidative stability measurement by Oxidograph [Vinter, 1991, Falch, 1999; Falch et al, 1999] are methods suitable for analysis of oil systems.

Recently, effort has been put into evaluation of new techniques for assessment of lipid oxidation [Frankel, 2005]. This includes free radical assessment by ESR spectroscopy (see chapter 3.7) and determination of primary and secondary lipid oxidation products by different chromatographic techniques. The GC-MS technique enables detection of a wide range of secondary volatile lipid oxidation products [Jonsdottir, et al., 2004; Olsen et al, 2005] while LC-MS is reported to determine also non-volatile compounds (including core aldehydes) in addition to specific hydroperoxides depending the chosen pre-treatment procedures and analytical conditions [Kuksis et al, 2003; Frankel, 2005]. More powerful and sensitive techniques are continuously being developed; however, these techniques generally require extraction and pre-treatment steps that may cause formation of derivatives.

Table 1. Overview of the available methods for determination of lipid oxidation

	Principle	Components Determined	Sample size	Advantages/ Disadvantages [2)]	Reference
FREE RADICALS:					
ESR spectroscopy Spin trapping Direct	Resonance spectroscopy by direct detections of free radicals or by assessment of spin trapped radicals	Free radicals directly or as spin adducts (trapped radicals)	1-5 g	Spin traps act as antioxidants and affects the lipid oxidation progress Direct measurement is dependent on the steady state concentration of radicals and the food matrix. Stability test and effect of antioxidants. Variable persistence of spin adducts.	Paper IV and V Andersen and Skibsted, 2002; Øverby, 2003;Falch, 2005b
PRIMARY REACTION PRODUCTS:					
Peroxide value (Ferrothiocyanate method/SIK)	Spectrophotometric method Fe (II) -> Fe(III) (Reaction due to hydroperoxides)	Hydroperoxides Primary reaction products (mEq/kg)	Micro (100ul)	During lipid oxidation the peroxide value generally reaches a maximum value before reduction. Sensitive to changes in temperature May give an incorrect picture of the oxidation level	Labuza, 1971; Undeland et al, 1998 Frankel, 2005
Peroxide value	Titration Released iodine from potassium iodine	As above	Macro (ca 5 g)	As above	AOCS Official method Cd 8-53
Conjugated dienes	Spectrophotometric method Dienes affect the absorbance in a metallosolution	Primary lipid oxidation with conjugated double bounds	Macro	Follow nearly the same levels as progress as the peroxide value and can be reduced after a certain oxidation level.	Halliwell and Gutterige, 1990
SECONDARY REACTION PRODUCTS:					
Thiobarbitureacid reactive substances (TBARS)	Spectrophotometric method Acid addition, heating and measurement of absorption (530-535 nm)	Secondary reaction products, aldehydes (alkanals, alkenals, 2,4 dieanls and dienals)	Micro (5-10mg) Macro (ca 0.5g)	Compounds such as sugars, urea and oxidized proteins may also form colour complexes with TBA. MDA may at neutral pH react with amino acids, proteins etc. and thereby not with TBA. Not a quantitative measurement As above	Ke and Woyewada, 1979; Addis, 1986; Halliwell and Gutterige, 1990; Frankel, 2005.
Anisidine value	Spectrophotometric method In the presence of acetic acid p-anisidine reacts with aldehydes and forms a yellow complex.	Secondary reaction products primarily 2-alkenals	Macro (0.5-4g)	Samples and reactants have to be free from water p-anisidine is toxic (carcinogenic). p-anisidine may react with other compounds than aldehydes	IUPAC, 1987 Warner and Eskin, 1995

	Principle	Components Determined	Sample size	Advantages/ Disadvantages[2)]	Reference
METHODS TO DETERMINE SEVERAL LIPID OXIDATION PRODUCTS					
High resolution NMR	Spectroscopic method (combination of ^{1}H and ^{13}C)	Chemical composition Specific hydroperoxides, Aldehydes, ketones, alcohols and Cyclic compounds etc.	30-50 mg	Provide the most important changes in chemical composition Can explain reaction mechanisms Low sensitivity for studying lipid oxidation Expensive equipment and required highly skilled personnel	Paper III and V (composition: VI, VII and VIII)
Chromatography[1)] **GC-MS**	Gas chromatography	Volatile oxidation products	Small	Sensitive but requires manipulation steps that might affect the composition. Varying recovery of lipid compounds	Kuksis et al, 2003
HPLC-MS	Liquid chromatography	Non polar and polar lipophilic secondary lipid oxidation products	Small	Sensitive and specific (also non-volatiles are detected)	Kuksis et al 2003
		Hydroperoxides		Sensitive, specific but requires manipulation steps such as solid phase micro extraction before analysis	
TLC	Chromatography	Mixture of oxidation products	Small	Qualitative not quantitative (however a matter of discussion with the recent method developments)	Frankel, 2005
Fluorescence spectroscopy	Spectroscopic method	Protein lipid interaction products	Small	Not suitable for pure oil systems Detects relatively late stages of lipid oxidation	Augourg, 1999
Sensory panel	Sensory evaluation by a panel	Reaction products affecting organoleptic properties	Macro (>1g)	Very sensitive, requires large amount of samples Labour expensive (trained panel) Direct and highly relevant for food products	Frankel, 2005
STABILITY TESTS:					
Oil stability Index (OSI) and Rancimat method	Conductivity changes due to formation of volatiles in heated oils.	Volatile compounds	Macro (ca 5g)	Gives the oxidation stability not the oxidation level Particularly valuable for measuring effect of antioxidants and comparison of stability between **oils**.	AOCS method Cd12b-92 Frankel, 2005
Oxidograph	Changes in oxygen pressure during heating of oil	Consumed oxygen during lipid oxidation	Macro (Ca 5g)	As above	
Oxygen electrodes	Electrodes for assessment of changes in oxygen during lipid oxidation	As above	0.01-2 ml	As above (applicability for emulsions)	Steinstø, 2000; Frankel, 2005 Mozuraityte et al, 2005

[1)] Different chromatographic techniques for determining lipid oxidation are reviewed by Frankel et al (2005). Chemiluminscence and fluorescence determination in combination with the chromatographic technique are also reported. MS, mass spectrometry enables the assignment of the compounds detected.

2) Advantages and disadvantages is basically found in Frankel [1998, 2005], in papers and experience from this thesis work and specified papers under the reference column in the table.

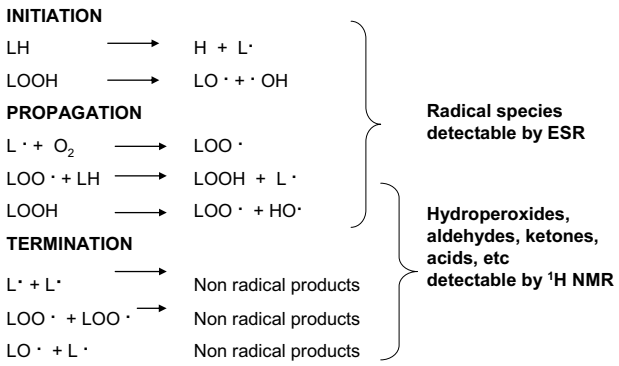

Fig. 5. Auto-oxidation of lipids showing which reaction products are detected by the two main analytical methods used to study lipid oxidation in this thesis work.

3.6 Nuclear Magnetic Resonance Spectroscopy

NMR in general

NMR spectroscopy is an analytical method that makes use of the fact that nearly all molecules contain magnetic nuclei and can therefore be detected in a strong magnetic field when irradiated with a specific radiofrequency. The NMR technique was discovered in 1945 [Purcell et al, 1946; Block et al., 1946] and since that time intensive research has resulted in improved knowledge and development of high resolution commercial equipment applicable for a wide range of disciplines. In the past decades high resolution (HR) NMR has developed into an important technique for instrumental analysis of organic compounds.

The most common magnetic nuclei are ^{1}H, ^{13}C, ^{31}P, ^{19}F, ^{14}N with natural abundances close to 100%, except ^{13}C which has 1.1% natural abundance. In lipid research ^{1}H, ^{13}C and ^{31}P are the most studied nuclei. Nuclei in different chemical environments have slightly different resonance frequencies. The resonances obtained from NMR are expressed as chemical shift values (δ) in ppm units relative to a reference compound (tetramethylsilane (δ = 0.0 ppm) for ^{1}H). While resonance frequency is given in Hz, which is dependent on the acquisition conditions, the chemical shift scale is a dimensionless quantity and a more convenient way of presenting the spectra. The chemical shift values are dependent on molecular structure [Hunter et al, 2005], but do also vary based on solvent, concentration [Gunstone, 2004] and pH [Fan, 1996]. Chemical shift values for different organic compounds are comprehensively reviewed by Fan [1996] and now also Spectral Databases for Organic compounds (SDBS). Techniques

for optimizing acquisitions and processing are comprehensively presented [Reynolds and Enriques, 2002; Berger and Braun, 2004].

The chemical shift values of ^{13}C normally ranges from 0 to 200 ppm and spectra are normally broadband, proton decoupled and therefore show the resonances as single lines. The lower abundance of ^{13}C compared to ^{1}H, makes it is 400 times less sensitive to the NMR phenomena. It therefore takes a longer time to acquire ^{13}C spectra, though they tend to look simpler. Overlap of peaks is much less common than for ^{1}H NMR which makes it easier to distinguish among different carbon atoms.

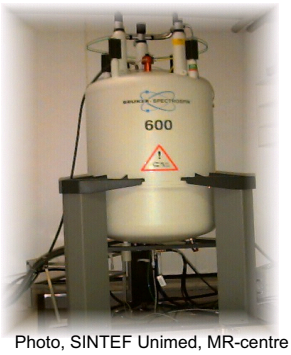

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Fig. 6. A 600MHz NMR magnet

New NMR technologies, such as CryoProbe technology, have lead to increased sensitivity of HR NMR by a factor of three to four [Serber et al, 2000], enabling better resolved spectra, smaller sample volumes and 16 times faster acquisition compared to the conventional HR NMR analysis [Colson, 2005].

Two-dimensional NMR techniques are valuable for peak assignment and the following three techniques are used in the present thesis work:

- ***COSY*** (H,H)-Correlated NMR spectroscopy yields NMR spectra in which ^{1}H chemical shifts along both frequency axes are correlated with each other [Aue et al, 1975].
- (C,H)-Correlation ***HETCOR*** (Heteronuclear correlation) yields cross signals for all protons and ^{13}C nuclei that are connected by a ^{13}C,H coupling over one bond [Berger and Braun, 2004].
- ***HMQC*** (Heteronuclear Multiple Quantum Coherence) is an inverse chemical shift correlation experiment that like HETCOR, is used to determine which protons of a

molecule are bonded to which ^{13}C nuclei [Berger and Braun, 2004]. The sensitivity of a HMQC spectrum is generally higher than a HETCOR spectrum.

Example of a HMQC MAS spectrum of cod roe is shown in Fig. 7 and an example of ^{1}H:^{1}H COSY of intact cod milt before and after storage in the magnet is shown in Fig. 8. These spectra show the high resolution that is possible to obtain from intact material with enlargement of the glycerol regions that also contains resonances from the polar head group of phospholipids.

A combination of different techniques such as LC NMR with cryoprobe has lead to an improved sensitivity [Lewis et al, 2005] and more powerful tools are now available.

HR Magic angle spinning (MAS)

High resolution magic angle spinning (MAS) NMR is a strong tool for studying heterogeneous systems [Rooney et al, 2003; Bollard et al, 2000]. NMR in chemical investigations has traditionally been limited to analysis of liquid samples. The MAS technique is now established for NMR analysis of intact sample material by spinning the sample at a magic angle (54.7^{o}) [Hennel and Klinowski, 2004]. Thus, partial averaging of chemical shift anisotropies and dipolar couplings thereby significantly reduce the line broadening effect [Andrew et al, 1959]. MAS requires high sample rotation (several kHz) speeds to prevent the appearance of satellite lines at integer multiplets of the rotation frequency in the spectrum [Lowe, 1959]. In some sample materials, such as biological materials, these high speeds may affect the chemical composition by disrupting cells resulting in chemical modifications, and special sequences enabling slow spinning have been recently developed [Wind et al, 2005]. The method has been used successfully to study different biological tissues including intact fish muscle [Sitter et al. 1999; Gribbestad et al, 2005; Aursand et al, 2005], microalgae [Broberg et al, 1998; Broberg and Lennart, 2000; Chauton et al, 2003] and intact tissue of cod roe [Falch et al, 2004] and liver [Paper VI]. It is possible to obtained well resolved spectra providing detailed information about a wide range of chemical compounds [Bollard et al, 2000; Broberg and Lennart, 2000; Chauton et al, 2003; Rooney et al, 2003; Chauton, 2005].

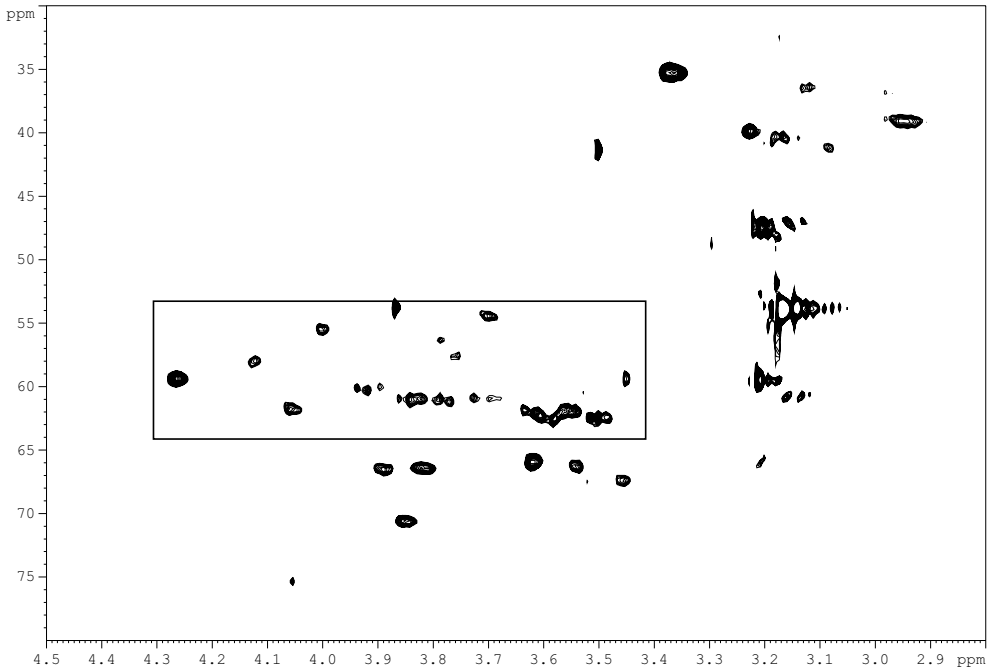


Fig. 7. 600 MHz HMQC MAS spectrum of the glycerol region of intact cod roe. Experimental conditions: 20 µg roe/30µl D_2O (incl. TSP) in a 50µl MAS rotor. Speed: 5 kHz MAS, 2048 data points in the F2 direction and 512 data points in the F1 direction. 48 scans obtained at a temperature of 4°C. The rectangle shows where the resonances from glycerols of *sn1,3* position of PLs are expected to appear.

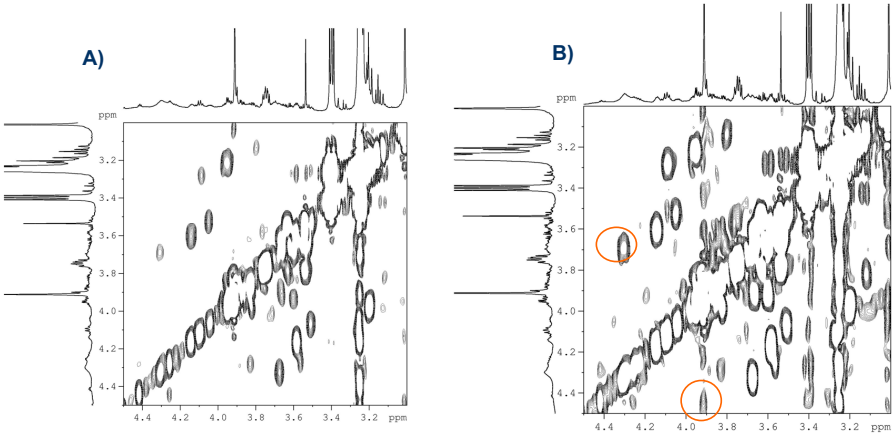


Fig. 8. 600 MHz MAS NMR spectra (^1H:^1H COSY) of the glycerol region of intact cod milt (A) before and after (B) experimental storage rotating in the magnet (4°C) for 2 days. Experimental conditions: 20µg roe/30µl D_2O (incl. TSP) in a 50µl MAS rotor. Speed: 5 kHz MAS, 2048 data points in the F2 direction and 512 data points in the F1 direction. 32 scans were obtained. The main changes during storage are marked with circles in the spectrum.

HR- NMR to study lipid oxidation

HR-NMR to study lipid oxidation of fish lipids is reviewed in Paper V. Table 2 gives an overview of chemical shift values of lipid oxidation products and also other compounds known to change during lipid oxidation. Among the NMR techniques, ^{1}H NMR has been most widely used to study lipid oxidation. Previous work has reported a decrease of the ratio between olefinic (∂ 5.1 – 5.6 ppm) to aliphatic protons (∂ 0.6 – 2.5ppm) and aliphatic to diallylmethylene protons (∂ 2.6 – 3.0 ppm) during lipid oxidation. These results were obtained from experiments on vegetable- [Wasasundara and Shahidi, 1993; Haywood et al, 1995; Silwood and Grootveld, 1999] and marine lipids [Saito, 1987, 1997; Saito and Udagawa, 1992a,b; Saito and Nakamura, 1990; Shahidi et al, 1994]. Additionally, a correlation has been found between this ratio and the peroxide value [Saito, 1987; Saito and Udagawa, 1992a,b; Wasasundara and Shahidi, 1993; Shahidi et al, 1994]. However, relatively high levels of lipid oxidation are reported before any detectable changes in the NMR spectra occur.

^{1}H NMR has been reported to be valuable for evaluating changes in lipids due to lipid oxidation in vegetable oils [Claxon et al, 1994; Silwood and Grootveld, 1999], and formation of hydroperoxides, conjugated diene hydroperoxides, saturated and unsaturated aldehydes, acids, ketones and polymerization are identified.

It has previously been stated that hydroperoxides of PUFAs are easily decomposed into a complex mixture of secondary oxidation products with the decrease of olefinic hydrogens [Saito, 1987]. Fig. 9 shows a typical ^{1}H NMR spectra of a lipid extract indicating regions that have been previously used to study changes during lipid oxidation. The ratio between olefinic and aliphatic hydrogen resonances (Ro) and diallylmethylene and aliphatic hydrogen resonances (Rm) has previously been reported to decrease during lipid oxidation [Saito, 1997]. Such changes were not found in the work in this thesis; however specific resonances from different regions of the spectra were obtained, particularly in the downfield regions between 8.0 and 12 ppm [Paper III]. Other compositional changes of the lipids such as decrease of signals originating from n-3 were also found. N-3 fatty acids are previously quantified from ^{1}H NMR spectra of oils [Igarashi et al, 2000, 2002].

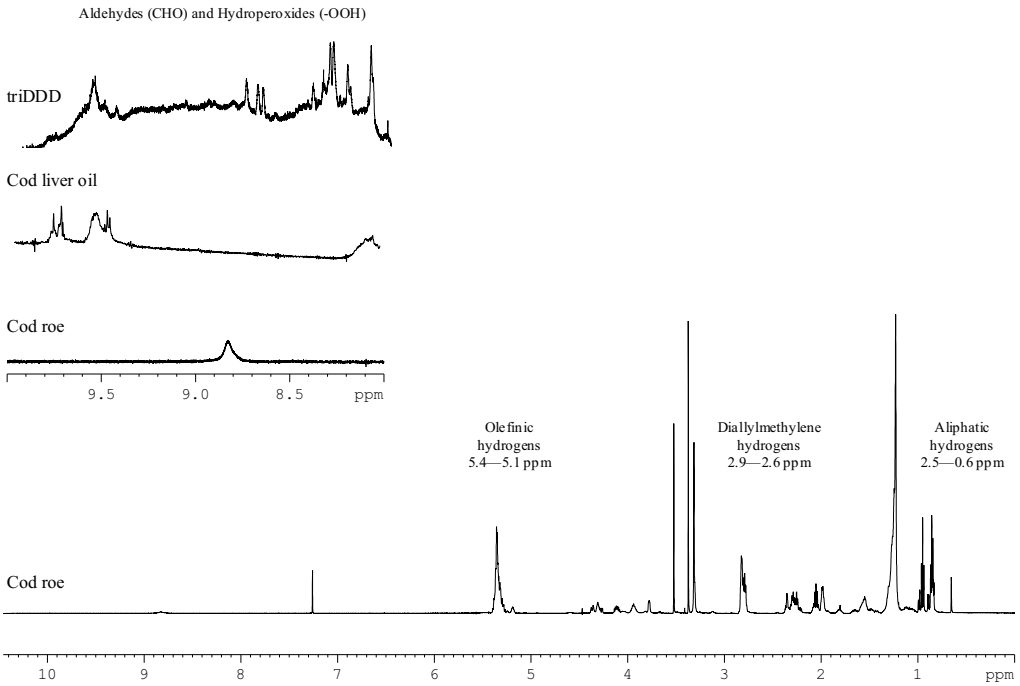
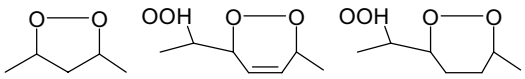


Fig. 9. ^{1}H NMR (600MHz) spectra of cod roe showing the main peaks associated with changes in during lipid oxidation (the ratio between there specified regions are reported to change during lipid oxidation). Enlargement are done on the downfield regions of spectra of cod roe, cod liver oil and tridocodahexaenoate (triDDD).

Table 2. Chemical shift assignment of components in the ^{1}H NMR spectra associated with changes of lipids [**Paper V**].

^{1}H substances	**Chemical shift values (ppm)**	**References**
Primary lipid oxidation products:		
Hydroperoxides (-OOH)	8.5 – 8.9	[Claxon et al., 1994]
	8.6 – 8.7	[Silwood and Grootveld, 1999]
Hydrogens on the peroxy bearing carbon (-CH(COOH))	4.1-4.3	[Neff et al, 1983; Shahidi et al, 1994; Claxon et al, 1994; Silwood and Grootveld, 1999]
Conjugated dienoic olefinic proton multiplets	5.4 – 6.7	[Claxon et al, 1994]
Secondary lipid oxidation products		
Aldehydes (-CHO)	9.0 – 10.0	[Silwood and Grootweld, 1999]
	9.3 – 9.8	[Claxon et al, 1994]
Saturated aldehydes	9.74	[Claxon et al, 1994]
Hexanal	9.75	[Paper III]
αß unsaturated aldehydes	9.48, 9.52, 9.63	[Silwood and Grootveld, 1999]
trans-2-heptenal, trans-2 octenal	9.48	[Silwood and Grootveld, 1999]
trans-2-pentenal, trans-2-octenal, trans-2-nonenal	9.5	[Paper III]
trans,trans-2,4-heptadienal	9.5 and 9.58	[Paper III]
Hexenal	9.74	[Claxon et al, 1994]
Unsaturated alcohols (-CH(OH)-)	4.5 – 5.0	[Vlahov et al, 1999]
1-penten-3-ol	5.8, 5.15, 4.0, 2.1, 1.5	[Paper III]
Cyclic compounds		
Cyclic peroxide methane hydrogens (epoxides)	4.5 – 4.7, 4.4	[Neff et al, 1983], [Paper III]
		
Groups of hydrogens		
Aliphatic hydrogens	0.6 – 2.5	[Saito and Nakamura, 1989; Wasasundara and Shahidi, 1993]
Diallylmethylene hydrogens (=C-CH$_2$-C=)	2.6 – 2.9	[Wasasundara and Shahidi, 1993]
	2.6 - 3.0	[Saito and Nakamura, 1989]
Olefinic hydrogens (-CH=CH-)	5.1 – 5.6	[Saito and Nakamura, 1989]
	5.1 – 5.4	[Wasasundara and Shahidi, 1993]
Unsaturated fatty acids		
Unsaturated fatty acids (-CH=CH-)	5.35	[Aursand et al, 1993]
Unsaturated fatty acids (CH$_2$-CH=CH-)	2.0	[Aursand et al, 1993]
Polyunsaturated fatty acids (=CH-CH$_2$-CH=)	2.81- 2.84	[Aursand et al, 1993, Paper III]
n-3 fatty acids (-CH$_3$)	0.896, 0.833	[Aursand et al, 1993]

HR- NMR to study lipid composition

HR-NMR is a non-destructive technique for analysis of lipid composition. D-chloroform, which is the most commonly used solvent [Gunstone, 2004] is easily evaporated and the sample may therefore be further analysed. The sample size of a routine lipid NMR analysis is 50-100 mg, but investment in a large number of scans makes it possible to decrease the sample amount. Previous work on vegetable oil has shown that ^{13}C NMR could be used for determination of fatty acid composition and positional distribution of fatty acids on the triacylglycerol backbone [Ng and Ng 1983; Ng, 1985]. Later, this technique was used for determination of fatty acids in marine oils [Aursand and Grasdalen, 1992; Aursand et al, 1993, 1995; Sacchi et al, 1993, 1994; Gunstone and Seth, 1994]. Table 3 presents the main applications of HR-NMR in the study of marine lipids. Information about the positional distribution of fatty acids (*sn1,3* or *sn2*) is primarily found in the carbonyl region (173.3 – 172.8 ppm), but also in other parts of the NMR spectrum. This is information not obtainable from GC analyses which are the most common methods for determination of fatty acid composition. The GC method requires labour-intensive methylation as a pre-treatment step [Park and Goins, 1994], however optimization of the pre-treatment has been reported [Indarti et al, 2003], resulting in simplification of the method and increased precision. Moreover, ^{13}C NMR is found to be valuable for providing resonances from a wide range of compounds in one single analysis. These compounds includes esters, phospholipids [Gunstone, 1994], wax esters, alcohols [Gunstone, 1993], sterols [Kapustina et al, 2002; Siddiqui et al, 2003], fat soluble vitamins [Siddiqui et al, 2003] and the hydrolysed counterparts of triacylclycerols (di- and monoacylglycerols and free fatty acids) [Dawe and Wright, 1988; Sacchi et al, 1989; Gunstone, 1991; Siddiqui et al, 2003]. ^{13}C NMR spectra can provide quantitative data when using instrumental conditions with sufficient relaxation times [Gillet and Delpuech, 1980; Aursand et al, 1993]. ^{1}H NMR is more commonly used for quantitative measurements and the levels of total n-3 fatty acids and levels of DHA have been successfully quantified [Igarashi et al, 2000, 2002]. Even though the ^{1}H NMR spectra generally provide broader peaks and more clusters than ^{13}C NMR spectra, the hydrogen resonances are reported to provide data on the individual phospholipids, cholesterol, cholesteryl ester and different diacylglycerols in human biological samples [Sze and Jardetzky, 1990].

Table 3. Applications of HR NMR in the study of marine lipids.

		Sample material	References
^{13}C	Fatty acids and acylstereospecific positions of fatty acids in the triacylglycerols molecule	Fish oil	[Aursand and Grasdalen, 1992; Aursand et al, 1995; 1997; Sacchi et al, 1994 Broadhurst et al, 2004]
	Fatty acids and acylstereospecific positions of fatty acids in the phospholipid molecules	Cod roe and milt Tuna	[Paper VII] [Medina et al, 1994a]
	Methylesters	Tuna and reference standards	[Sacchi et al, 1994]
	Formation of free fatty acids during lipolysis of fish	Tuna Canned tuna	[Sacchi et al, 1993; Medina et al, 1994b] [Medina et al, 1995]
		Cod roe and milt	[Paper VI and VIII]
	Oxidation during thermal stress	Salmon Atlantic Mackerel	[Medina et al, 1998] [Saeed and Howell, 1999]
	Plasmalogen (alk-1-enyl-phosphatidylethanolamine)	Different fish samples	[Sacchi et al, 1995]
	Individual Phospholipids (PC and PE) Cholesterol	Cod roe and milt Cod roe and milt Commercial n-3 concentrates	[Paper VII] [Paper VII and VIII] [Siddiqui et al, 2004]
	Authentication/origin testing of fish	Wild and farmed salmon	[Aursand et al, 2000]
	Cis/trans ratio of fatty acids	Salmon and trout	[Aursand et al, 1997]
	Glycerol-, methyl-and wax esters, acids, alcohols, nitriles, amides and acetates	Different sample materials	[Gunstone, 1993]
	Mobility of fatty acids at different freezing temperatures	Salmon muscle	[Grasdalen et al, 1995]
^{1}H	Quantification of n-3 and DHA	Fish lipids Cod roe and milt Salmon muscle	[Sacchi et al, 1993; Igaraschi et al, 2000, 2002] [Paper VII] [Aursand et al, 2005; Gribbestad et al, 2005]
	Lipid oxidation changes in ratio	Fish meal Fish oil	[Saito and Udagawa, 1992] [Saito and Nakamura, 1990; [Saito, 1997]
	Specific oxidation products	DHA/fish oils	[Paper III]
	Changes in lipids during cooking	Different fish species	[Cengarle et al, 1999]
	Cholesterol/cholesteryl esters	Cod roe and milt	[Paper VI and VIII]
^{31}P	Quantification of phospholipids Quantification of PE and PC	[1)]	[Helmerich and Koehler, 2003] [Olson and Cheung, 1990] [Diehl and Ockels, 1995]

[1)] These quantifications are not performed on fish in the referred papers

3.7 Electron Spin Resonance Spectroscopy

The ESR technique in general

The fundamental physics and basic principles of ESR are similar to NMR however, while NMR studies the paramagnetic nuclei, the ESR technique measures the absorbance of electromagnetic irradiation by an electron spin system. The other main difference between these two methods is that ESR operates at a fixed frequency and commonly extends over a wide range of magnetic field, while the opposite is the case for NMR [Eaton and Eaton, 1997]. Unpaired electrons spins like small magnets but the sign of the magnetic moment is opposite to that of a proton moment. ESR analyses are generally performed in the X-band region at about 9.5 GHz [Rosenthal, 1999]. The microwave absorption during analysis results in changes in the frequency which is the resonance that is measured. The free electrons resonances generally occur at about 3200 to 2400 Gauss [Eaton and Eaton, 1997]. The ESR method enables assessment of unpaired electrons such as organic free radicals or transition metal ions [Kemp, 1986] and the spectrum can be used for identification of paramagnetic substances and to study chemical reactions through unpaired electrons and their interactions with their closest environment. ESR was first developed in Russia in 1944 and the method is today applied in physics, chemistry, biology, and medicine for quantitative and qualitative measurements of radical species. The method is generally non-destructive and can therefore be used in both in vivo and in vitro analysis.

ESR spectroscopy enables detection of free radicals that are formed during the early stages of lipid oxidation. It has been reported to be one of the most direct and sensitive methods for detecting free radicals [Dikalov and Mason, 2000], but the method is relatively new in the study of lipid oxidation in food systems [Thomsen et al, 2000 a,b].

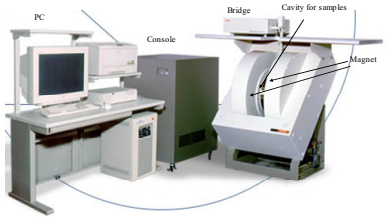


Fig. 10. The ESR instrument with specifications of the different parts

ESR in lipid oxidation

The detection limit of free radicals using ESR techniques are reported to be between 10^{-6} and 10^{-10} M [Davis, 1987; Thomsen et al. 1999; Chen et al., 2000] depending on the conditions. This is sensitive compared to the other traditional method used in lipid oxidation assessment. For powder samples, the free radicals will be trapped in the food matrix and the method has shown promising results in different dried food samples [Stapelfeldt et. al. 1997; Kristensen et. al. 2000; Nissen et. al. 2000, 2002; Jensen et al, 2005]. The free radicals in liquids and emulsions are labile and non detectable during their short half-life at normal conditions (steady state concentration). This challenge may be conquered by techniques such as rapid freezing, lyophilisation (freeze drying), continuous flow systems or by the use of spin traps [Davies, 1987]. The spin trapping technique has become a valid technique for evaluating the lipid oxidation in such food systems [Thomsen et al., 2000a,b; Velasco et al, 2004]. Spin traps are a group of substances that react with radicals to form stable radical compounds (spin adducts) that are detectable by ESR. Since some free radicals will react further and never reach detectable levels, the spin traps can trap them and make detectable. A presentation of the current literature using the ESR techniques to evaluate lipid oxidation is found in Paper V in this thesis. The same paper also includes a discussion of the selection of the different spin traps and limitations by using spin traps. One of the drawbacks of using spin traps is that information identifying the radical may be difficult to obtain. Another drawback is that the spin traps act as antioxidants and the spin trapping will therefore affect the lipid oxidation [Falch et al, 2005b].

Direct trapping of free radicals may be obtained by using rapid freezing (liquid N_2) since bimolecular reactions are so slow that lipid-derived radicals become longer lived [Andersen et al, 2005]. An example of freeze detected radicals from a vegetable oil is shown in Fig 11. We have performed some direct trapping experiments on fish oils with different oxidation levels (PV values of 1.5, 36 and 150 mE/kg), however detectable ESR signals were only obtained from the vegetable oils (rapeseed- and soybean oil) studied.

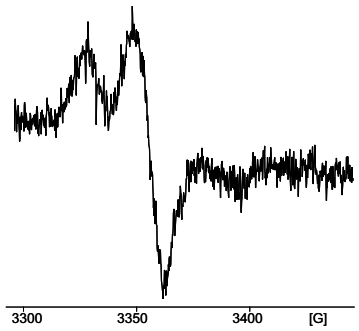


Fig. 11. ESR spectrum from freeze trapping (liquid N_2) of rapeseed oil stored for 14 days at 40°C (Peroxide value of 6.5 mEq/kg). Experimental conditions: power: 10mW; frequency: 100 kHz; center field: 3370 G; temperature: 77 K, sweep width: 150 G, conversion time: 168 ms; and time constant: 168 ms. (ESR analysis performed by E. Falch – unpublished results Falch, Velasco and Andersen)

4. OBJECTIVES

The main objective of this work is to provide data on the quality and composition of lipids in by-products from cod species (gadiforms). Biochemical processes such as lipolysis and lipid oxidation are studied with advanced analytical techniques such as HR NMR and ESR.

Sub-goals:

1. Provide data on the composition and quality of lipids in by-products from gadiform species including calculation of the amount of available lipids, and lipid composition from by-products generated during a total catch of gadiforms.
2. Study the applicability of different ESR spectroscopy techniques for assessment of the early stages of lipid oxidation in residual raw materials from fish
3. Study the applicability of ^{1}H NMR to evaluate lipid oxidation of marine oils.
4. Generate more knowledge of lipid composition and deterioration of cod by-products by adapting HR NMR applications for analysis of heterogeneous lipid extracts

5. DESCRIPTION OF THE WORK

The research activities performed during this PhD work are divided into four topics following each of the sub-goals presented above.

5.1 Composition and quality of lipids in cod by-products

Paper I: E. Falch, T. Rustad, and M. Aursand, By-products from gadiform species as raw material for production of marine lipids as ingredients in food or feed. Process Biochemistry. 2006, 41, 666-674.

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Paper II: E. Falch, M. Sandbakk, and M. Aursand, On-board handling of by-products to prevent microbial spoilage, enzymatic reactions and lipid oxidation, In book: Maximizing the value of marine by-products (Prof. F. Shahidi, Ed) – accepted manus - invited authors

Description of the work

The main activities in this part of the study were (1) to establish data on the variation of lipids and composition of lipids from residual raw material generated during processing of gadiform species [Paper I], (2) to study lipid deterioration in visceral organs of cod (unpublished data) and (3) to evaluate the importance of conservation and early processing to retain the quality of these materials [Paper II].

Paper I contains data on amounts of lipids and lipid composition of residual raw materials from four gadiform species (cod, saithe, haddock and tusk) collected in three different seasons. The effect of species, season and fraction (liver, viscera and trimmings) were studied and the potential of producing lipids and n-3 fatty acids from these fisheries were calculated. Biochemical changes in visceral organs from cod during one week of storage were studied by oxidation methods and thin layer chromatography (lipid class distribution) while cod roe and milt during storage additionally were studied by HR NMR in the analytical part of this thesis [Paper VI –VIII].

Paper II reviews important aspects regarding utilisation of fish by-products in general, with a special focus on the on-board activity to prevent microbial growth, enzymatic reactions and

lipid oxidation. This paper also discusses the availability of neutral and polar lipids from residual raw materials.

5.2 Applicability of ESR spectroscopy to evaluate lipid oxidation

Paper IV E. Falch, J. Velasco, M. Aursand, M. Andersen, Detection of radical development by ESR spectroscopy techniques for assessment of oxidative susceptibility of fish oils. 2005, Eur. J. Food Res. 2005, 221(5) 667-674.

Paper V E. Falch, and M. Aursand, Resonance spectroscopy to study lipid oxidation in fish and fish products, Handbook of modern magnetic resonance, Edited by Graham Webb, Kluwer Academic/Plenum Publisher, New York, USA (2006). In Press

Description of the work

ESR spectroscopy detects radical species at the early stages of lipid oxidation (see chapter 3.7), but limited studies have been done on systems containing fish lipids. It was therefore interesting to study the applicability of ESR techniques to evaluate lipid oxidation of marine lipids. The sample materials used in Paper V consisted of pure oils originating from residual raw materials from processing of cod and salmon (cod liver oil and salmon viscera oil). Due to the low steady state concentration of free radicals in liquids, two different techniques were tested (1) the spin trapping technique using α-Phenyl-N-*tert*-butylnitrone (PBN) as spin trap and (2) the spin scavenging technique using a stable nitroxyl radical. For comparative purposes, the susceptibility of oxidation was determined by more traditional method such as peroxide value and TBARS and Oil Stability index.

Paper IV presents a short review of different aspects of the ESR techniques applied on the study of lipid oxidation of marine lipids and food components. This paper includes a discussion of the effect of commercial available spin traps and it also presents examples of ESR spectra of different marine sample materials such as emulsions and powders.

5.3 Applicability of ^{1}H NMR spectroscopy to study lipid oxidation

Paper III E. Falch, H. Anthonsen, D. Axelson, and M. Aursand, Correlation between ^{1}H NMR and traditional analytical methods for determining lipid oxidation in ethylesters of Docosahexaenoic acid. Journal of the American Oil Chemist Society. 2004, 81(12) 1105-1109

Paper V E. Falch, and M. Aursand, Resonance spectroscopy to study lipid oxidation in fish and fish products, Handbook of modern magnetic resonance, Edited by Graham Webb, Kluwer Academic/Plenum Publisher, New York, USA (2005). In Press

Description of the work

Reference standards of reaction product typically found during oxidation of marine lipids were recorded to study the chemical shift values and detection limits. Paper III include a storage trial on ethyl docosahexaenoate (22:6n-3) which is the ester of the characteristic DHA found in marine lipids. This sample was stored in the dark at 20^{o}C with and without α-tocopherol as antioxidant for up to 10 days. Samples were withdrawn daily for analysis of peroxides, aldehydes and changes in NMR spectra. Results are also presented from analysis by the new NMR Cryoprobe to study the sensitivities. Tri docosahexaenoate was analysed by ^{1}H NMR both on the regular 600 MHz magnet and also by using a Cryoprobe to study whether or not it gave improved sensitivity (unpublished data). Paper V presents a short review of the literature on NMR to study lipid oxidation in marine samples and discusses its applications.

5.4. Lipid composition and deterioration through HR NMR spectroscopy analysis

Paper VI E. Falch, T. R. Størseth, and M. Aursand, HR NMR to study quality changes in marine by-products, Magnetic Resonance in Food Science. The Multivariate Challenge. SB Engelsen, PS Belton and HJ Jakobsen (eds.), The Royal Society of Chemistry, Cambridge, 2005, 11-19.

Paper VII E. Falch, T. R Størseth, and M. Aursand, High resolution NMR for assessment of lipid classes and acyl stereospecific position of fatty acids in marine phospholipids, Submitted to Chemistry and Physics of lipids, November 4th

Paper VIII E. Falch, T. R. Størseth, and M. Aursand, High resolution NMR for studying lipid deterioration in cod (*Gadus morhua*) gonads.

Description of the work

The aims of these studies was to (1) apply HR-NMR techniques and interpret spectra to determine composition of heterogeneous lipid extracts including positional distribution of fatty acids in phospholipids and (2) evaluate the applicability of using the NMR techniques

to study biochemical changes in marine lipids derived from cod by-products. The sample material consisted of visceral fractions from cod, in particular roe, milt and liver. In Paper VII ^{13}C NMR was used to assign the positional distribution of PUFAs in PC and PE. This work included analysis of several reference standards of different PL and TAG. NMR techniques such as ^{1}H, ^{13}C, and ^{13}C:^{1}H HETCOR correlation analysis were used to help assign the main peaks.

Paper VIII included a storage trial of the same samples as used in Paper VII to generate data on NMR observable changes in these lipids. The samples were stored for one week at 4^{o}C before extraction of lipids followed by analysis of lipid class composition by using TLC and lipid composition and changes by using ^{13}C and ^{1}H NMR. Paper VI presented some different applications for HR NMR in the study of marine lipids and it also demonstrated examples of MAS NMR spectra of cod liver with resolution enabling quantification of n-3 fatty acids on intact material.

6. RESULTS AND DISCUSSION

6.1 Composition and quality of lipids in cod by-products

Amounts and composition of available lipids (Paper I)

Residual raw materials generated during fillet production of gadiform species were shown in this study to make up ⅔ of the round weight of fish. Based on the compositional data obtained in this survey, our calculations demonstrated that these materials are good sources of health beneficial marine lipids with 1 000 kg lipids available from the by-products generated during an average daily production of 10 000 kg cod fillet. The n-3 content in the lipid fractions were 30% on an average basis which is comparable to commercial fish oils [Haraldson and Hjaltason, 2001; Falch, 2005, personal experience]. Fig. 12 illustrates calculations of amounts and lipid composition from the main groups of residual raw materials: liver, viscera and trimmings. Depending on species, the liver contained between 43 and 69% with the lowest levels found in tusk. The viscera contained between 2 and 9% lipids and the trimmings contained <1% lipids. No significant seasonal differences were found in the levels of total lipids in liver and viscera which were the main lipid containing sources. No seasonal differences were found in the groups of fatty acids in liver while the viscera contained lower levels of PUFA during the winter catch. The PUFA levels in haddock liver were significantly higher than the corresponding levels in cod, saithe and tusk. The effect of fishing ground on the gadiform species was published in a recent paper [Falch et al, 2005a] showing significant differences in weight proportion of liver and PUFA levels between fishing grounds. Paper I showed that the lipid classes were highly different among the three fractions liver, viscera and trimmings. Principal component analysis showed that it was possible to classify what organ the lipids originate from depending on their (1) lipid class composition or (2) fatty acid composition.

Traditionally, the marine lipids were recommended as nutrients due to their content of fat soluble vitamins; however, today the n-3 fatty acids are the main selling points [Gunstone, 2004]. The current practice of collecting and utilising liver is generally limited to cod (*Gadus morhua*) above a certain size (>4 kg). This present work also shows that also other raw material from gadiform species may be potential sources of nutritious lipids. Our data from

Paper I show that independent of species and fractions, the gadiform species provide lipids with high levels of the nutritious n-3 fatty acids and the composition is therefore a valuable resource as ingredient in food, feed and healthcare products. It is hoped that this knowledge and these data will be a valuable contribution to reaching the goal of maximizing the value of gadiform fish species.

LIVER	VISCERA	TRIMMINGS
4-6 % of round weight 43-69 % total lipids	5-8 % of round weight 2-9 % total lipids	49-55 % of round weight Approx. 1 % total lipids
700-1000 kg lipids and	30-120 kg lipids and	120-170 kg lipids and
170-250 kg n-3 fatty acids pr 10 000 kg fillet	11-25 kg n-3 fatty acids pr 10 000 kg fillet	34-52 kg n-3 fatty acids pr 10 000 kg fillet

Fig. 12. Different by-products from cod and determination of available total lipids and n-3 fatty acids from an average daily production of 10 000 kg cod fillets. [Paper I]

Due to the increased demand for high quality fish oils, the present work has particularly focused on the available amounts and composition of marine lipids in these residual raw materials and the long-chain n-3 fatty acids in particular. Liver, which is the organ containing the highest levels of lipids, made up 5.2 ± 0.5% (N=135) of the round weight of wild cod [Paper I]. The increased farming of cod leads to larger quanta of fresh residual raw materials available for processing onshore [Børresen, 2001; Morais et al, 2001]. The intensively farmed cod are shown to generate larger proportions of fatty liver which are partly explained by the feed composition [Morais et al, 2001]. The fatty acid profile in the diet has also been reported to be partly reflected in the liver lipids of farmed cod [Morais et al, 2001]. Our unpublished data has shown that the liver made up, respectively, 13.6 ± 1.4 (N=40) and

11.2% (N=30) of the round weight of intensively farmed and wild captured farmed cod. The viscera of intensively farmed cod made up 17.6% of the round weight.

High value utilization is highly dependent on the lipid deterioration (see chapter 3.4). The levels of free fatty acids found in the viscera and trimmings in present study were higher than guideline specifications for crude fish oils [Young, 1987; Codex, 1999] and are above the levels that are reported to affect the sensory properties in frozen stored salmon [Refsgaard et al, 2000]. Only the liver was within or below these guideline levels. Using trimmings and viscera in the process line for generating food grade qualities therefore requires optimal handling immediately post mortem to retain the quality, probably in addition to neutralisation of the extracted lipids [Paper II].

Biochemical deterioration of visceral organs of cod (unpublished data)

The lipid composition of different visceral organs from cod (N=30, average weight: 3.5 kg) is shown in Table 4, demonstrating the coherence between the low lipid content, high content of polar lipids (primarily phospholipids) and high levels of PUFAs (approx. 45% in gonads). During one week of dark storage, at 4^{o}C (100 g, homogenates), the lipid class distribution changed dramatically (Fig 13). The levels of polar lipids decreasing to half of the original levels, while the FFA and cholesteryl esters increased correspondingly (in accordance with Paper VI). The changes occurred particularly in the gonads. Due to the lack of reference standards of lysophospholipids and hydrolysed acylglycerols, these peaks were not assigned in the current TLC chromatograms. NMR analysis of similar samples is shown in Paper VI and VIII. The levels of FFA analysed in the gonads were much higher than guideline specification of crude fish oils [Young, 1987; Codex, 1999].

The lipid oxidation during the same storage trial showed that gonads and viscera oxidized between two to four times faster than liver and cod liver oil. The peroxide values after one week of storage were in coherence with the PUFA levels. Recent studies have further shown that cod liver contains higher levels of the antioxidative α-tocopherol than cod viscera [Malone et al, 2004] which surely affect oxidative stability. On a general basis, these oxidation levels (peroxide values between 3-9 mEq/kg oil; TBARS 0.3-1.2 μmol/g oil) were regarded as within the guideline specifications for crude fish oils [Codex, 1999] and the oxidative changes were regarded as less significant compared to the corresponding changes in lipid class composition due to lipolysis [Fig 13 and Paper VIII]. Results from storage of salmon muscle have recently shown that also the enzymatic hydrolysis has a key role in the sensory

deterioration [Refsgaard et al, 2000]. A discussion of biochemical spoilage and suitable handling and processing for retaining the quality are discussed in Paper II.

Table 4. Fatty acid composition (% of total fatty acid) and lipid class composition (% of total lipids) of visceral fractions. Homogenized samples from wild captured and ongrown cod (N=30, pooled samples, average weight of fish: 3.5 kg cod). The lipids were extracted after 1 week of dark storage at 4°C. (Falch, Unpublished data)

	Liver	Cod liver oil [1)]	Roe	Milt	Viscera
Fatty acids (% of total fatty acids) [2)]					
C14:0	3.7 ± 0.1	4.3 ± 0.0	0.7 ± 0.0	1.6 ± 0.0	2.0 ± 0.0
C16:0	11.6 ± 0.1	10.9 ± 0.3	15.5 ± 0.2	17.3 ± 0.3	14.9 ± 0.1
C16:1n7	6.9 ± 0.0	7.8 ± 0.0	1.3 ± 0.0	4.3 ± 0.0	4.3 ± 0.1
C18:0	2.5 ± 0.0	2.5 ± 0.0	4.4 ± 0.1	2.4 ± 0.0	4.1 ± 0.1
C18:1n9	21.8 ± 0.1	19.4 ± 0.1	21.0 ± 0.0	16.4 ± 0.1	18.7 ± 0.1
C18:1n7	6.7 ± 0.1	6.1 ± 0.0	4.8 ± 0.1	4.8 ± 0.0	5.2 ± 0.0
C18:2	1.8 ± 0.0	1.9 ± 0.0	0.7 ± 0.0	1.1 ± 0.0	1.3 ± 0.0
C18:3n3	0.3 ± 0.0	0.4 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.0
C18:4n3	1.1 ± 0.0	1.4 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.5 ± 0.0
C20:1n9	17.6 ± 0.2	19.1 ± 0.2	6.8 ± 0.0	4.1 ± 0.0	9.0 ± 0.1
C20:1n11	0.3 ± 0.1	0.5 ± 0.1	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.0
C20:2	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.2 ± 0.0
C20:4n6	0.3 ± 0.1	0.3 ± 0.1	1.4 ± 0.0	2.1 ± 0.0	2.0 ± 0.0
C20:4n3	0.4 ± 0.0	0.3 ± 0.0	0.2 ± 0.0	0.3 ± 0.0	0.3 ± 0.0
C20:5n3	7.2 ± 0.1	6.7 ± 0.0	14.0 ± 0.0	16.0 ± 0.1	11.4 ± 0.1
C22:1n11	4.2 ± 0.0	5.5 ± 0.1	0.4 ± 0.0	0.6 ± 0.0	2.1 ± 0.1
C22:1n9	0.7 ± 0.0	0.9 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.4 ± 0.0
C22:5	0.8 ± 0.0	0.8 ± 0.0	1.1 ± 0.1	0.9 ± 0.1	1.0 ± 0.0
C22:6n3	11.4 ± 0.1	10.5 ± 0.1	26.3 ± 0.1	25.8 ± 0.1	21.2 ± 0.1
C24:1n9	0.2 ± 0.0	0.3 ± 0.0	0.2 ± 0.0	1.2 ± 0.0	0.6 ± 0.0
SFA	17.8	17.6	20.7	21.3	21.0
MUFA	58.3	59.6	34.9	31.9	40.5
PUFA	23.6	22.4	44.2	46.5	38.1
n-3	20.4	19.2	40.7	42.4	33.6

Lipid class composition (% of total lipids) [2)]

	Fresh	Stored[4)]	Fresh	Stored[4)]	Fresh	Stored[4)]	Fresh	Stored[4)]	Fresh[3)]	Stored[3)]
Triacylglycerols	98.0	97.0	99.4	98.0	8.9	8.9	0.2	0	-	-
Polar lipids	0.4	0.4	0.0	0.0	56.3	25.0	76.9	23.2	-	-
Free fatty acids	0.0	2.0	0.0	0.0	2.2	40.1	0.4	40.5	-	-
Cholesterol	0.14	0.14	0.14	0.14	17.6	2.1	17.1	2.5	-	-
Steryl esters	0.0	0.1	0.46	0.50	1.3	18.0	0.0	31	-	-
Others	1.46	0.36	-	1.36	13.8	5.8	5.4	3.1	-	-
Lipid content (weight %)	73.8 ± 0.0		100 ± 0.0		2.7 ± 0.0		2.2 ± 0.1		2.0 ± 0.1	

1) Cod liver oil were extracted (separated after thermal treatment) from the liver samples (at day 0) and stored similar to the other samples.

2) Analytical methods as described in Paper I

3) Missing data due to difficulties in separation from the TLC chromatogram

4) Samples were stored in 100 g portions wrapped in a thin plastic film and aluminum foil around.

Handling and processing (Paper II)

Retaining the quality of the fresh raw materials immediately post mortem is fundamental for producing food grade quality lipids (Paper II). As presented previously (chapter 3.4 and introduction), the biochemical processes are known to affect the sensory properties of the lipids and problems due to off-taste have been suggested to be one of the main reasons for the limited use of marine n-3 as food ingredients [Frankel et al, 2002; Kolanowski and Laufenberg, 2005] in a demanding market. Recent consumer studies have shown that taste is one of the most important influences of their food choice [Glanz et al, 1998; Kearny et al, 2000]. Taste properties are, in Paper II, discussed with the aspects of handling and processing in utilization of marine residual raw materials. To produce high quality products some important aspects are provided: (1) a market demand for the products (2) knowledge about the available amounts and the variations in quality and composition, (3) systems for efficient sorting of the valuable, parts and (4) suitable conservation and gentle processing. It is a growing prerequisite for high quality fish oils as ingredients in functional food that they provide acceptable organoleptic quality and stability before and after incorporation in a food system [Frankel et al, 2002]. Compositional data from Paper I and from a survey of gadiform species from three European fishing grounds [Falch et al., 2005a] enable prediction of availability and composition of residual raw materials. These data have been applied by SINTEF to develop a data program for calculating the value of by-products from different catches of gadiform species. The program is called MaxFish and should be a help tool for the fishing vessels that wish to utilize residual raw materials. The program serves weight estimates for the different by-products and it contains product suggestions from the different residual parts (see example in Paper II). The next important aspect is to efficiently sort the valuable fractions. It has previously been necessary to do manual gutting in order to bring out and sort the valuable parts from whitefish and salmon. Today, these operations are performed by recently developed gutting machines [Einarsson, 2004] securing successful recovery of visceral organs. The new and gentle gutting operations enable differentiation between quality levels and end uses and thereby organize individual optimal handling and preservation of the different fractions. Gentle and non-destructive gutting also makes it possible to get reasonable prices for the most valuable parts. The traditional automatic gutting machines, consists of a vacuum-system that is homogenising and destroying the viscera and thereby preventing efficient separation of the different organs. The sorting procedures will therefore prevent contamination among visceral organs and allows for further processing of the valuable

fractions. Some of the fish organs might be used for human consumption (Fig. 12), or alternatively used for extraction of valuable components such as lipids and proteins.

Cod liver oil has been produced for centuries [Curtis et al, 2004]. Liver lipids may be extracted gently by low temperature processes [Barrier and Rosseau, 1998; Jansson and Elvevoll, 2000], however, harsh processes by direct steam heating are still widely in use. The lipids may be further processed into n-3 concentrates or structured lipids. Recently, there has been a lot of research interest in the processing of cod (*Gadus morhua*) by-products [Slizyte et al, 2004, 2005a,b, Dauksas et al, 2005, Aspmo et al, 2005] with the use of enzymatic technology (commercial proteases) generating protein and lipids as end products. Our study on mass distribution of lipid components in cod [Dauksas et al, 2005] showed that the phospholipids were found in the protein-rich fractions after hydrolysis, particularly in the sludge (water non-soluble phase) which therefore might be a basis for further extraction of phospholipids. In order to find the most suitable methods to conserve these raw material knowledge of the early stages of lipid oxidation and methods to detect these changes are valuable. Different processing technologies, onboard handling and conservation are further discussed in Paper II.

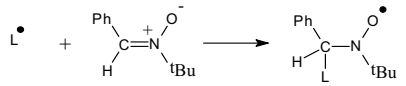
Concluding remarks:

- Residual raw materials generated during an average daily production of 10 000 kg fillets from gadiform species generate more than 1 ton of marine lipids containing approximately 30% n-3 fatty acids.
- There were significant differences among seasons, fractions and species, however, the lipids contained high levels of n-3 fatty acids.
- Free fatty acids were higher than guideline specifications for crude fish oils and a storage trial of the visceral fractions demonstrated a major change in lipid classes due to formation of free fatty acids and cholesteryl esters.

6.2 ESR spectroscopy to evaluate lipid oxidation

Paper IV demonstrated that the spin trapping technique was very sensitive and that it was possible to assess free radicals in a reference standard of DHA during the first minutes of lipid oxidation by the use of PBN as spin trap. This method detects the very early stages of

lipid oxidation in fish oils as have been found in other food systems [Andersen and Skibsted, 2002]. The reaction of PBN with the lipid radicals is shown in Fig. 13 and examples of ESR spectra obtained by spin trapping of cod liver oil, salmon oil and DHA is shown in Fig 14. Recording of PBN spin adducts in cod liver oil and salmon oil during experimental storage at mild conditions (40°C) showed an earlier trapping of radicals in the salmon oil that had higher oxidation levels and lower oxidative stability compared to cod liver oil (Fig 15). The results from using the spin trapping technique were consistent with the traditional methods and the ESR spin trapping method is therefore a potential method for evaluating the oxidative stability of lipids under mild conditions (40°C).



(1) **(2)** **(3)**

Fig. 13. Spin trapping reaction of radicals involved in lipid oxidation (**1**) with the spin trap PBN (**2**) to form PBN spin adducts (**3**). (PBN - α–phenyl- N-tert-butylnitrone)

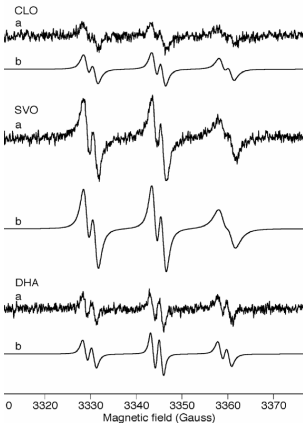


Fig. 14. Representative examples of ESR spectra recorded in cod liver oil (CLO), salmon viscera oil (SVO) and methyl docosahexaenoate (DHA) containing PBN and corresponding spectra obtained by computer simulation.

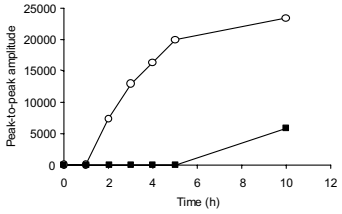


Fig. 15. PBN spin-adduct formation during heating at 40°C in the dark in salmon viscera oil (○) and cod liver oil (■). The ESR signal intensity was considered as the peak-to-peak amplitude.

Papers IV and V demonstrate the importance of choosing the right sampling time, since the persistence of PBN adducts varied significantly among the samples investigated. The variable decay of PBN spin adducts found in the present study is in accordance with our recent findings [Falch et al, 2005b]. Paper V gives an overview of different applications of the ESR technique for evaluation of oxidation of different marine products. It also discusses limitations and pitfalls during the analysis, in particular the effect of different spin traps and what specific radicals they are trapping. Challenges regarding the use of spin trapping in combination with natural and added substances are further discussed in Falch et al [2005b] focusing on the oxidative stability of cod liver oil with and without natural antioxidants such as tocopherol isomers (α-, γ-, and δ-tocopherol), tea cathechin and rosemarine extracts added. One of the main limitations found in using this method for evaluating oxidative stability were that the persistence of PBN spin adducts were highly variable between oils with different additives.

Results obtained by ESR spin scavenging were not conclusive in establishing the different oxidative susceptibility of the two oils found by other determinations applied.

Concluding remarks:

- ESR is very sensitive and determines early stages of lipid oxidation in oil. The spin trapping technique was successful in trapping radicals and the spin trapping during the first 5 hours of oxidation were in coherence with conventional methods for assessing lipid oxidation and oxidative stability.
- The persistence of ESR detectable spin adducts were highly variable among the oil samples investigated, which underlines the importance of time of sampling and detailed knowledge about the lipid composition.

This work has shown that ESR detects free radicals at the early stages of lipid oxidation. In order to use ESR spin trapping as a method to assess oxidative stability of marine oils the decay of ESR visible spin adducts and how the persistence is dependent on other substances in the oil should be further investigated. ^{13}C NMR might be a possible method to study the reaction products after the decay of spin adducts. Some preliminary trials has shown changes in the olefinic region of the ^{13}C NMR spectra of cod liver oil containing PBN, however a

simple model system of these reaction products from the decay may provide valuable information making us able to study free radicals and early stages of lipid oxidation in marine oils.

6.3 NMR spectroscopy to study lipid oxidation

Fig. 16 presents the ^{1}H NMR spectra of ethyl docosahexaenoate with assessment of the main resonances. The spectra revealed both primary and secondary reaction products and also indicated formation of cyclic compounds such as epoxides, which are not regularly analysed by conventional methods. In addition, Paper III showed that ^{1}H NMR separated samples with and without antioxidant (α-tocopherol) based on the lack of signals in the aldehyde region (9-10.5 ppm) of the samples with added antioxidant. The detection limits of oxidation products were found to be ~0.01mM [Paper III].

NMR has, in present work, been shown to provide unique information about the reaction products from lipid oxidation of ethyl docosahexaenoate, which is the ethyl ester of one of the most representative fatty acids that first undergoes oxidation in fish oils. Lipid oxidation is generally measured by determination of the primary and secondary lipid oxidation products such as peroxide value and TBARS. This paper indicates that NMR may provide information about oxygen bridges forming cyclization (epoxidation) of fatty acids. Polymerization of fish oils, leading to changes in physical properties (increased viscosity) and changes in nutritional values of the lipids, is a challenge for the fish oil industry and should be taken into account when finding suitable methods to stabilise lipids.

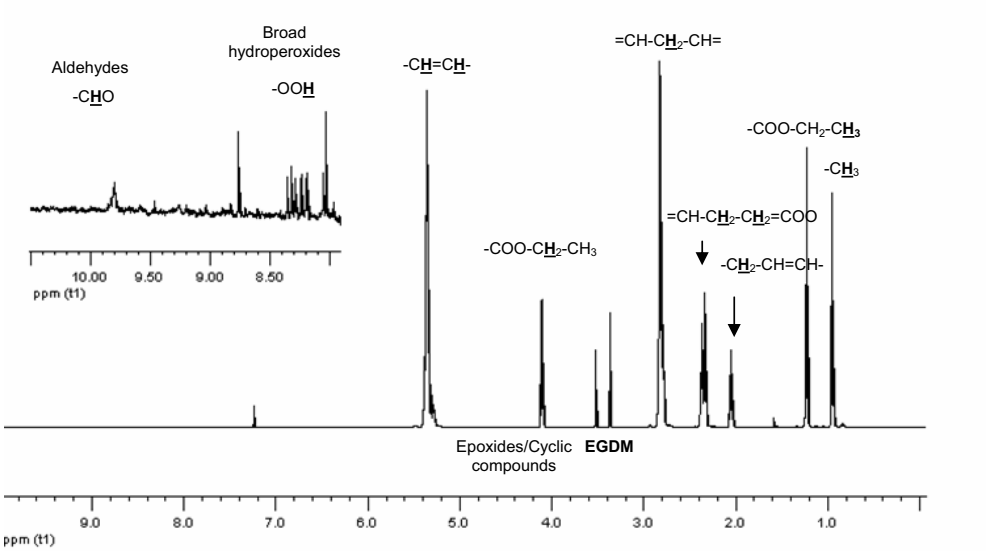


Fig. 16. 600 MHz ^{1}H NMR spectra of ethyldocosahexaenoate with assignment of the main peaks. The enlarged downfield region (10.5 – 8.0 ppm) show the development of hydroperoxides (-OOH) and aldehydes (-CHO)

In the present work it has been shown that it is possible to use NMR to study many chemical reactions in the same material, providing data on changes in double bounds, reduction of n-3 fatty acids and a broad range of specific oxidation products. However, the sensitivity is low at this stage. Improved methodology and equipment increases the sensitivity and thereby the information obtained by using ^{1}H NMR [Paper I]. Use of the CryoProbe technology is reported to enhance the sensitivity by a factor of four [Colson, 2005]. This was therefore investigated by comparison between conventional ^{1}H 600 MHz and recording of the same samples by ^{1}H 600 MHz magnet equipped with a cryoprobe. Fig. 17 presents the downfield region of tridocosahexaenoate (triDDD) before and after experimental storage for one week at 4^{o}C. An improved sensitivity was found and Fig. 17C showed well resolved resonances originating from hydroperoxy bearing protons (-OOH). The sensitivity could be further improved by higher magnetic strength.

Paper III includes an investigation of lipid oxidation in ethyl docosahexaenoate using ^{1}H NMR. Many of the same reaction products as are generally found during lipid oxidation of oils were detected. Spectra of oxidized fish oils show clear development of peaks in the downfield region of the spectra (8-10.5 ppm). One of the main benefits of using NMR is that the spectra might provide information on a broad range of compounds, including not only the reaction products formed during lipid oxidation, but also the chemical composition in

general and how it is affected by these reactions (e.g. unsaturated fatty acids). Oxidisability between different lipid components is reported to vary due to differences in: unsaturation of fatty acids, position of double bonds, positional distribution of fatty acids within the glycerides [Neff and El- Agaimy, 1996] and amount of free fatty acids (hydrolysed phospholipids and triglycerides) which is all information that is possible to obtain from ^{13}C and ^{1}H NMR spectra [Medina et al, 1994b; Paper VI]. NMR can provide information on both lipid composition and how the composition changes due to lipid oxidation and lipolysis. It is a requirement that the compounds are at detectable levels, which is one of the limitations at this stage. The paper also discusses the applicability of NMR analysis of intact raw material by using HR-MAS. This method is shown to provide well resolved spectra (see Fig. 7 and 8) from intact cod liver and gonads. However, the down-field region of the ^{1}H NMR spectrum where we have investigated the oxidation products, contain other resonances such as those originating from phospho-metabolites (degradation of ATP) [Wang et al, 1997], and this complicates the assignments.

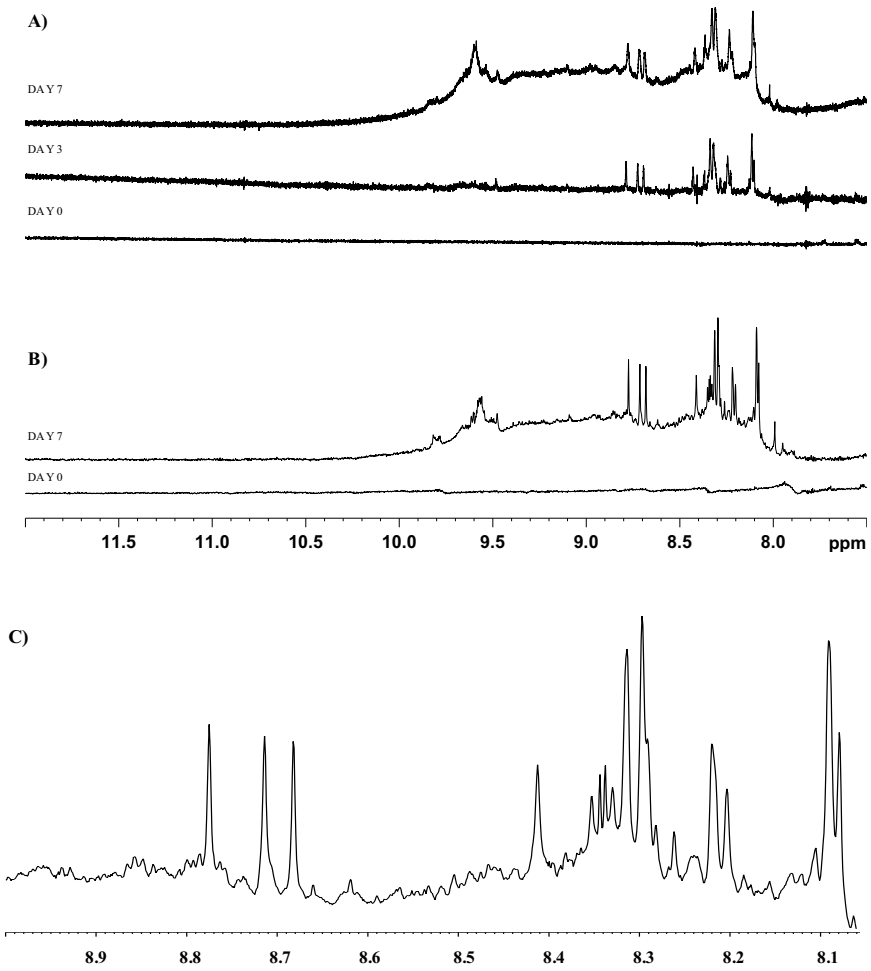


Fig 17. Enlargement of the downfield region of the ^{1}H NMR spectra of tridocosahexaenoate recorded during experimental storage at 3 and 7 days 25^{o}C. (A) Recorded at 600 MHz using a BBO probe, (B and C) Recorded at 600 MHz using a cryoprobe. Experimental conditions (as in Paper III). 0.1 ml sample in 0.6 ml $CDCl_3$. Centre frequency: 7.2 ppm; spectral width, 16.2 ppm; Acquisition time: 3.4 sec; 64K time domain data points; pulse angle: 50 degree; recycle delay: 4.4 sec, and 256 scans.

Concluding remarks:

- ^{1}H NMR has in the present study been shown to present a total picture of the lipid oxidation (non-selective) showing specific primary and secondary lipid oxidation products along with other oxidative changes such as cyclisation and reduction of olefinic signal intensity.

- Correlation between traditional oxidation methods and changes in specific regions of the spectra were found, however the sensitivity were low.
- The ^{1}H NMR spectra of DHA with and without α-tocopherol were easily separated due to the lack of resonances originating from the secondary lipid oxidation products (aldehydes) in the samples containing antioxidant.

6.4. Lipid composition and deterioration through HR NMR spectroscopy analysis

The present work has proven that it is possible to use NMR to study a wide range of lipid constituents of marine residual raw materials. Fig. 18 gives an overview of ^{13}C NMR and the main results from assignment of lipid compounds and demonstrates what information is possible to grain from the specific chemical shift regions. Paper VII demonstrated that it was possible to assign the positional distribution of PUFAs in PC and PE by using ^{13}C NMR. Further, the method enabled detection of cholesterol, TAG and spectra of the stored samples of roe and milt [Paper VIII] revealed different changes in lipids such as esterification of cholesterol and lipolysis of PL by formation of free fatty acids and hydrolysed acylglycerols and phospholipids. Paper VI presents different applications of HR NMR in the study of marine lipid and it also shows examples of MAS NMR spectra of cod liver with resolution enabling quantification of n-3 fatty acids on intact material.

The ^{13}C NMR spectra not only showed development of free fatty acids in these materials, it was also possible to distinguish the resonances originating from DHA and EPA from the other free fatty acids. Study of the corresponding changes in the carbonyl region made it possible to obtain information of acyl positions hydrolysed. The spectra of the stored samples of gonads showed that free fatty acids were esterified to cholesterol, forming cholesteryl esters (see also chapter 6.1). One of the most important parameters for classifying the quality and freshness of fish oils is the amount of free fatty acids. On a general basis, determination of the levels of FFA as a measure of lipolysis will not give a complete picture of the chemical changes due to lipolysis since there are more fatty acids hydrolysed from the phospholipids and triacylglycerols than those determined by measuring FFA in this material. This is shown by the formation of cholesteryl esters that esterifies the free fatty acids that are hydrolysed from the acylglycerols or phospholipids. I will therefore again stress the importance of having reliable methods, and use of analytical methods focusing on specific compounds might hide important information of the composition and compositional changes. In this context NMR is unique and provides information on the total lipid composition.

Composition of roe and milt analysed by NMR:

The compositional analysis of roe and milt [Paper VII] showed that these materials contained high levels of long chain PUFAs. In roe these fatty acids were primarily esterified in the *sn-2* position of the phospholipids, while in milt they were more equally distributed between the *sn-1* and *sn-2* position. These samples were rich in phospholipids but in the roe resonances originating from TAG were also detected. ^{13}C NMR showed that the ratio between the levels of PC and PE in roe were much higher than in milt. Considerable levels of cholesterol were found in both raw materials.

The NMR spectra obtained after one week of storage ($4^{o}C$) changed dramatically compared to the spectra of fresh lipid extracts [Paper VIII]. A clear formation of free fatty acids and other hydrolysed derivatives of PLs was found in roe and milt. These changes are explained by the activity of endogenous lipases in the raw material [Søvik, 2005]. Free fatty acids should generally be kept at low levels to prevent off-taste and lipid oxidation (se chapter 3.4). In addition, both ^{1}H and ^{13}C NMR spectra showed a clear formation of cholesteryl esters.

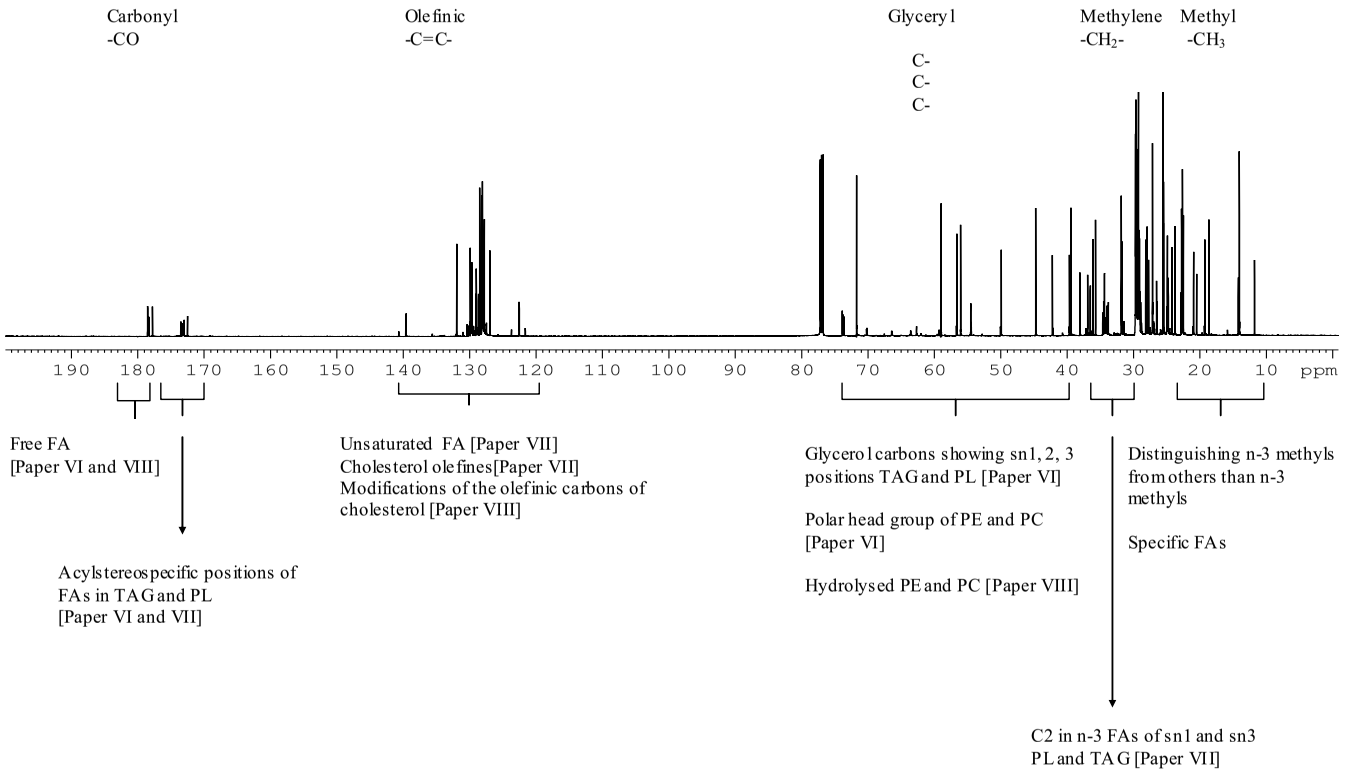


Fig. 18. ^{13}C NMR spectra of cod milt with illustrations of what lipid species are presently detected in freshly extracted raw material and detectable components formed during storage of the targeted cod residuals.

The main advantages of the HR NMR techniques compared to other methods, is the multi-component determination. Evaluation of both lipid composition and deterioration of heterogeneous lipids is possible without too much prior knowledge about the composition.

Concluding remarks:

- NMR has in the present work been found to be a unique and non-selective method for studying the lipid composition and deterioration in heterogeneous lipid extracts.
- Interpreting the ^{13}C NMR spectra was done by combining different HR NMR techniques and results from this work showed that it was possible to find the positional distribution of PUFAs in the phospholipids. The PUFAs were unequally distributed in acyl positions in PLs in roe compared to milt. It was also possible to find the ratio between PE and PC in addition to the relative composition between PL and TAG in these heterogeneous lipid extracts.
- The main deterioration of lipids of roe and milt were found by ^{1}H and ^{13}C NMR and were classified as: formation of specific free fatty acids and hydrolysed PLs and esterification of cholesterol.

7. CONCLUSIONS

Residual raw materials generated during processing of cod species make up considerable amounts of the health beneficial marine lipids. These current calculations show that an average daily production of 10 000 kg cod fillet will generate 1 tonne of marine lipids containing approximately 30% omega-3 fatty acids. These raw materials are very prone to biochemical deterioration leading to formation of degradation products (free fatty acids and lipid oxidation products) that effect organoleptic and nutritional properties. This shortens the shelf life and prevents high value food applications. The present work has demonstrated the importance of inactivating lipases soon after slaughtering, but also lipid oxidation should be reduced at early stages. To meet the demands for production of food grade qualities the analytical methodology for assessment of the changes occurring is fundamental. This is also important in order to find the best suitable methods of stabilizing these materials particularly concerning lipid oxidation.

Conventional approaches to analyse detailed lipid composition are laborious and often involve elaborate sample preparation for detection of a limited group of substances. In this work NMR has shown to be a method that has the advantages of providing information of a broad range of lipid compounds such as fatty acids, lipid classes (triacylglycerols, cholesterol, phospholipids, free fatty acids and cholesteryl esters), specific phospholipids, positional distribution of fatty acids in the molecules of TAG and PL, hydrolysed derivatives and new compounds that were not obviously formed in the raw materials studied.

ESR is shown to detect the very early stages of lipid oxidation in these marine materials and correlation with conventional lipid oxidation methods was found. However, some drawbacks should be investigated to show the further potential of the method (marine lipids are complex and endogenous chemical constituents may affect the spin trapping). The marine lipids are also shown to act different than vegetable oils in many analytical studies. What methods are suitable for vegetable oils may show different results when applied on marine lipids. This present work highlights some of the precautions important to take in order to gain successful and trustworthy results by using ESR.

^{1}H NMR was, in the present study, found to provide valuable information about the oxidative changes occurring in reference standards of DHA. It was possible to study specific lipid oxidation products such as different hydroperoxides, aldehydes and also cyclic

compounds, which is information that is not usually obtained by single conventional analytical methods. The spectra also demonstrated the effectiveness of α-tocopherols. Multivariate data analysis demonstrated changes in spectra during storage and showed a reduction of resonances originating from n-3 fatty acids during oxidation. However, the sensitivities were low, but were shown to be improved by using the new cryoprobe technology. The method may be used to gain information about reaction mechanisms and effect of antioxidants and can be a valuable tool in combination with traditional methods for studying lipid oxidation.

Results from interpretation of NMR spectra of lipid extracts of cod by-products have shown that a combination of various NMR techniques may provide detailed information on the lipid substances and the deterioration of lipids during storage. This work contains interpretation of PL spectra that are not previously reported for these materials.

This knowledge is valuable for optimizing processing and conservation in order to increase the food grade utilisation of these health beneficial lipids.

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Paper I

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By-products from gadiform species as raw material for production of marine lipids as ingredients in food or feed

Eva Falch [a,b,*], Turid Rustad [b], Marit Aursand [a]

[a] SINTEF Fisheries and Aquaculture Ltd., 7465 Trondheim, Norway

[b] Department of Biotechnology, Norwegian University of Science and Technology, Trondheim, Norway

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Abstract

An average production of 10,000 kg cod fillets (gadiform species) will generate by-products with more than 1000 kg marine lipids. More than 30% of these lipids are the health beneficial n-3 fatty acids, which have commercial value. To increase the industrial utilization of these lipids different sources of raw material need to be evaluated in respect to available amounts and chemical composition. The present work presents such data on four gadiform species caught in the Barents Sea (*Gadus morhua* (cod), *Pollachius virens* (saithe), *Melanogrammus aeglefinus* (haddock) and *Brosme brosme* (tusk)) evaluated at three seasons during 1 year. Both seasonal and inter-species differences were found in the amount of by-products and the lipid composition. The levels of polyunsaturated fatty acids were significantly higher in haddock liver and significantly lower in tusk liver compared to saithe and cod. However, regardless of the variations found, the lipids from all samples analysed contained significant quantities of health beneficial fatty acids. Liver was the best lipid source containing between 43 and 69% total lipids. The viscera contained between 2 and 9% lipids and trimmings contained approximately 1% lipids. While the lipids from liver generally contained more than 90% triacylglycerols, the lipids from the other by-products contained higher levels of phospholipids making up more than 60% of the total lipids in muscle and gonads.

Keywords: By-products; Fatty acids; Lipid classes; Cod; Viscera; Liver; Trimmings; Gadiforms

1. Introduction

Globally, more than 130 million tonnes of fish and shellfish are caught each year [1], of this, about 1/4 is discarded. Some of the by-products are utilized, but the main part is dumped to waste. In Norway, about 46% of by-products (viscera, liver and filleting residuals) from the cod fisheries are utilized [2], and only 1/3 of this amount is utilized for human consumption. It is therefore a great potential for the fishing industry to land and utilize a greater part of the total catch for higher value products. A particularly valuable fraction in marine biomass is the marine lipids, which have well documented beneficial health effects [3,4]. These effects are mainly associated with the long chain highly unsaturated fatty acids EPA (20:5 n-3, eicosapentaenoic acid) and DHA (22:6 n-3, docosahexaenoic acid) which are omega-3 fatty acids found in significant quantities in marine lipids. Other valuable lipid components found in marine raw material are phospholipids, lipid soluble vitamins, sterols and colour components. Cod liver oil has been a commercial health care product in Northern Europe for centuries, and cod liver oil as a nutraceutical can be traced back to 1783 [5]. Today, by-products that are utilized for human consumption are mainly liver and roe from relatively large cod (*Gadus morhua*). However, small cod, other gadiform species and fractions of by-products are also potential sources of valuable marine lipids. In order to utilize the marine lipids found in marine by-products, it is necessary to have knowledge about the possible variations in content and composition of the lipids. It is common knowledge that the chemical composition of cod may vary with factors such as age, stage of sexual maturity and diet [6–10] and seasonal differences in proximate and lipid composition in fish are previously suggested to be partly due to differences in water temperature [11,12]. In lean fish species, such as gadiform species, liver is the main lipid depot and the lipid content and composition is shown to vary among seasons [6,7,13]. While the liver lipids consist primarily of triacylglycerols and the muscle lipids consist of phospholipids, the viscera lipids are less explored as commercial products and investigations of the variation in available lipids are needed.

Recently, there has been interest in the processing of cod (*G. morhua*) by-products [15–17] by enzymatic hydrolysis.

* Corresponding author. Tel.: +47 40 00 53 50; fax: +47 93 27 07 02.
E-mail address: Eva.Falch@sintef.no (E. Falch).

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However, previous research on variation in chemical composition of cod by-products is limited and a scientific overview presenting the amounts of the different by-product fractions in different gadiform species are lacking. Some studies have investigated composition of specific fractions of by-products, but in contrast to our present studies, the majority of these studies have not focused on the industrial utilization of these raw materials. To utilize more than the fish fillet for human consumption, data on weight composition of by-products are needed [18]. Some data are established on Fishbase (www.Fishbase.org) for a number of species, however, data on the gadiform species analysed in the present study are generally not currently established in this database. The present data will be valuable for predicting amounts and lipid composition in by-products from various catches of these gadiform species for further processing.

The gadiform species used in the present study were caught in the Barents Sea, in an area with some of the most productive biomasses in the world. Gadiform species make up approximately one-third of the annual catch in these waters [14]. The present study includes seasonal catches of cod (*G. morhua)*, haddock (*Melanogrammus aeglefinus*), saithe (*Pollachius virens*) and tusk (*Brosme brosme*). By-products were weighed and lipids were analysed enabling calculation of available lipid constituents from the different gadiform species.

2. Materials and methods

2.1. Samples and treatment

In this survey, cod (Atlantic cod; *G. morhua*), haddock (*M. aeglefinus*) and saithe (*P. virens*) were collected from the Barents Sea and tusk (*B. brosme*) was collected from the North Sea. The sample collection started in winter (February/March) in 2001 and was repeated in summer (June) and autumn (September–November) the same year. In order to minimize the influence of size, size requirements were included in the trial. The size was measured as nose to fork of tail and the size specifications were chosen due to common industrial grading of gadiform species (Nordic countries). Only cod was used to study the influence of size. The cod was therefore collected in 3 size groups (50–60, 60–70 and 70–80 cm). The other species had the following size requirements: haddock: 45–55 cm, saithe: 70–80 cm and tusk: 40–50 cm. For each group of fish, a collection of 15 individuals was used. After bleeding, the fish were frozen (ungutted) and stored at <-24 °C until thawing. Before gutting and filleting, the fish was thawed in water with a fish to water ratio 1:4.5. The starting temperature of the water by immersion of the fish was 20 °C. Thawing time was approximately 22 h, to a core temperature of 0 ± 1 °C. After gutting, visceral components, liver, roe, milt, viscera (stomach and gut), trimmings (v-cut and belly flap/nape), heads and backbone were weighed. The filleting parts specified (v-cut, belly flap, head and backbone) were cut corresponding to industrial filleting of gadiform species (BAADER Food Processing Machinery, market leader of fish processing equipment) for whitefish (www.baader.com). The total weight of viscera consisted of all parts except liver. The weight of round fish, length and the weight of head were also determined. The liver, viscera (stomach, gut) and trimmings from each group of fish (a pool from 15 individuals) were homogenized using a Waring Blender with a knife that was sharp enough to cut the hard wall of the stomach. Homogenization of the samples was done within 14 days after catch. The fish were not split in groups according to sex. The aim of this study was to establish a scientific basis for commercial bulk production and the differences between sexes in the material are therefore not important. Another reason for this decision is that separation of the sexes is difficult for immature cod [19].

In addition to the work characterizing saithe described above, one catch (spring/April 2000) of saithe were characterized in more detail studying roe and milt. A collection of 30 kg viscera (all visceral cavity) from saithe were frozen on-board (−20 °C) a freeze trawler before transportation to the laboratory. The samples were thawed before sorting and homogenising the gonads. A characterization of total lipids, lipid classes and fatty acids are described below.

2.2. Lipid analysis

The lipids were extracted using a modified Bligh and Dyer [20] method. Fatty acid methyl esters were prepared according to Metcalfe et al. [21], and analyzed on a Carbo Erba HRGC 5160 gas chromatograph equipped with an Omegawax 320 (Supelco) glass capillary column, employing on-column injection and flame ionization detector. Peaks were reported by a Shimadzu Chrompac C-3R computing integrator, identified by comparison with known standards (Nu-Chek Prep, MN, USA) and quantified by means of the response factor to the internal standard C21:0. Lipid classes were separated by an Iatroscan thin layer chromatography-flame ionization detector system (TLC-FID) (Iatron Laboratories, Tokyo, Japan). Chromarods SIII (Iatron Laboratories, Tokyo, Japan) were first scanned twice through the Iatroscan FID immediately before sample application in order to remove possible contaminants from the rods. Chloroform solutions of the extracts (1 ml, concentration 20 mg/ml) were spotted on the Chromarods SIII by use of a single spotting action and a 10 ml chromatographic syringe. After spotting, the rods were conditioned in a constant humidity chamber for 8 min over a saturated NaCl solution and then transferred immediately to the developing tank. The solvent system consisted of hexane/diethyl ether/formic acid (85:35:0.04).

2.3. Data treatment

Weight of the fractions, the total lipid content, amount of individual fatty acids and lipid classes were evaluated and compared. Multivariate statistical analysis was performed using analysis of effect (ANOVA) and principal component analysis (PCA) in Guideline™ [Camo, Oslo, Norway]. For the weight fractions, results from all individuals in the groups ($N = 15$) were included. Some of the statistical work has been performed using the ANOVA, Tukey's family error rate ($\alpha = 0.05$) in MINITAB. The fatty acids are presented as percentage of total fatty acids, but when appropriate, quantitative values (mg/g lipids) are given. Using percentages is the more common way to present the fatty acid composition and are therefore easier to compare with previous findings.

3. Results and discussion

3.1. Available amounts of by-products

On an average, production of cod fillets will generate 2/3 of the whole body weight as by-products. The present data show that the viscera (all inner fractions) makes up 12–15% of the whole body weight of the four gadiform species analysed, the head 15–20% and the backbone and trimmings make up 18–30% (Table 1). An average daily catch of gadiforms from a trawling vessel (10,000 kg fillets) will generate 17,000–21,000 kg by-products depending on species (Table 2). The commercial value of these by-products is varying, and the present data show significant differences in lipid content and composition in the by-products investigated (Figs. 1 and 2, Tables 3–5). The main lipid depot is found in the liver with the amount of total lipids ranging from 54 to 69% in cod saithe and haddock and 43% in tusk (Table 3). The viscera and trimmings contain lower amount of total lipids (Table 3), ranging from 2 to 9% in viscera and as low as 1% in trimmings. The head, trimmings, backbone and viscera make up more than 60% of the available catch (93%) of the by-products, however, it only

Table 1
Weight distribution (percent of whole body weight) of the different by-product fractions from *Gadus morhua* (cod), *Pollachius virens* (saithe), *Melanogrammus aeglefinus* (haddock) and *Brosme brosme* (tusk)

	Cod (c)[a] ($N = 135$)	Saithe (s)[a] ($N = 36$)	Haddock (h)[a] ($N = 44$)	Tusk (t)[a] ($N = 5$)	Significant differences (significance level: 0.05)	
					Species	Season
Head	20.2 (±0.5)	15.3 (±0.2)	18.9 (±0.2)	17.9 (±1.6)	c > s > h	a > s > w
Roe[b]	0.7 (±0.8)	0.3 (±0.3)	0.7 (±0.5)	2.0 (±4.0)	ch > s	w > as
Milt[b]	1.3 (±2.4)	0.2 (±3.0)	0.1 (±0.2)	0.0 (±0.0)	c > sh	w > as
Liver	5.2 (±0.5)	6.0 (±0.3)	4.2 (±0.2)	5.3 (±5.5)	s > ch	n.s.[c]
Stomach	2.2 (±1.4)	3.9 (±2.3)	1.4 (±0.5)	2.0 (±0.3)	s > ch	w > a
Gall bladder	0.2 (±0.1)	0.1 (±0.1)	0.1 (±0.0)	0.4 (±0.9)	s > ch	m[d]
Intestines	1.3 (±0.1)	1.1 (±0.0)	2.7 (±0.2)	0.9 (±0.2)	s > ch	w > as
Blindsack	1.5 (±0.1)	1.8 (±0.0)	1.9 (±0.0)	0.5 (±0.3)	hs > c	ws > a
Viscera	5.6 (±1.2)	7.2 (±2.0)	6.2 (±0.3)	5.4 (±0.9)	h > sc	a > s > w
Guttet weight	66.0 (±3.8)	70.4 (±1.2)	67.9 (±0.7)	63.5 (±18.2)	m[d]	m[d]
Back bone	9.7 (±0.3)	9.9 (±0.3)	10.6 (±0.3)	8.4 (±0.6)	h > sh	w > a > s
Trimmings	8.2 (±0.3)	8.8 (±1.3)	9.3 (±0.3)	21.2 (±0.9)	s > h > c	a > ws
Filet	33.6 (±0.5)	36.9 (±0.7)	31.9 (±0.5)	32.3 (±4.2)	m[d]	m[d]
Skin	4.2 (±0.1)	4.8 (±0.1)	4.5 (±0.3)	6.4 (±1.0)	s > hc	a > s > w

The viscera consist of all inner part except liver, roe and milt. The gutted weight is gutted and de-headed. AV, average value calculated as an average of the averages in each season; w, winter; s, summer; a, autumn. The samples that are significantly different are specified.
[a] Abbreviations for the species used to present the statistics.
[b] Average in, respectively, female and male cod over the year.
[c] n.s., non-significant data.
[d] m, missing data.

accounts for approximately 1% of the available lipids (240 kg lipids from an average daily catch) (Table 2). Sorting out liver as a separate product will therefore generate a residual bulk fraction with low amounts of total lipids.

Both seasonal and inter-species differences are found in the amount of by-products showing generally higher amounts of viscera (stomach, intestines and liver) in saithe. In the winter catch, the gonadosomatic indexes (milt and roe weight as a percentage of the whole body weight) are higher due to the spawning that takes place from January to April in the fishing grounds investigated [14]. This is the most intensive fishing period in these waters. Studying the three size categories of cod, a higher gonadosomatic index (roe) was found in the winter catch, and analysis of variance within the winter catch alone, revealed a higher gonadosomatic index in the large female cod compared to the other groups of samples investigated. Our data

Table 2
Mass distribution of by-products and lipids calculated from an average daily catch of gadiform species (*Gadus morhua* (cod), *Pollachius virens* (saithe), *Melanogrammus aeglefinus* (haddock)) (producing 10,000 kg fillet)

Species	Daily catch (kg)	By-products	Total quantity		Lipids		n-3[a]
			%	kg	%	kg	kg
Cod	29762	Total viscera (except liver)	8.6	2560	0.3	61	14
		Liver	5.2	1548	4.8	946	222
		Trimmings, head and backbone	52.6	15655	0.8	157	46
		By-products	66.4	19762	5.9	1164	282
Saithe	27100	Total viscera (except liver)	8.3	2249	0.4	124	25
		Liver	6.0	1626	6.0	1024	254
		Trimmings, head and backbone	48.8	13225	0.8	132	34
		By-products	63.1	17100	7.1	1280	313
Haddock	31348	Total viscera (except liver) liver trimmings	9.0	2821	0.3	59	16
		Total viscera (except liver) liver trimmings	4.2	1317	3.7	795	229
		Total viscera (except liver) liver trimmings	54.9	17210	0.8	172	50
		By-products	68.1	21348	4.8	1027	295
Tusk[b]	30928	Total viscera (except liver)	5.4	1670	0.1	30	11
		Liver	5.3	1639	2.3	710	172
		Trimmings, head and backbone	53.9	16670	0.4	117	52
		By-products	64.6	19979	2.8	856	235

Calculation of average data.
[a] Based on quantitative analysis of total lipids.
[b] Calculations based on few samples ($N = 5$).

Table 3
Lipid classes (triacylglyserols, free fatty acids, cholesterol, sterylester and phospholipid) (percent of total lipids) in the liver, viscera, and trimmings from *Gadus morhua* (cod), *Pollachius virens* (saithe), *Melanogrammus aeglefinus* (haddock) and *Brosme brosme* (tusk)

Lipid classes	Cod small[c]			Cod medium[c]			Cod large[c]			Saithe[s]			Haddock[h]			Tusk	Significant differences (significance level: 0.05)	
	w[a]	s[a]	a[a]	w[a]	s[a]	a[a]	w[a]	s[a]	a[a]	w[a]	s[a]	a[a]	w[a]	s[a]	a[a]	s[a]	Species (csh)	Seasonal
Liver																		
Lipid content	53.6	61.1	57.9	64.2	60.4	64.1	65.4	63.7	59.7	58.1	61.8	69.2	60.8	58.0	62.3	43.3	n.s.	n.s.
Triacylglycerol	92.0	96.1	95.5	95.0	95.8	95.6	95.5	96.2	95.6	91.2	93.2	95.1	98.6	96.7	97.7	89.7	h > sc	n.s.
Phospholipids	0.5	0.3	0.6	–	0.3	0.5	0.4	0.4	0.4	0.7	0.4	0.4	0.2	0.1	0.2	–	cs > h	n.s.
Free fatty acids	3.3	1.4	1.5	2.4	1.7	1.3	1.2	1.6	1.3	3.1	2.5	1.9	1.0	0.9	0.6	2.2	s > c > h	w > a
Cholesterol	2.3	1.4	1.3	1.4	1.3	1.3	1.5	1.2	1.1	3.0	2.3	2.3	0.4	1.0	0.7	1.7	s > c > h	n.s.
Steryl ester	0.4	0.0	0.0	0.4	0.0	0.0	0.0	0.0	0.0	0.4	0.0	0.0	0.0	0.0	0.0	0.5	n.s.	w > sa
Viscera																		
Lipid content	2.1	2.6	2.5	3.0	3.0	1.9	2.3	2.1	1.9	9.1	4.6	2.7	1.8	2.5	2.1	1.7	n.s.	n.s.
Triacylglycerol	7.1	15.7	3.3	29.3	26.1	5.5	25.9	10.5	10.4	69.1	12.6	2.0	4.9	15.7	23.4	8.9	n.s.	w > a
Phospholipids	30.8	38.1	29.6	19.7	30.7	32.7	23.6	40.1	25.3	13.2	46.8	47.3	40.7	37.0	36.8	31.1	n.s.	sa > w
Free fatty acids	42.0	25.6	46.9	30.3	23.2	40.8	30.4	29.0	44.5	12.1	21.7	33.7	36.1	30.0	23.8	37.4	c > h > s	a > ws
Cholesterol	19.1	19.7	19.7	16.2	19.3	20.1	18.4	19.0	18.4	4.6	16.7	16.0	17.1	16.6	15.7	21.2	c > h > s	n.s.
Steryl ester	1.0	0.4	0.6	4.5	0.8	0.9	1.7	1.5	1.4	1.0	2.1	1.0	1.3	0.7	0.4	1.4	n.s.	w > a
Trimmings																		
Lipid content	1.0	1.1	0.8	1.0	1.0	1.0	1.0	1.0	1.0	1.5	1.1	1.1	1.1	1.0	0.7	0.7	s > ch	n.s.
Triacylglycerol	3.8	5.2	4.3	9.5	5.1	4.5	8.0	4.9	10.4	3.9	1.5	2.9	2.5	2.1	1.3	4.2	c > sh	n.s.
Phospholipids	69.4	75.7	79.6	63.7	76.3	79.1	65.1	77.0	25.3	81.5	86.4	84.9	78.1	78.4	82.4	70.5	sh > c	a > w
Free fatty acids	11.6	4.8	4.8	12.3	5.3	5.6	12.0	5.0	44.5	5.8	3.2	3.4	5.9	6.6	5.0	9.6	c > s	w ≫ sa
Cholesterol	11.6	10.5	9.4	11.0	10.2	8.5	10.4	9.5	18.4	7.4	7.6	7.3	10.0	10.5	9.6	12.8	ch>s	n.s.
Steryl ester	3.7	4.1	1.9	3.5	3.2	2.3	4.5	3.8	1.4	1.4	1.3	1.5	3.5	2.4	1.8	2.9	ch > s	ws>a

[c,s,h,t] are abbreviations for the fish species used to presenting the results from the analysis of variance. n.s.: not significant.
[a] Season: w, winter; s, summer; a, autumn.

indicate that the small cod are premature, which is in accordance with observations on the length of cod at first maturity 70–85 cm [14]. The gonadosomatic indexes in mature female cod in the present study were between 3 and 4, which is in accordance with previous findings on roe from spawning cod (*G. morhua*) (gonadosomatic index 3.5) [22]. The gonadosomatic index for mature cod in a previous Norwegian study were reported to be lowest in August followed by an increase to reach a maximum of 9–10 in March [9]. The roe which is one of the two by-product fractions that are sorted out as food products therefore makes up an average of 5% of the by-products in female cod during the spawning season. The liver, which is the

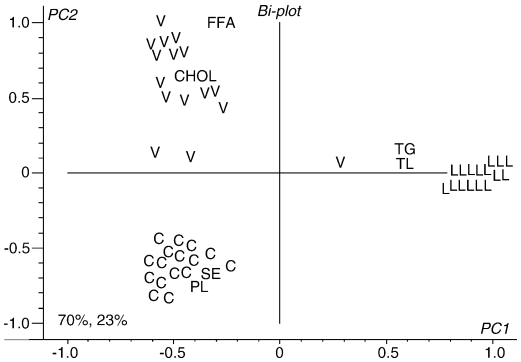

Fig. 1. PCA plot (bi-plot containing the scores and loadings) of the following lipid classes: TG, triacylglycerols; PL, phospholipids; FFA, free fatty acids; Chol, cholesterol; SE, steryl esters; and TL, total lipids. The scores represents samples of: V, viscera; L, liver; and C, trimmings (cut-offs) in *Gadus morhua* (cod), *Pollachius virens* (saithe), *Melanogrammus aeglefinus* (haddock) and *Brosme brosme* (tusk). The plots are presenting the inter-relationship between the response variables in the PC1 and PC2 plane. PC1 plane explains 70% of the variance while PC2 explains 23%. The cluster formation shows the similarities in the specific lipid classes among the three different fractions (viscera, liver and trimmings).

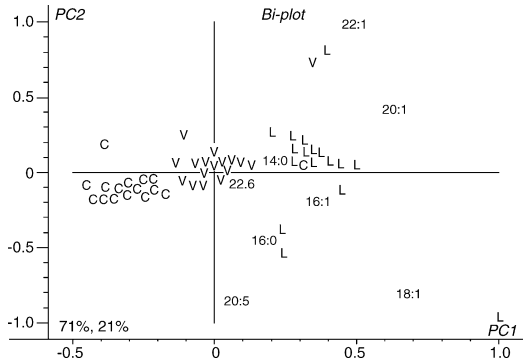

Fig. 2. PCA plot (bi-plot containing the scores and loadings) where the loadings are the fatty acids and the scores represents samples of: V, viscera; L, liver; and C, trimmings (cut-offs) in *Gadus morhua* (cod), *Pollachius virens* (saithe), *Melanogrammus aeglefinus* (haddock) and *Brosme brosme* (tusk). The plots are presenting the inter-relationship between the response variables in the PC1 and PC2 plane. PC1 plane explains 71% of the variance while PC2 explains 21%. The cluster formation shows the similarities in the specific fatty acids among the three different fractions (viscera, liver and trimmings). The fatty acids that are most significantly different between the fractions are illustrated.

Table 4
Fatty acid composition (percent of total fatty acids) in by-products from *Gadus morhua* (cod), *Pollachius virens* (saithe), *Melanogrammus aeglefinus* (haddock) and *Brosme brosme* (tusk)

Fame%	Cod small[c]			Cod medium[c]			Cod large[c]			Saithe[s]			Haddock[h]			Tusk[t]	Significant differences (significance level: 0.05)	
	w[a]	s[a]	a[a]	w[a]	s[a]	a[a]	w[a]	s[a]	a[a]	w[a]	s[a]	a[a]	w[a]	s[a]	a[a]	s[a]	Species (csh)	Seasonal
(A) Liver																		
C14:0	5.2	4.4	4.2	4.1	4.0	5.0	4.9	3.4	3.9	–	3.8	3.9	–	4.5	3.8	4.8	n.s.	n.s.
C16:0	9.2	6.8	7.5	6.7	7.3	8.0	7.9	6.4	7.3	–	7.3	8.5	–	9.4	7.7	7.2	n.s.	n.s.
C18:0	2.6	3.0	2.8	2.8	3.0	2.8	2.6	3.5	2.8	–	3.9	4.3	–	3.8	3.7	2.4	sh > c	n.s.
C20:0	0.0	0.1	0.1	0.0	0.1	0.0	0.1	0.1	0.1	–	0.1	0.0	–	0.1	0.1	0.1	n.s.	n.s.
C22:0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	–	0.0	0.0	–	0.2	0.0	0.4	n.s.	n.s.
C16:1	8.1	6.4	7.2	6.6	6.6	8.1	7.7	5.9	7.1	–	5.9	6.5	–	7.6	7.1	5.7	n.s.	n.s.
C18:1 n-9	19.8	18.9	20.8	17.5	19.0	21.8	19.8	18.5	20.6	–	18.5	20.0	–	21.3	21.6	16.5	n.s.	n.s.
C20:1	12.9	14.9	14.7	14.5	14.5	15.7	14.8	14.3	15.1	–	12.8	11.7	–	10.6	10.4	16.6	c > sh	n.s.
C22:1	7.4	10.2	8.6	8.4	10.8	8.3	8.2	10.1	8.9	–	10.7	9.2	–	1.5	4.4	19.4	cs > h	n.s.
C18:2 n-6	2.2	3.5	3.0	2.7	3.4	3.5	2.4	3.1	3.2	–	2.6	2.3	–	3.3	2.3	1.3	n.s.	n.s.
C18:3 n-6	0.2	0.2	0.3	0.3	0.2	0.2	0.3	0.3	0.1	–	0.2	0.2	–	0.5	0.3	0.2	n.s.	n.s.
C20:2 n-6	0.4	0.4	0.4	0.4	0.4	0.4	0.3	0.4	0.4	–	0.4	0.3	–	0.5	0.6	0.4	h > cs	n.s.
C20:3 n-6	0.1	0.1	0.2	0.1	0.1	0.1	0.1	0.1	0.1	–	0.1	0.0	–	0.1	0.0	0.0	n.s.	n.s.
C20:4 n-6	0.6	1.3	0.7	0.9	0.8	0.7	0.7	0.8	0.7	–	0.8	0.6	–	1.3	1.4	0.7	h > cs	n.s.
C18:3 n-3	1.0	1.0	1.1	1.1	2.9	1.0	1.0	1.1	1.0	–	1.1	1.1	–	1.6	1.3	0.9	n.s.	n.s.
C18:4 n-3	2.7	3.2	2.7	4.2	1.0	2.5	3.0	3.2	2.7	–	3.9	3.1	–	3.7	3.4	2.1	n.s.	n.s.
C20:4 n-3	0.8	0.8	0.7	0.9	0.7	0.7	0.8	0.9	0.6	–	0.7	0.5	–	0.7	0.7	0.7	ch > s	w > a
C20:5 n-3	10.0	9.6	9.9	11.4	9.1	8.6	9.3	10.4	9.6	–	10.3	11.5	–	13.1	14.8	5.0	h > cs	n.s.
C22:5 n-3	1.2	1.0	1.0	1.2	1.1	0.8	1.1	1.3	1.0	–	0.9	0.8	–	1.2	1.3	1.4	ch > s	n.s.
C22:6 n-3	15.7	14.3	14.2	16.1	15.0	11.8	15.0	16.2	14.6	–	15.9	15.5	–	15.2	15.2	14.2	n.s.	n.s.
SFA	17.0	14.2	14.5	13.6	14.3	15.8	15.5	13.4	14.2	–	15.1	16.6	–	17.9	15.3	14.9	n.s.	n.s.
MUFA	48.2	50.4	51.2	47.0	50.9	54.0	50.5	48.8	51.8	–	47.9	47.3	–	41.0	43.4	58.2	c > h	n.s.
PUFA	34.8	35.4	34.3	39.4	34.8	30.2	34.0	37.7	34.1	–	36.9	36.0	–	41.0	41.3	26.9	h > cs	n.s.
n-3	31.4	29.9	29.6	35.0	29.9	25.3	30.2	33.1	29.5	–	32.9	32.6	–	35.4	36.6	24.3	h > cs	n.s.
n-6	3.4	5.6	4.7	4.3	4.9	4.9	3.8	4.6	4.6	–	4.0	3.4	–	5.6	4.7	2.6	ch > s	n.s.
(B) Viscera																		
C14:0	4.0	3.4	3.6	4.3	3.3	2.3	3.7	2.5	2.9	5.6	4.9	2.9	–	4.3	4.1	2.8	n.s.	w > a
C16:0	9.4	8.0	7.9	8.9	7.4	9.1	8.3	7.6	7.6	8.2	9.6	7.9	–	8.5	8.2	8.4	n.s.	n.s.
C18:0	4.3	4.1	4.4	3.5	3.9	5.0	4.4	5.0	5.0	1.7	3.1	6.2	–	3.9	4.4	6.2	n.s.	a > w
C20:0	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.2	0.1	0.1	0.2	–	0.1	0.1	0.2	n.s.	n.s.
C22:0	0.0	0.1	0.0	0.0	0.0	0.0	0.1	0.1	0.0	0.1	0.0	0.0	–	0.1	0.0	0.3	n.s.	n.s.
C16:1	5.6	5.8	5.5	7.1	5.8	4.9	5.5	4.9	5.2	7.3	5.9	4.6	–	4.7	4.4	5.0	n.s.	w > a
C18:1 n-9	17.0	18.5	17.5	17.8	18.2	19.0	17.2	17.7	17.6	15.4	18.8	17.8	–	16.3	16.0	17.2	n.s.	n.s.
C20:1	9.7	11.7	11.4	11.7	12.1	10.2	10.6	10.8	11.3	17.3	9.7	8.8	–	9.5	8.2	11.6	n.s.	w > a
C22:1	6.5	4.4	6.7	6.9	6.2	3.5	6.2	3.7	4.6	18.7	8.8	4.7	–	8.5	7.3	6.3	n.s.	n.s.
C18:2 n-6	1.6	2.2	2.1	1.7	2.2	1.8	1.7	2.0	2.4	2.7	1.9	2.5	–	1.7	1.7	1.2	s > ch	n.s.
C18:3 n-6	0.1	0.2	0.2	0.1	0.2	0.3	0.2	0.2	0.3	0.3	0.2	0.2	–	0.2	0.2	0.1	n.s.	n.s.
C20:2 n-6	0.3	0.3	0.4	0.3	0.4	0.4	0.3	0.4	0.5	0.3	0.3	0.4	–	0.4	0.5	0.4	h > cs	n.s.
C20:3 n-6	0.1	0.1	0.0	0.1	0.1	0.1	0.0	0.1	0.1	0.1	0.1	0.1	–	0.1	0.0	0.1	n.s.	n.s.
C20:4 n-6	2.2	3.1	2.5	1.8	2.4	3.1	2.4	3.9	2.7	0.6	2.2	3.1	–	2.4	3.2	4.3	n.s.	as > w
C18:3 n-3	0.7	0.6	0.8	0.8	0.7	0.5	0.7	0.5	0.5	0.7	0.6	0.7	–	0.7	1.1	0.5	h > cs	n.s.
C18:4 n-3	1.7	1.6	2.3	2.3	1.8	1.1	1.8	1.3	1.4	3.0	2.3	1.6	–	1.5	3.1	0.9	n.s.	n.s.
C20:4 n-3	0.7	0.6	0.6	0.7	0.6	0.4	0.6	0.5	0.5	0.4	0.5	0.5	–	0.6	0.7	0.4	h > s	n.s.
C20:5 n-3	10.7	10.9	10.6	10.9	11.2	11.6	11.5	12.6	12.5	7.3	12.7	13.6	–	11.7	12.0	7.0	n.s.	sa > w
C22:5 n-3	1.0	1.0	1.2	1.1	1.2	1.0	1.1	1.3	1.3	0.6	0.7	1.0	–	1.1	1.4	1.6	ch > s	n.s.
C22:6 n-3	24.3	23.3	22.0	20.0	22.0	25.6	23.6	24.9	23.3	9.5	17.6	23.2	–	23.7	23.2	25.5	ch > s	n.S.
SFA	17.7	15.6	16.1	16.8	14.8	16.5	16.6	15.4	15.6	15.8	17.7	17.2	–	16.8	16.9	18.0	n.s.	n.s.
MUFA	38.7	40.4	41.1	43.5	42.3	37.6	39.6	37.0	38.7	58.7	43.2	35.9	–	39.0	36.0	40.1	n.s.	w > a
PUFA	43.5	44.0	42.8	39.7	42.9	45.9	43.9	47.6	45.6	25.5	39.0	46.9	–	44.2	47.1	41.9	ch > s	sa > w
n-3	39.2	38.1	37.5	35.7	37.6	5.8	39.3	40.1	39.6	21.5	34.4	40.6	–	39.3	41.5	35.9	ch > s	n.s.
n-6	4.4	5.9	5.3	4.0	5.3	40.1	4.6	6.6	6.1	4.0	4.6	6.3	–	5.6	4.9	6.0	n.s.	sa > w
(C) Trimming																		
C14:0	2.3	2.6	–	2.5	2.3	1.9	2.2	2.1	2.1	4.2	2.4	1.6	1.9	1.8	1.6	2.1	n.s.	w > a
C16:0	9.2	8.3	–	8.5	8.6	9.5	7.8	8.1	9.5	8.7	9.4	9.5	9.6	10.0	9.7	10.7	n.s.	w > a
C18:0	4.4	3.7	–	4.2	3.8	3.8	4.7	4.1	3.8	4.2	4.3	5.1	3.9	3.8	4.9	5.2	n.s.	n.s.
C20:0	0.0	0.1	–	0.0	0.1	0.2	0.1	0.1	0.1	0.1	0.5	0.1	0.1	0.1	0.1	0.1	n.s.	n.s.
C22:0	0.0	0.0	–	0.0	0.1	0.0	0.1	0.1	0.0	0.0	1.8	0.0	0.2	0.1	0.0	0.2	n.s.	n.s.

Table 4 (*Continued*)

Fame%	Cod small[c]			Cod medium[c]			Cod large[c]			Saithe[s]			Haddock[h]			Tusk[t]	Significant differences (significance level: 0.05)	
	w[a]	s[a]	a[a]	w[a]	s[a]	a[a]	w[a]	s[a]	a[a]	w[a]	s[a]	a[a]	w[a]	s[a]	a[a]	s[a]	Species (csh)	Seasonal
C16:1	3.4	3.9	–	4.5	3.8	3.4	4.0	3.7	3.7	7.5	2.5	3.0	3.3	2.8	2.9	3.5	n.s.	w > a
C18:1 n-6	15.6	15.1	–	15.9	16.0	16.4	15.1	15.6	16.5	21.9	16.2	17.1	16.1	16.1	15.3	18.0	n.s.	w > sa
C20:1	5.8	7.7	–	7.4	7.7	6.1	8.0	7.5	6.4	14.4	4.5	4.6	5.1	3.6	4.1	6.8	n.s.	n.s.
C22:1	2.6	3.0	–	3.2	3.2	2.3	3.3	2.8	2.7	8.8	0.0	2.0	2.4	1.7	1.6	3.6	n.s.	n.s.
C18:2 n-6	1.6	1.8	–	1.6	1.7	2.3	1.9	1.8	1.5	2.1	1.5	1.5	1.4	1.3	1.5	1.1	n.s.	n.s.
C18:3 n-6	0.1	0.1	–	0.2	0.1	0.3	0.2	0.1	0.2	0.2	0.1	0.2	0.1	0.1	0.3	0.1	n.s.	n.s.
C20:2 n-6	0.3	0.3	–	0.3	0.3	0.4	0.3	0.3	0.3	0.3	0.3	0.2	0.4	0.3	0.4	0.3	n.s.	n.s.
C20:3 n-6	0.1	0.1	–	0.1	0.1	0.2	0.1	0.1	0.1	0.1	0.1	0.0	0.1	0.1	0.1	0.1	n.s.	n.s.
C20:4 n-6	2.8	3.6	–	2.5	2.7	2.4	3.4	3.6	2.4	0.6	2.4	1.9	3.9	3.7	4.1	4.2	h > c	n.s.
C18:3 n-3	0.5	0.4	–	0.5	0.4	0.6	0.5	0.4	0.3	0.9	0.5	0.5	0.4	0.4	0.4	0.4	n.s.	n.s.
C18:4 n-3	1.1	1.1	–	1.5	1.0	1.0	1.2	1.0	0.9	2.8	1.0	0.9	0.9	0.9	0.9	0.5	n.s.	w > a
C20:4 n-3	0.7	0.5	–	0.6	0.5	0.6	0.6	0.5	0.4	0.6	0.6	0.4	0.6	0.4	0.5	0.4	n.s.	w > a
C20:5 n-3	14.2	14.7	–	14.9	14.3	15.7	14.6	15.0	16.4	10.5	15.0	17.1	14.6	15.8	16.6	6.8	n.s.	n.s.
C22:5 n-3	1.3	1.4	–	1.3	1.5	1.1	1.4	1.5	1.2	0.9	1.4	1.1	1.8	1.5	1.6	1.3	n.s.	ws > a
C22:6 n-3	33.8	31.8	–	30.4	32.1	31.8	30.5	31.4	31.5	11.3	35.5	33.2	33.3	35.7	33.5	34.8	n.s.	n.s.
SFA	16.0	14.6	–	15.3	14.8	15.4	14.8	14.5	15.5	17.2	18.4	16.2	15.7	15.8	16.3	18.2	n.s.	w > a
MUFA	27.5	29.7	–	31.0	30.7	28.2	30.5	29.7	29.3	52.5	23.2	26.8	27.0	24.1	23.9	31.9	n.s.	n.s.
PUFA	56.5	55.7	–	53.8	54.5	56.3	54.8	55.9	55.2	30.2	58.4	57.0	57.4	60.1	59.9	49.9	n.s.	n.s.
n-3	51.7	49.9	–	49.2	49.6	50.8	48.9	49.9	4.5	26.9	54.0	53.2	51.5	54.6	53.4	44.2	n.s.	n.s.
n-6	4.9	5.9	–	4.5	4.9	5.6	5.9	6.0	50.7	3.3	4.4	3.8	5.9	5.5	6.4	5.7	ch > s	n.s.

(A) liver; (B) viscera; and (C) trimmings. SFA, sum saturated fatty acids; MUFA, sum monounsaturated fatty acids; PUFA, sum polyunsaturated fatty acids.[c,s,h,t] are abbreviations for the fish species used for presenting the results from the analysis of variance. n.s.: not significant.

[a] Season: w, winter; s, summer; a, autumn.

other main fraction commercially utilized from large cod (*G. morhua*) (>4 kg) constitute on an average 5% (1.5–14.9%) of the total weight of fish (Table 1). Tusk is different from the other three species both in amount of rest raw material, and in chemical composition in general, so the analysis of variance is therefore calculated with respect to cod, saithe and haddock. The hepatosomatic index (liver weight as a percentage of the whole body weight) is higher for tusk, followed by saithe, a lower index are found in cod and haddock. The lower hepatosomatic index recorded in the autumn and summer collection of cod in this study is in accordance with previous findings on pre-spawning cod [6] and the hepatosomatic index found in the gadiform species from the present study are in agreement with values found in a long term study on cod caught in the Barents Sea [23]. The lipid content found in liver, are also in accordance with previous findings on cod (*G. morhua*) [24]. When studying the lipid content in liver of the mature cod (*G. morhua*) separately, higher levels were found in the winter collection. However, no seasonal effect was found when analyzing the variance in total lipids in all species included in our study. The largest cod (70–80 cm) had higher hepatosomatic index and lower lipid levels in liver compared to the medium and small size cod. The lipid content in roe and milt from saithe caught in April (spawning period) (Table 5) was 5.7 and 3.3%, respectively.

Table 5
Chemical composition of roe and milt from a spring collection of *Pollachius virens* (saithe)

		Roe	Milt
Lipid (%)		5.7	3.3
Lipid classes	Triacylglycerols	6.2	9.4
(percent of total lipids)	Phospholipids	84.5	70.7
	Free fatty acids	1.3	4.6
	Sterol esters	0.4	0.0
	Cholesterol	7.5	15.3
Fatty acids	EPA	12.0	12.2
(percent of total fatty acids)	DHA	31.7	26.2
	SFA	25.6	24.4
	PUFA	26.0	31.8
	MUFA	48.4	43.8
	n-3	46.2	41.0

SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids.

3.2. Variations in lipid classes and fatty acid composition

The content of triacylglycerols and phospholipids were clearly different among liver, viscera and trimmings (Fig. 1 and Table 3) in all species evaluated. Fractions rich in triacylglycerols (liver) contain low levels of phospholipids. There is a positive correlation between amount of free fatty acids and cholesterol (both compounds are higher in viscera) and between phospholipids and steryl esters (both compounds are higher in trimmings). Inter-species and seasonal differences in lipid classes are found (Table 3). The lipids in milt and roe (Table 5) consist of more than 70% phospholipids, which is higher than what is found in viscera.

The fatty acid composition was also clearly different among viscera, liver and trimmings (Fig. 2) independent of species studied. The PCA plot (Fig. 1) shows that the monounsaturated fatty acids (MUFA) (18:1, 20:1, 22:1 and 16:1) are the main contributors to the differences among fractions, and high levels

of these fatty acids are found in the liver lipids. DHA is located close to the origo of the plot and therefore does not contribute to the differences among fractions in general. The most dominating fatty acids in liver and viscera are 18:1, 20:1, 22:1 and 20:5 (Table 4A and B). In trimmings, the same fatty acids are dominating, but generally lower concentrations of 20:1 and 22:1 are found (Table 4C). With the exception of 18:0, 20:0, 22:0 and EPA, the levels of all identified fatty acids are significantly different between the fractions. Generally, higher levels of saturated and monounsaturated fatty acids and lower levels of highly unsaturated n-3 fatty acids were found in liver lipids compared to the lipids in viscera and trimmings. Minor seasonal differences are found among the classes of different fatty acids in liver and trimmings, while the fatty acids in viscera collected in winter contained lower levels of PUFA and n-6 fatty acids and higher levels of MUFA. Inter-species differences were found in liver and viscera (lower levels of PUFA in saithe) while the fatty acids in trimmings were more similar between species. The EPA and DHA, which have beneficial health effects, vary between the fractions and species. The levels of EPA and DHA were higher in the lipids from trimmings compared to the other two fractions (except from tusk). These findings are in agreement with what is usually reported for fatty acids in fractions rich in phospholipids (like the muscle tissue) compared to the neutral lipid fractions [7,25]. Among the fractions analysed, the trimmings are the fraction with the lowest lipid content and the highest content of polar lipids. Additionally, higher levels of EPA and DHA were found in liver and trimmings compared to viscera. In all three fractions of tusk, the EPA is found to be lower than in the other species evaluated, which is in agreement with the lower levels of PUFAs found in the gadiform species at increasing depth [26]. The fatty acid composition in the trimmings is consistent with previous findings [7], however the present study shows higher levels of all the MUFAs. The PUFA levels ranging from 27 to 41% in the liver lipids found in present study are comparable to previous studies performed on cod liver [27]. Although differences are found among gadiform species, fishing grounds and seasons in these samples, the levels of EPA, DHA and total PUFA in the lipids are nutritionally regarded as high.

The differences found in liver size, total lipid content of liver and the lipid composition of the by-products among species and seasons might be due to differences in feeding behaviour and the composition of prey [14] since the feed is highly reflected in liver and muscle lipids [28]. Consistent with the present study, a seasonal difference in 22:1 in cod liver has been reported [7]. The highest fat content in the present study was found in the liver in winter collection, while the 22:1 were significantly higher in the summer.

3.3. Mass distribution of lipids

A calculation of the mass distribution of lipids based on available amounts and composition of by-products from cod, saithe, haddock and tusk is given in Table 2. By-products from an average daily catch of cod species can provide approximately 1000 kg of marine lipids containing 300 (±20) kg n-3 fatty acids depending on the variables discussed in this paper. A major part of the available lipids in gonads, viscera and trimmings are phospholipids (Fig. 1, Tables 3 and 4) and are therefore not easily extracted by traditional thermal treatment followed by a centrifugal separation [29] or enzymatic hydrolysis by commercial proteases [16]. The oil fraction generated from these processes will consist mainly of neutral lipids (e.g. triacylglycerols) [29], which are found in high amounts in liver (>95% of lipids, Table 3) but also in minor amounts in viscera. Using optimal processing technology, large parts of the neutral lipids might be separated and utilized, however, since there are generally no solvents used in such processes, the polar lipids (phospholipids) will follow the protein containing fractions, particularly the sludge (the water insoluble phase after centrifugation), when using enzymatic hydrolysis of cod by-products [16] or thermal treatment. When sorting out liver as a separate high value commercial food product the residual amount available for bulk processing have relatively low lipid content consisting primarily of phospholipids (Table 3). Processing and handling of these fractions are dependent of the end use and are discussed in Falch et al. [17].

3.4. Lipolysis

Free fatty acids are generally undesirable in food products and should be kept at low levels. The relatively high levels of free fatty acids found in trimmings and viscera of these samples are due to lipase activity [30] and might generally be reduced by optimal handling procedures on-board the fishing vessel [17]. According to Young [31], the free fatty acids in the viscera and trimmings samples analysed are above the limits in guidelines specifications for crude fish oils, which is 2–5%. Only the liver lipids are within or below these levels of free fatty acids. Using trimmings and viscera into the process line for generating marine lipids might require alkali refining to decrease the levels of fatty acids to levels acceptable for feed and human consumption [32].

3.5. Applications

These marine raw materials are resources that might be utilized for applications in food, health–care products, and pharmaceuticals or as specialty feed for fish and animals. The highly unsaturated n-3 fatty acids are found in relatively high concentrations in all samples analysed in this study. These lipids will therefore be valuable ingredients for functional foods and as diet supplements. For pharmaceutical applications, the European Pharmacopoeia [33] specifies different products as: cod liver oil, fish oils rich in omega-3 acids, omega-3 ethyl esters and omega-3 acid triacylglycerols. The cod liver oil products should originate from fresh livers of gadiform species and both composition of fatty acids and quality parameters are specified in the monograph. Comparing the fatty acid profile specified for cod liver oil [33] with the profile found in liver of cod, saithe, haddock and tusk showed that the levels of fatty acids are within the specifications except for higher values for 22:1 and lower values for EPA found in tusk. The fatty acid profile in the

viscera have higher levels of 18:0 and DHA and lower levels of 22:1 in some species compared to the specifications for cod liver oil, while the same fatty acids and in addition 18:0 and 16:1 are different in the trimmings.

The phospholipids remaining in the sludge [16] might be further extracted to generate products for dermatological purposes or used as speciality feeds.

4. Conclusions

On an average, production of fillets of gadiform species will generate 2/3 of the round weight as by-products. An average production of 10,000 kg fillets will generate by-products with more than 1000 kg marine lipids. More than 30% of these lipids are the health beneficial n-3 fatty acids with commercial applications. Amount of liver, viscera and trimmings and their lipid composition varied significantly among species. The levels of polyunsaturated fatty acids were significantly higher in haddock liver and significantly lower in tusk liver compared to saithe and cod. However, regardless of the variation found, the lipids from all samples analysed contain significant quantities of health beneficial fatty acids. Liver was the best lipid source containing between 43 and 69%. The viscera contained between 2 and 9% lipids and the trimmings contained approximately 1% total lipids. The phospholipids that are the major lipid fractions in the gonads and trimmings (>60% of total lipids) requires chemically extractions to be isolated. An optimization of the bulk processing that separate proteins and lipids might concentrate the phospholipids in the sludge (heavy layer). From this source a further extraction of phospholipids might be performed. Alternatively, the phospholipid rich fraction can be utilized for other applications in food or feed.

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Paper II

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Paper VI

HR NMR TO STUDY QUALITY CHANGES IN MARINE BY-PRODUCTS

E. Falch[1,2], T. R. Størseth[1] and M. Aursand[1]

[1] SINTEF Fisheries and Aquaculture, Trondheim, Norway
[2] The Norwegian University of Science and Technology, Department of Biotechnology, Trondheim, Norway

1 INTRODUCTION

Marine raw materials contain health beneficial lipids[1,2] with applications in food, healthcare and pharmaceutical products. However, marine lipids are highly susceptible to lipid oxidation and to chemical reactions caused by endogenous enzymes. The lipid composition in fish tissues is a complex mixture of fatty acids esterified in neutral and polar lipids, but also sterols, vitamins and other lipid components are found. Lipids in fish muscle are relatively well characterised. However, more effort is needed to characterise the chemical composition of potential by-products such as head, trimmings and visceral fractions to make utilisation of the total catch of fish feasible[3]. Recently, a number of studies on lipid composition in cod by-products are reported [4,5,6,7,8,9].

Several analytical methods to determine lipid composition are available. Traditional methods are generally based on lipid extraction which may be prone to incomplete extraction of the desired components. High resolution (HR) NMR may provide chemical information about all NMR visible lipid classes and thereby changes in lipids and their reaction products. HR- NMR has been used to characterise a wide range of chemical compounds found in marine lipids[10]. The omega-3 content in muscle lipids extracted from Atlantic salmon[11] and in different fish oils[12] as well as the content of docosahexaenoic acid in different fish oils have been quantified using ^{1}H NMR. Moreover, ^{1}H-NMR has also been successfully used to study lipid oxidation in ethyl docosahexaenoate[13]. The ^{13}C spectra may provide detailed information about the fatty acid composition, positional distribution of fatty acids in acylglycerolmolecules[14,15,16] and phospholipids[15]. The phospholipids can be identified by ^{1}H and ^{13}C NMR due to their differences in the structure of the polar head groups, whereas ^{31}P NMR has been more widely used to differentiate individual phospholipids including lysophospholipids[17].

Magic angle spinning (MAS) NMR was first applied on lipids in 1972[18]. Since then, the method has been gradually improved. MAS experiments can be performed on intact tissues and spinning the samples at the angle of 54.7°. Thus, partial averaging of chemical shift anisotropies and dipolar couplings thereby significantly reduce the line broadening effect[19]. The method has been used successfully to study different biological tissues and intact rat liver. The obtained spectra were well resolved providing detailed information about a wide range of chemical compounds in these materials[20].

2 MATERIALS AND METHODS

2.1 Raw material

Among the by-products from cod (*Gadus morhua*), roe, milt and liver are presently commercial food products. Roe and milt are generally rich in phospholipids. In our study, the lipids in milt and roe contained 60-77% phospholipids, 17-18% cholesterol, and 0-9% triacylglycerols. Examples of endogenous seafood enzymes catalysing hydrolysis of phospholipids are shown in Fig. 1 whereas the formation of lysophosphatidylcholine and free fatty acids from phosphatidylcholine is illustrated in Fig. 2.

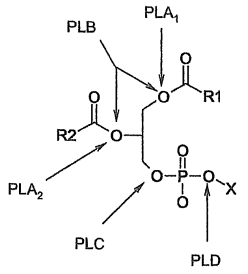

Figure 1 *Various enzymes catalysing phospholipid hydrolysis. PLA_1, PLA_2 and PLB are phospholipases belonging to the group acyl hydrolases together with lysophospholipases while PLC and PLD are phosphodiesterases. R_1 and R_2 denotes fatty acids and X represents the polar head group*[21]

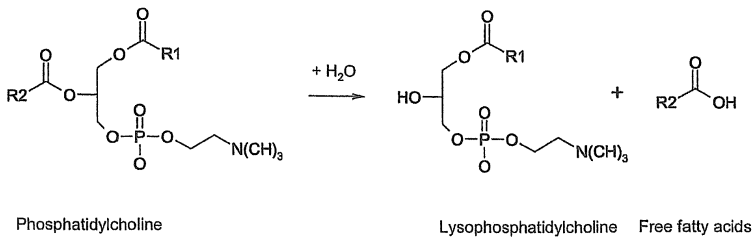

Figure 2 *Hydrolysis of phosphatidylcholine*[22].

The lipids in cod liver consist primarily of triacylglycerols (>95% in our study)[4] with a level of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) at respectively 7% and 11% respectively. Comparatively, lipids in roe and milt contain 15% EPA and 25% DHA.

The by-products were collected from a batch of fresh cod and immediately frozen and stored at -80°C until NMR analysis. Immediately after gutting a storage experiment was started with fresh milt packed in dark plastic bags (10g) and stored at 4°C up to one week. Samples were withdrawn at time specific intervals. Lipids from samples at time 0 and after one week (extracted by the method of Bligh and Dyer[23]) were analysed by thin layer chromatography[24]. The lipid extracts (100 mg) were also transferred into 5 mm NMR tubes and dissolved in 50 ml deuterated chloroform ($CDCl_3$) for ^1H, ^{13}C and 2D (^1H,^1H COSY) NMR analysis.

Additionally, samples at time 0 and after 1 week storage were analysed by HR-MAS at 4°C. The samples were weighed in the rotor (50μl) and added deuterium oxide (D_2O) with trimethylsilylpropionic acid (TSP) as a reference.

2.2 NMR procedures

The NMR experiments were carried out on a Bruker DRX 600 spectrometer (Bruker Biospin GmBH, Germany) resonating at 600 MHz (proton frequency).

For liquid state experiments, a 5 mm BBO probe was used at 298 K. For HR-MAS experiments, a 4 mm MAS probe was used with a MAS spin rate of 5 kHz. All ^1H experiments were recorded using a 90° pulse, 256 scans with 64 K data points were recorded over a spectral width of 7 kHz.

In the HR-MAS spectra the water resonance was suppressed by a pulse sequence using presaturation in the interscan delay. Zero filling and 0.3 Hz exponential line broadening was applied before Fourier transformations. ^{13}C spectra were recorded using a 90° pulse. 8 k scans were accumulated with 64 k data points with a spectral width of 30303 Hz and 3Hz exponential line broadening was applied before Fourier transformation. The ^1H,^1H-COSY NMR measurements were carried out using a 45° polarization transfer pulse to record 2048/1024 data points for the F2/F1 directions. Before Fourier transformation both dimensions were zero-filled and apodized by a squared sine bell function. The HR-MAS ^1H,^1H-COSY spectra were recorded using presaturation of the water resonance in the interscan delay. Chemical shifts were referenced to TSP in the HR-MAS experiments where the samples were added D_2O as a field frequency lock. A series of 1D (^1H and ^{13}C) and 2D (^1H-^1H COSY) spectra were acquired at 4°C by HR-MAS over a 3 day period.

3 RESULTS AND DISCUSSION

The levels of phospholipids were halved during one week of storage at 4°C. The levels of cholesterol were initially high but decreased during storage. Simultaneously, the levels of steryl esters and free fatty acids increased from 0 up to significant levels during storage (Fig. 3).

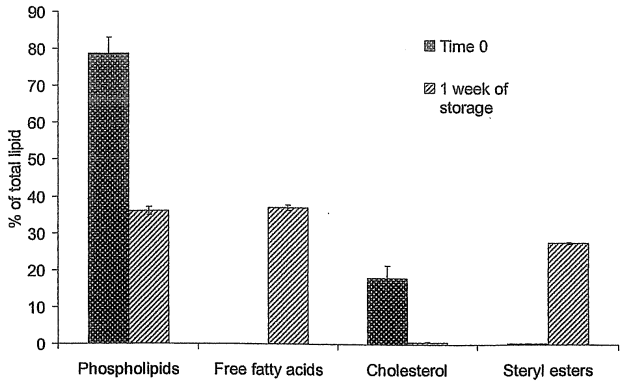

Figure 3 *Lipid classes in milt samples (% of total lipid content).*

^{13}C NMR spectra of lipid extracts provided particularly valuable information about the changes occurring. By studying the whole ^{13}C NMR spectra of milt extracts at time 0 and after 1 week storage, the differences in the amount of cholesterol were clearly recognizable (Fig. 4).

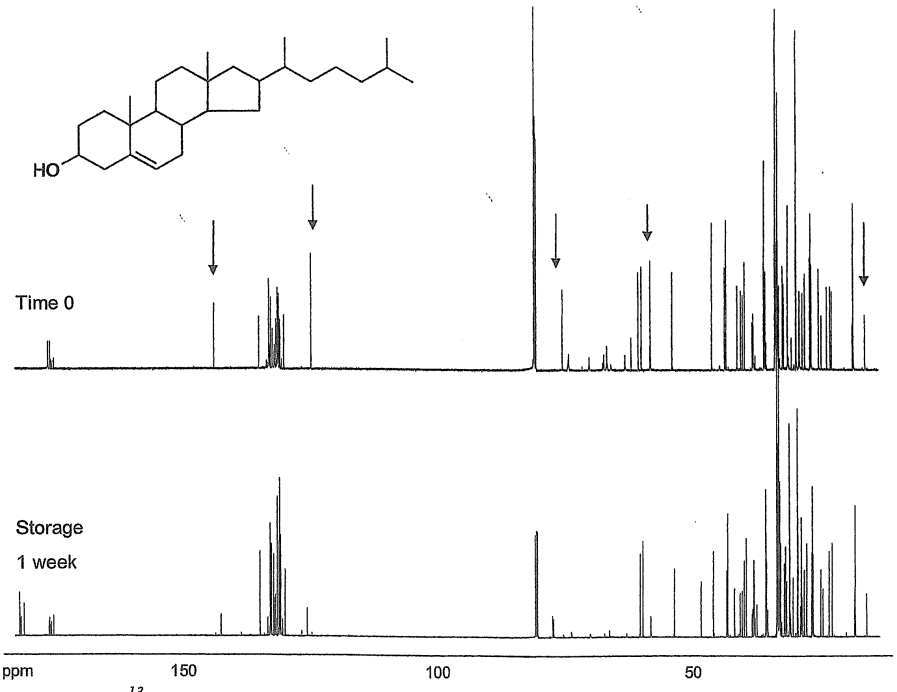

Figure 4 *^{13}C NMR spectra of milt lipids extracts of milt at time 0 and after 1 week storage at 4°C. The structure of cholesterol which is a major constituent in this material and some peaks from the molecule are illustrated.*

The signals from cholesterol carbons were detected as narrow singlets. It was obvious that the hydroxyl group of cholesterol had changed since the signal of the C3 carbon (71.81 ppm) decreased. Also, the disappearance of double bound signals from cholesterol (121.7 and 140.8 ppm (singlets)) were found while new peaks appeared in the olefinic region. Changes due to esterification of cholesterol were observed in the ^{1}H spectra around 1.0 ppm (Fig.5) and at 3.8 and 4.5 ppm (Fig. 8).

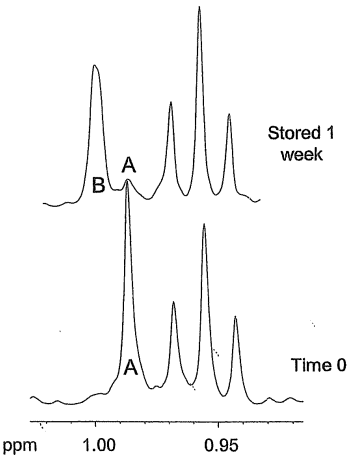

Figure 5 *^{1}H NMR spectra of milt lipids extracted at time 0 and after 1 week storage at 4°C. The ratio between peak A and B as affected by storage and are similar to reported cholesterol and cholesteryl esters chemical shift values[25].*

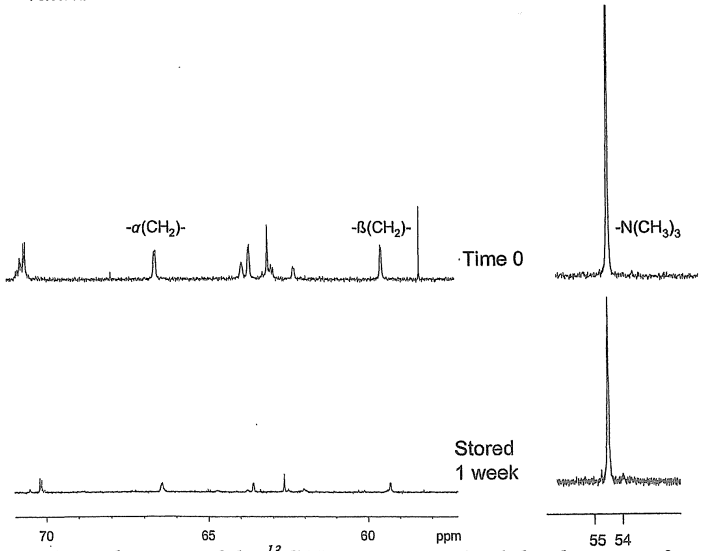

Figure 6 *Glycerol region of the ^{13}C NMR spectra of milt lipids at time 0 and after 1 week storage at 4°C. The phospholipids region is shown. The assigned peaks originate from carbons in the phosphogroup of the phospholipids.*

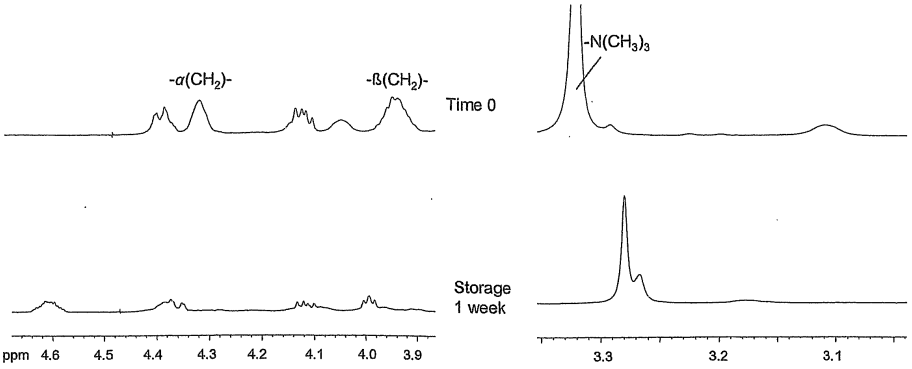

Figure 7 *^{1}H NMR spectra of milt lipids extracted at time 0 and after 1 week storage at 4°C. The signals originate from hydrogens in the phosphogroups of phospholipids.*

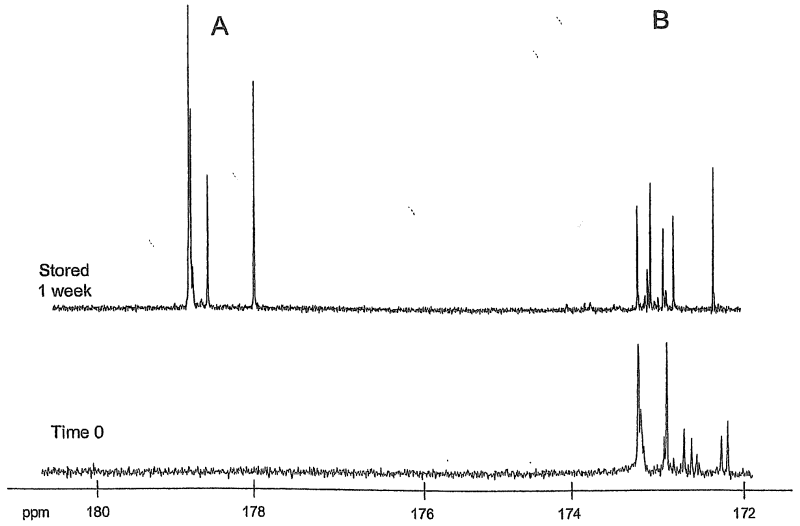

Figure 8 *^{13}C NMR carbonyl spectra of milt lipid extracted at time 0 and after 1 week storage at 4°C. (A)Free fatty acid region, and (B) region containing information about positional distribution of unsaturated fatty acids in the glycerol molecule.*

The free fatty acids in the lipid extracts of milt were significant higher in the samples stored for one week compared to the samples at time 0 which were at non detectable levels. The spectra were also well resolved concerning the carbonyl region. This differentiated between the fatty acids released based on their levels of unsaturation. ^{13}C NMR spectra also provided information about the glycerol position (in the tri, di monoacyl phospholipids or in the lysophospholipid) from which the fatty acids were released. Our

spectra showed that in the milt samples, the EPA and DHA are mainly located at glycerol-carbon atom 2 (sn-2) position and that a major fraction of these fatty acids were hydrolysed during storage. By studying the glycerol region (Fig. 6), a decrease of signals from phospholipid carbons was found without a corresponding increase due to lysophospholipids signals at similar levels. This may indicate that also other enzymes than phospholipases were active hydrolysing the polar head groups of phospholipids. The ^{13}C NMR spectra provided information about the specific fatty acids, position of fatty acids in the triacylglycerol and phospholipids molecules. Fig. 8 illustrates the formation of free fatty acids during 1 week storage. The ^{1}H NMR spectra provided information about changes caused by hydrolysis of phospholipids (3.2 – 4.6 ppm) (Fig. 7).

HR-MAS analysis provided well resolved ^{1}H NMR spectra (Fig. 9 presents results from liver). In milt, time dependent differences in region at 3.0-4.5 ppm were found after relative short time. Possible influence of high spinning rate, pH variations and peak assignments when using MAS should be investigated further before drawing any further conclusions.

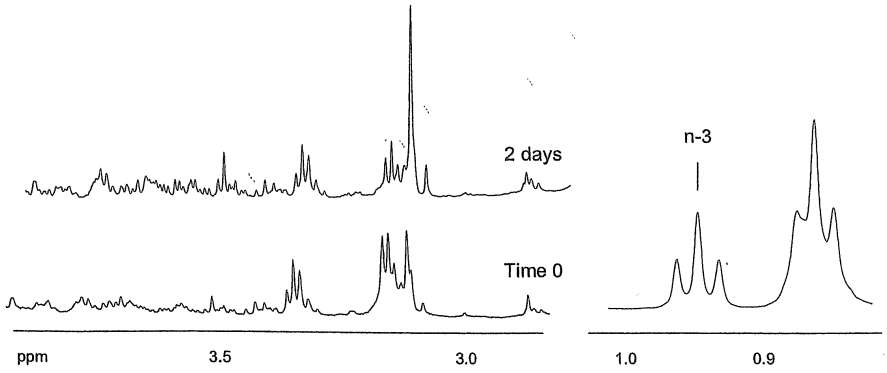

Figure 9 *HR-MAS ^{1}H NMR spectra of cod liver stored at 4°C.*

The marine lipids in by-products were labile and different biochemical processes occur simultaneously during chilled storage. It has previously been reported that due to difficulties in isolation and characterisation of lipases in fish tissues, limited information about the enzymology of digestive lipases are available[26]. NMR can be a valuable tool for such studies. The level of free fatty acids – due to lipid hydrolysis - is widely used industrially as an indicator of lipid quality. However, our results show this might not be the whole answer. Clearly, there was an increase of the levels of free fatty acids in milt during one week of storage. The strong indication of esterification of free fatty acids to the cholesterol molecule might be a hidden effect since the degree of lipolysis was higher than expected. NMR could also provide detailed molecular information about new compounds being formed. These compounds may play a significant role regarding potential health effects and sensory properties of food.

Reaction products from the lipid oxidation and lipolysis (free fatty acids) are known to affect the sensory acceptance of the products. Possible health effects due to the reaction products being formed should also be given attention. Some of oxidation compounds are toxic at high concentrations and studies have shown that hydroperoxides and aldehydes might cause damage of DNA[27].

4 CONCLUSIONS

Results from the early stages of this work have shown that a combination of various NMR techniques may provide a more detailed understanding of the chemical and biochemical reactions occurring in the lipids of marine by-products. Detailed information about lipolysis and other changes in lipid classes were found. However, the used methods need further optimisation including more extensive assignments of unknown peaks.

Acknowledgements

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Paper VII

High resolution NMR spectroscopy for assessment of lipid classes and acyl stereospecific positions of fatty acids in marine phospholipids.

E. Falch[1,2] T. R. Størseth[1], and M. Aursand[1]

[1] SINTEF Fisheries and Aquaculture Ltd., Trondheim, Norway
[2] The Norwegian University of Science and Technology, Department of Biotechnology, Trondheim, Norway

Corresponding author:

Eva Falch

SINTEF Fisheries and Aquaculture Ltd., 7465 Trondheim, Norway

Tel: + 4740 0053 50, Fax: + 47 932 70 701

Eva.Falch@sintef.no

ABSTRACT

High resolution NMR has been applied for assessment of lipid classes and acyl stereospecific positions of fatty acids in marine phospholipids and triacylglycerols. 1D and 2D NMR techniques in combination with recording of a number of reference standards have been used to interpret the ^{1}H and ^{13}C NMR spectra of fish gonads. ^{13}C NMR spectra gave information regarding the polyunsaturated fatty acids (PUFAs) in phosphatidylcholine (PC) and phoshatodyletanolamine (PE). The carbonyl resonances showed that n-3 PUFAs primarily were esterified in the *sn-2* position of PC and PE. The glycerol resonances showed that the PC/PE ratio was higher in roe than in milt and that roe comprised more triacylglycerols that milt. Thin layer chromatography showed that milt contained 2.4 times more cholesterol than roe, which was also found by integrating the ^{1}H spectra. Concentration (mole%) of n-3 fatty acids were calculated from the ^{1}H NMR data and showed 44.8 and 36.3% in roe and milt respectively.

Key Words: Phospholipids, acyl stereospecific position, NMR, fish oil, n-3, PUFA, DHA, EPA, cholesterol

1 INTRODUCTION

Phospholipids in fish constitute an important source of health beneficial n-3 polyunsaturated fatty acids (PUFA) (Takahashi, 2004, Falch et al., 2005a). Higher levels of eicosapentaenoic acid (EPA, 20:5) and docosahexaenoic acid (DHA, 22:6) are concentrated in the phospholipids (PL) compared to triacylglycerols (TAG). In fish, these fatty acids are mainly located in the *sn*-2 position of the TAG, while saturated- and monounsaturated fatty acids are located primarily in the *sn*-1 and *sn*-3 position (Ackman, 1980). Aursand et al. (1995), who used ^{13}C NMR to study positional distribution of TAG in lipid of cod and salmon, found the majority of DHA in the *sn*-2 position while EPA was more randomly distributed on all three positions. Traditional analytical methods for obtaining the positional distribution of fatty acids in TAG and PL are comprised of different destructive chromatographic techniques that are, in some cases, combined with a pre-treatment step consisting of hydrolysis by specific lipases (López-López et al., 2001). ^{13}C NMR offers the advantages of being non-destructive to extracted samples and the positional distribution of fatty acids in TAG may be detected directly (Aursand et al., 1995; Aursand et al., 1993; Gunstone and Seth, 1994; Medina et al., 1994). The polar head groups of different marine PLs have been previously assessed by ^{13}C NMR (Gunstone, 1994; Jimeno et al., 2002) and assignment of resonances in model systems of phosphatidylcholine (PC) containing DHA were previously performed (Everts and Davies, 2000). However, ^{31}P NMR has been more widely used to differentiate PLs into phosphatidylcholine, phosphatidyletanolamine (PE), phosphatidylinisitol (PI) and also different lysophospholipids (LPL) (Haraldsson et al., 1999, Schiller and Arnold, 2002). Previous ^{31}P NMR studies have succeeded in quantifying PL (Helmerich and Koehler, 2003). By studying ^{31}P, information on positional distribution of fatty acids is not obtained. ^{13}C NMR has been used in previous studies to detect compounds other than fatty acids in lipid extracts, such as alcohols, wax esters (Gunstone 1993), cholesterol and other lipid classes (Falch et al., 2005b).

Roe and milt are suitable model systems for studying marine PLs, as the PLs comprise more than 70% of the total lipids (Tocher and Sargent, 1984; Falch et al., 2005a). Today, cod roe is used commercially in food products such as caviars or as other spreads (Bledsoe et al., 2003). The fish gonads have been used as a basis for production of pure marine lipids for different applications, mainly in pharmaceuticals, cosmetics, photographic industry or as speciality feed. Furthermore, the interest in using PLs as carriers of n-3

PUFAs in the *sn-2* position has received commercial interest (Guo et al., 2005). PL with DHA in the *sn-2* position have received attention because of their beneficial effects in metabolism (Takahashi, 2004) and studies of infant nutrition have demonstrated a higher absorption of DHA in phospholipids from eggs than DHA in breast milk (Makrides et al., 2002). Previous studies have shown that EPA and DHA in the *sn*-2 positions are more readily absorbed compared to the structured lipids where these fatty acids are in one of the other positions in the TAG (Christensen et al., 1995). The positional distribution of the natural forms of these lipids is therefore of importance and additionally as it relates to end products from production of structured lipids and concentrates of n-3 PUFAs for health improvement. This growing awareness of human health results in a demand for reliable methods for mapping lipid molecular species 'lipidomics' (www.lipidmaps.org), preferably in a non-destructive way.

The present work has applied $CDCl_3$ as solvent aiming to assess lipid species, such as PL and TAG, in the same spectra without any pre-treatment of the pure lipids. We have used ^{13}C and ^{1}H NMR to assess the main lipid components in samples rich in marine PLs. A number of reference standards of lipid classes (PLs, TAG and cholesterol) were analysed and roe and milt from cod (*Gadus morhua*) were used as model systems of samples rich in marine PLs. The chemical shift values in $CDCl_3$ were identified, describing the positional distribution of EPA and DHA in the individual PL. Other lipid constituents such as PLs and cholesterols were also identified and assigned.

2 EXPERIMENTAL

2.1 Sample preparation

Roe and milt were withdrawn from spawning cod (*Gadus morhua*), less than 2 hours (<4^{o}C) after catch, and frozen at -80^{o}C until analysis. Lipids were extracted by the method of Bligh and Dyer (1959). The outer membranes of the roes were not used in order to get homogeneous samples. The lipid extract was introduced (50 mg in 0.6 ml $CDCl_3$) into 5 mm NMR sample tubes (Wilmad lab-glass, Buena, USA). Ethylene glycol dimethyl ether (EGDM, 99.5%) purchased from Fluka (Buchs, Switzerland) was used as an internal standard (Igarashi, et al., 2000).

Stearoyl-2-docosahexaenoyl-sn-glycero-3-phosphoetanolamine (SDPE), 1–stearoyl-2-docosahexaenoyl-sn-glycero-3-phosphocholine (SDPC), 1 –stearoyl-2-aracinodyl-sn-

glycero-3-phosphoetanolamine (SAPC), 1,2-diarachidonyl-sn-glycero-3-phosphocholine (AAPC), 1,2-diarachidonyl-sn-glycero-3-phosphoethanolamine (AAPE), 1-stearoyl-2-arachidonyl-sn-glycero-3-phosphocholine (SAPC), 1,2-didocosahexaenoyl-sn-glycero-3-phosphocholine (DDPC), L-α-Lysophosphatidylcholine (Egg-chicken) (LPC), and a synthetic PL blend with dioleoyl (DO) (DOPE:DOPS:DOPC (5:3:2)) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). Cholesterol, reference solution of lipid classes (triacylglycerols, free fatty acids, cholesterol, phospholipids and cholesteryl esters) and tridocosahexaenoate (DDD) were purchased from Nu-Check-Prep. Inc. (Elysian, NM, USA).

2.2 NMR analysis

The NMR experiments were carried out on a Bruker DRX 600 spectrometer (Bruker Biospin GmBH, Germany), resonating at 600.13 MHz for ^{1}H and 150.90 for ^{13}C, using a 5 mm BBO probe at 297 K. For each sample ^{1}H, ^{13}C, and ^{13}C-^{1}H heteronuclear correlation (HETCOR) spectra were obtained with standard pulse programs from the Bruker pulse-library. ^{1}H experiments were recorded with a center frequency of 4.5 ppm; spectral width 11.97 ppm; acquisition time 4.56 sec.; 64 K time domain data points; 90^{o} pulse angle relaxation delay 3 sec. 256 free induction decays (FIDs) were collected for each sample. Zero-filling and an exponential window function of 0.3 Hz was applied before Fourier transform. ^{13}C spectra were recorded with a center frequency of 100 ppm, spectral width 200.79 ppm, acquisition time 1.08 sec., 128 K time domain data points, relaxation delay 3 sec. Proton decoupling was used. 64 K FIDs were collected for each sample. Zero filling and 0.3 Hz exponential line broadening were applied before Fourier transform. For HETCOR experiments, 4096 (t_2) x 512 (t_1), complex data points were acquired, and 36 scans were averaged for each FID with a relaxation delay of 3 sec. The FIDs were zero filled to 1024 for t_1 and were apodized by exponential (F2) and squared sine bell (F1) functions.

NMR (^{13}C and ^{1}H) spectra of a series of reference standards of lipid components, that are generally constituents of fish, were obtained using $CDCl_3$. In addition to analysis of the individual reference standards following combinations of standard were analysed; SDPE/SDPC, SDPE/SDPC/DDD to enable the peak assignment. Roe was spiked by

adding the individual PLs to observe what resonances increased in the complex lipid and also to enable the peak assignments.

2.3 Lipid classes by TLC

Composition of lipid classes was determined by thin-layer chromatography using an Iatroscan TLC Flame ionisation detector (TH-10 MK-IV, Iatron Laboratories, Tokyo, Japan) (Rainuzzo et al., 1992). Chromarods SIII were scanned twice through the Iatroscan FID after separation with neutral and polar solvents. The neutral solvent system (hexan: dietylether:formic acid / 85:15:0.04) separated the groups of lipid classes (sterylesters, acylglycerols (mono-, di- and tri-), free fatty acids, cholesterol and phospholipids. The polar solvent system (chloroform:methanol:distilled water / 70:35:3.5) separated neutral lipids from the specific phospholipids. Lipid classes were identified by the comparison of their retention times with those of a reference solution run at identical conditions. Analyses were performed in duplicates or triplicates dependent on the standard deviations.

2.4 Fatty acid composition by GC

Fatty acid composition was analysed by gas-liquid chromatography after derivatisation to fatty acid methyl esters according to Metcalfe et al. (1966). Fatty acid methyl esters were analysed on a Fison 8160 capillary chromatograph (Fisons Instruments S.pA., Milano Italy) equipped with an on-column injector, a fused silica capillary column (Omegawax 320 capillary column, 30m x 0.32mm I:D: and 0.25 μm film thickness)(Supelco Inc., Bellefonte, PA) and a flame ionisation detector. The oven temperature was increased for 80^{o}C to 180^{o}C at 25^{o}C min^{-1} and held for 2 min, followed by an increase of 2.5^{o}C min^{-1} to 205^{o}C (held for 8 min). Hydrogen was used as a carrier gas using a flow rate of 1.6 mL min^{-1}. Fatty acid methyl esters were identified by the comparison of their retention times for those of a reference solution run at identical GC conditions. GC analysis was performed in duplicates and results were expressed as percent of total fatty acids, as a mean value.

2.5 Processing of data

NMR spectra were recorded using an internal standard enabling comparison of relative intensities between spectra. Processing of NMR spectra was done using Topspin v 1.3 software (Bruker Biospin GmBH, Germany). NUTS - NMR Utility Transform Software

(Acorn NMR Inc., Livermore, USA) was used for line fitting to enable quantification of n-3 fatty acids (Igaraschi et al, 2000). For the chemical data (fatty acids and lipid classes) average values ± standard deviations (Microsoft Exel) are given.

3 RESULTS AND DISCUSSION

3.1 Lipid class compositions

The lipid class composition is given in **Table 1**. Both roe and milt contained high levels of phospholipids making up 79 and 74 % of the total lipids respectively. These findings are in agreement with other studies on cod gonads (Kaitaranta and Ackman, 1981; Falch et al., 2005a). The higher levels of neutral lipids found in milt consisted primarily of cholesterol and monoacylglycerols, while the neutral lipids in roe consisted of triacylglycerols, cholesterol and monoacylglycerols.

3.2 Fatty acid composition by GC

The fatty acid composition (**Table 2**) in roe and milt showed that more than 40% of the fatty acids were n-3 fatty acids. The polyunsaturated fatty acids made up nearly half of the amount of fatty acids in both samples. Some differences were found between the two samples showing higher levels of saturated and lower levels of monounsaturated fatty acids in roe compared to milt. The fatty acid composition found in the present study is comparable to what has recently been found in gonads of cod species (Falch et al., 2005a).

3.3 ^{13}C NMR

^{13}C NMR spectra of the lipid extracts from cod roe and milt (**Fig. 1A** and **B** respectively) present a wide range of resonances from specific fatty acids, lipid classes and cholesterol. These values were used to interpret the ^{13}C NMR spectra of roe and milt.

3.3.1 Glycerol region

The chemical shift values of the reference standards (**Table 3**) showed that the glycerol region provides useful information about the composition of PL and TAG. The polar head group of PL contains aliphatic carbons bonded to heteroatoms (O, N), which are detected in the 40–67 ppm regions. The signals from the polar head group of PC were assigned to 54.3-54.4 ppm (-N($\underline{C}H_3)_3$), 59.3-59.4 ppm ($\underline{C}H_2N$) and 66.3 ppm ($\underline{C}H_2O$). The

signals from the head group of PE were clearly different from those of PC and were assigned to 64.3-64.4 ppm ($\underline{C}H_2O$) and 40.4-5 ppm ($\underline{C}H_2N$).

Resonances from the glycerol carbons were divided into different regions depending on whether they were originating from the *sn-1* (62.6-63.0 ppm), *sn-2* (70.3-70.6 ppm) or *sn-3* (63.3-63.7 ppm) of PL. The resonances from glycerol carbons of *sn-2* (68.0-68.1 ppm) and *sn-1,3* (62.0-62.1 ppm) of TAG were clearly different from those originating from PL.

The resonances from glycerol carbons of the *sn-3* position of PL easily distinguished PC (63.3-63.5 ppm) from PE (63.7 ppm), while the corresponding carbons from the *sn-1* position were more challenging as objects for separating of individual phospholipid species. The most likely explanation for this is the shorter distance to the polar head group of the glycerol carbons in position *sn-3* and that the fatty acids esterified to position *sn-1* and *sn-2* are of greater importance for the chemical shift values of the glycerol carbons of *sn-1*. The chemical shift value of a carbon atom is influenced by the chemical environment and is reported to be generally affected to a distance of six atomic centres and therefore esterified fatty acids and PL head group will in this system affect the shift values of glycerol carbons (Gunstone, 2004). The chemical shift values of the glycerol carbon resonances are previously reported to differentiate between fatty acids esterified at the TAG (Aursand et al, 1995). The carbonyl region was, however, a better region to study with the purpose of obtaining data on the positional distribution of fatty acids.

The glycerol region of the ^{13}C NMR spectra of roe and milt is illustrated in **Fig. 2** with corresponding chemical shift values shown in **Table 4.** The spectra showed that phospholipids (PL) were the dominating lipid class compared to TAG in both samples. An example of this can be seen by a comparison of intensity of the $\underline{C}H$ signals from the *sn-2* position of PL (multiplet at 70.5 ppm) and TAG (68.7-68.9 ppm) and also from the *sn-1,3* resonances at 62.0-63.6 ppm. Furthermore, roe contained higher levels of TAG (62.0 ppm and 68 ppm) compared to the milt, which was in agreement with the TLC results (**Table 1**). The glycerol region also revealed that the ratio between PC and PE was higher in roe compared to what was observed in milt, which was in agreement with the TLC determination (**Table 1**) that showed a PC: PE ratio of 7:1 in roe and 2:1 in milt. Higher levels of PC compared to the other PL are previously reported in fish roe (Tocher and Sargent, 1984)

It was possible to distinguish PC from PE in roe and milt by studying the glycerol resonances from the *sn-3* position, but it was more challenging to clearly assign these

phospholipids using resonances from the *sn-1* glycerol carbon. These materials contained several fatty acids which naturally affected the chemical shift values and thereby complicated the separation due to differences in the polar head group in these complex lipids.

Spiking the roe with SDPC demonstrated that two of the middle peaks in the cluster of peaks were assigned as DHA in *sn-2*. These signals had lower intensity in milt compared to in roe which indicated lower levels of DHA in *sn-2* position in the milt than in roe. This finding was in agreement with what was found in the carbonyl region of the same spectra (**Fig. 4.**).

An excerpt of the glycerol region of the HETCOR plot of cod roe is shown in **Fig. 6,** and it enables one to differentiate among resonances of the different PLs and between PL and TAG. In addition, the 2D spectra of the reference standards of PL and cod liver (rich in TAG) were important for assignment of resonances from each individual PL

3.3.2 Olefinic region

The olefinic region (140-120 ppm) gave rise to signals from various double bonds present in the fatty acids of the lipid samples (**Fig. 3**). The fatty acids in roe and milt (**Table 2**) contained high levels of n-3 polyunsaturated fatty acids, of which a large fraction comprised DHA and EPA. Among the fatty acids present, DHA was found in highest concentrations making up 24 and 30% of the total fatty acids in milt and roe respectively. EPA made up approximately 15% of the total fatty acids in these materials. The high concentration of n-3 fatty acids in these lipids (41-47% of total fatty acids) was reflected in the high intensity signals at 126.9 and 131.9 ppm that were assigned to the two olefinic n-3 carbons, assigned by NMR spectra of reference standards of the individual PL and by comparison of chemical shift values with those reported in the literature (Aursand et al., 1993; Siddiqui et al., 2003). Due to the high concentration of DHA, which contains 6 double bounds, the majority of the high intensity olefinic resonances that were present in this region (126.9-128.6 and 129.2 ppm) originated from this fatty acid. Resonances from other unsaturated fatty acids were also found in this region, and two resonances at 128.8 ppm were assigned to carbons from 20:5n-3 (C5, C6) (Aursand et al., 1993). The monounsaturated fatty acids, which made up relatively high concentrations in roe and milt, (27 and 47% of total fatty acids) were assigned in the region downfield of 129.5 ppm (129.6 – 129.9 ppm). The higher levels polyunsaturated fatty acids (PUFA) and lower

levels of monounsaturated fatty acids (MUFA) in roe compared to in milt (Table 2) were verified in the olefinic regions of the spectra. The intensity of the resonances at 129.6 ppm (C8, 18:1) and 129.9 ppm (C10, 18:1) in milt were markedly higher than in roe which were in agreement with the TLC results in Table 2.

3.3.3 Carbonyl region

The carbonyl region of the reference samples (Table 3) points out the uniqueness of ^{13}C NMR when studying acyl stereospecific positions of fatty acids in TAG and PL. Spectra obtained from a reference standard containing DHA in *sn-2* and *sn-1* (DDPC) contained carbonyl resonances at 172.46 (*sn-2*) and 172.80 (*sn-1*) ppm, a difference of 0.34 ppm. A reference standard containing arachidonyl in *sn-2* and *sn-1* (AAPC) showed carbonyls at 172.90 and 173.25 ppm respectively, a distance of 0.35 ppm. For the reference standard containing DHA in all three positions (*sn1,2,3*) the carbonyls were detected at 172.09 (*sn-2*) and 172.47 (*sn-1,3*) ppm, a difference of 0.38 ppm. This information was applied during peak assignments in the raw material studied (roe and milt).

The carbonyl resonances from the *sn-2* position of PL were located at higher field than the corresponding *sn-1* carbonyl resonances. Furthermore, the resonances appeared in the order: DHA<AA<MUFA<SFA from high to low field in each of the acyl positions. The TAG carbonyl resonances were high field of the *sn-2* of PL and were clearly different from those originating from carbonyls of the *sn-2* PL.

Spectra of the reference standards of PL showed that it was possible to distinguish carbonyl resonances of specific fatty acids based on differences in the polar head group. Using SDPC and SDPE as an example, the resonances from DHA in *sn-2* position PC (172.43 ppm) were high field of the corresponding resonances from PE (172.39 ppm).

The carbonyl region of roe and milt is presented in **Fig. 4** with peak assignments in **Table 5.** The high intensity resonances at 172.3-172.4 ppm demonstrated that DHA was primarily esterified in the *sn-2* position of the PL both in PC (172.4 ppm) and PE (172.3 ppm) in roe. This was also found in milt, but in these spectra resonances were also found for DHA in *sn-1*. Resonances from the *sn-1* position of EPA in PL were expected to resonate around 173.20 ppm (Table 3) where the other FAs in *sn-1,3* position of TAG were located in the roe spectrum. It could therefore theoretically be an overlap of resonances. The up-field region of the spectra (33-34 ppm, **Fig. 4**) revealed that minor signals were located at approx. 33.5 ppm, where EPA in *sn-1* are expected to be found (Aursand et al,

1992). It therefore supported the observation of EPA primarily esterified in the *sn-2* position. TLC showed that 40% of the total fatty acids were n-3 PUFAs, and ^{13}C NMR showed that these were esterified to the *sn-2* position of PLs, most of the other fatty acids were therefore necessarily located in the *sn-1* position. This was observed in the carbonyl region of roe. However, the milt spectra also showed that a considerable amount of fatty acids other than n-3 PUFAs were esterified to *sn-2* position (173.1 ppm) and resonances from DHA in the *sn-1* position of both PE and PC were detected in milt.

The carbonyl carbon from DHA in *sn-2* position of TAG provided detectable resonances in the roe spectrum while no detectable resonances for *sn-2* TAG were found in the milt spectrum. This showed that the levels of TAG were lower in milt than in roe. EPA were mainly esterified to the *sn-1,3* position of the TAG in roe, while non detectable levels of EPA are found in the TAG of milt.

Results, both from analysis of reference standards and the gonad lipids, showed that it was possible to observe differences in the chemical shift values of EPA/20:4 in *sn-2* depending on whether it was part of a PE, PC or TAG. The resonances of the *sn-1* position of PL and TAG were located downfield of the *sn-2* carbonyls. As seen in the glycerol region of roe and milt spectra, the majority of the fatty acids were esterified in phospholipids, and PC and PE were the main phospholipids present. In milt, the resonances from PC and PE were of equivalent intensity, while in roe the signal intensity of PC was significantly higher than the PE. This was in agreement with findings in the glycerol region and by TLC. These assignments were found through evaluation of the ^{13}C NMR spectra recorded from reference standards of PLs, tridocosahexaenoate and by ^{13}C:^{1}H correlation. The assignments were also supported by unpublished data from cod liver oil (TAG). Analysis of a reference standard of lyso PC showed that the carbonyls resonated at 174.01 ppm. However, no detectable signals were found in the spectra of roe or milt.

3.3.4 Up-field aliphatic region

Specific fatty acids such as 22:6n-3, 20:5n-3/20:4n-3, total n-3, and total n-6 were found in the upfield region (50-0 ppm) of the ^{13}C NMR spectra for roe and milt (spectra not shown here). Resonances from mono- and diene saturated fatty acids were also observed. A comparison between the two spectra indicated that the roe contained higher levels of n-3 (-$\underline{C}H_3$ 14.2 ppm) fatty acids and lower levels of n-6 (-$\underline{C}H_2$- 31.5 ppm) fatty acids compared to milt. This was in agreement with the n-3/n-6 ratios and the contribution of n-3 fatty

acids in the samples found by TLC. The spectra also showed higher intensity of resonances assigned as mono- and diene- unsaturated fatty acids (-$\underline{C}H_2$-CH=CH, 27.2 ppm) in milt compared with what was found in roe. This was supported by the GC results in Table 2.

Since semiquantitative ^{13}C NMR was recorded in this study, comparison between intensities of resonances within the same spectra should be performed with care since the T1 relaxation times vary among the different resonances. Comparison of signals among different spectra obtained at similar conditions is however possible.

3.3.5 Cholesterol

^{13}C NMR spectra of the lipid extracts of roe and milt showed distinct signals from the carbons of cholesterol distributed through regions from 12 to 140 ppm (**Fig. 1, Table 4**). The olefinic carbon resonances (C1 and C2) were found at 140.8 and 121.6 ppm and were easily differentiated from olefinic carbons originating from fatty acids. This simplified the assessment of cholesterol in such lipid systems. Milt contained higher levels of cholesterol than roe (21% and 9% of the total lipids respectively), which was clearly demonstrated by the higher intensity of the cholesterol carbon resonances in the spectra (**Fig. 1**).

The cross peak at 77/4.5 ppm was assigned to cholesterol through HETCOR analysis of reference standard. Due to overlap with the solvent resonance ($CHCl_3$), this signal was not detectable in a 1D ^{13}C NMR spectrum.

3.4 ^{1}H NMR

The high-field region of the ^{1}H spectra of roe and milt are presented in **Fig. 7** with assignment of methyl protons and resonances from cholesterol. A comparison of the intensity of CH_3 resonances of n-3 fatty acids (0.95 ppm) and non n-3 fatty acids (0.85 ppm), in roe and milt, showed that the n-3 fatty acids made up a higher proportion of the total fatty acids in roe than in milt. This was in agreement with what was found in the ^{13}C NMR spectra and by TLC (**Table 1**). A calculation of the relative levels of n-3 fatty acids by peak fitting of methyl resonances at 0.95 and 0.85 ppm (Sacchi et al., 1993; Igarashi et al., 2000) demonstrated n-3 levels of 44.8 mole% in roe and 36.3 mole% in milt. Representative values, calculated as relative values from the GC spectra (percentage of total fatty acids in area % in the chromatogram), showed that the n-3 fatty acids comprised 46.9 and 41.1% of the fatty acids in roe and milt. Similar comparison of tuna lipids and

salmon oil has also shown an underestimation of n-3 fatty acids by ^{1}H-NMR (Sacchi et al., 1993; Aursand et al, 2005).

The same region of the ^{1}H NMR spectra (1.0-0.5 ppm) contained the C24, C25 and C26 resonances from cholesterol. A comparison of the ratio between the methyl resonances originating from fatty acids (0.85 and 0.95 ppm) with the methyl signals from cholesterol showed the higher levels of cholesterol found in milt. Calculation of the relative intensities (by peak fitting comparison) of methyl resonances from C25 of cholesterol and methyl resonances of fatty acids showed a ratio of 2.37 times more cholesterol in milt compared to roe. This was an identical ratio to those found by TLC. Cholesterol has previously been quantified by ^{1}H NMR analysis (Pollesello et al., 1992) from the resonance at 0.67 ppm (CH_2 at C26). The methyl cholesterol carbon resonances in the ^{13}C NMR spectra, obtained in the present study were well resolved without overlap from other resonances and could thereby easily be quantified from the lipids of roe and milt. Protons from the head group of PL resonated at 4.0 – 3.35 ppm (2D spectrum in Fig. 6). The trimethyl group of PC ($-N(CH_3)_3$) resonated at 3.4 ppm, while the other protons in the head group resonated at 3.86 and 4.0 ppm. Proton resonances from PE were detected at 3.17 and 3.96 ppm.

3.5 Applicability of these methods

In this study, chloroform was used as the only solvent in order to employ the large knowledge base edified in this research field during assignment of the lipid resonances. Chloroform has frequently been used as solvent of lipids during NMR measurements (Aursand et al., 1993; Gunstone, 1993, Gunstone and Seth, 1994; Sacchi et al., 1993, 1995) and a large number of resonances have previously been assigned for marine lipids. A combination of chloroform and methanol in combination is recommended when studying PLs due to the polarity (Schiller and Arnold, 2002; Foss et al., 2004). This solvent combination has previously been used to study hydrolysed marine phospholipids (Medina et al., 1994). However, when analysing sample materials containing other compounds than PL, it might be difficult to assign peaks since most studies published in the literature are performed with chloroform as the only solvent. In the present work we have assigned the most important resonances originating from PL, and the method is shown to enable a first screening of the composition of relatively unknown lipid samples. Moreover, this work has shown the possibility of NMR for quantitative measurements in well resolved spectra. The

chemical shift values will however vary on the basis of concentration, pH and temperature (Diehl et al., 1995).

To the authors knowledge, high resolution ^{13}C NMR analysis of lipids from fish gonads has not been previously published. In a study by Medina et al. (1994) phospholipids from tuna were analysed by ^{13}C NMR, however, hydrolysis of the polar head group as a pre-treatment step made it difficult to compare chemical shift values of PLs from total lipids obtained in the present study.

The positional distribution of fatty acids both in triacylglycerols and phospholipids presented in the present study is also of interest in nutritional studies and development of functional food and health care products. Although roe and milt contain low amounts of lipids, these lipids comprise high levels of health beneficial fatty acids, with DHA attached to the favourable *sn-2* position. Assessment of changes of these lipids due to deterioration or processing should be analysed with this NMR methodology with the same solvent system and no pre-treatment steps of the sample. Our present work demonstrates the relevance of using NMR methodology in the study of food components affecting human health. Cholesterol has gained increasing focus due to its health consequences. Not only cholesterols are assigned in these spectra but also reaction products from deterioration of cholesterols are previously detected by NMR (Falch et al., 2005b).

In conclusion, ^{13}C-NMR is a non-destructive method to analyse the positional distribution of fatty acids in extracts of fish products with high levels of phospholipids. Also other valuable lipid compounds in the same material are observed such as cholesterol and TAG. After obtaining the NMR spectra, the same sample material is available for further analyses.

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Table legends

Table 1. Lipid class composition in roe and milt based on neutral and polar solvent systems before TLC separation.

Table 2. Fatty acids composition in roe and milt presented as percentage of total fatty acids. SFA: Saturated fatty acids, MUFA: Monounsaturated fatty acids, PUFA: Polyunsaturated fatty acids.

Table 3. Chemical shift values (ppm) of ^{13}C-NMR resonances from a selection of references standards of different phospholipids with different fatty acids attached in *sn-1* and 2 position and a symmetric triacylglycerol (tridocosahexaenoate DDD). SDPE, 1 –stearoyl-2-docosahexaenoyl-*sn*-glycero-3-phosphoetanolamine; SDPC, 1–stearoyl-2-docosahexaenoyl-sn-glycero-3-phosphocholine; SAPC, 1 –stearoyl-2-aracinodyl-sn-glycero-3-phosphoetanolamine; AAPC, 1,2-diarachidonyl-sn-glycero-3-phosphocholine; AAPE, 1,2-diarachidonyl-sn-glycero-3-phosphoethanolamine, SAPC, 1-stearoyl-2-arachidonyl-sn-glycero-3-phosphocholine and DDPC, 1,2-didocosahexaenoyl-sn-glycero-3-phosphocholine.

Table 4 Chemical shift values (^{13}C, ^{1}H and ^{13}C:^{1}H correlated) of cholesterol resonances of lipid extracts of cod roe and milt and reference standard of cholesterol.

Table 5 Chemical shift values (ppm) of ^{13}C-NMR resonances from the glycerol region of lipids extracted from roe and milt.

Table 6 Chemical shift values (ppm) of ^{13}C-NMR resonances from the carbonyl region and parts of the up-field region of lipids extracted from roe and milt demonstrating positional distribution of fatty acids in phospholipids (PL) and triacylglycerols (TAG).

Table 1.

	Roe (% of total lipids)	Milt (% of total lipids)
Steryl ester	0.4 ± 0.1	0.0
Triacylglycerols	8.1 ± 0.4	0.9 ± 0.0
Free fatty acids	0.0	0.0
Diacylglycerols	0.2 ± 0.4	0.0
Cholesterol	8.7 ± 0.3	20.6 ± 1.0
Monoacylglycerols	3.6 ± 1.7	4.6 ±1.1
Phospholipids	79.0 ± 1.0	74.0 ± 1.9
PE [a)]	8.1	35.2
PC [a)]	69.5	43.2
Total lipids (%)	4.6 ± 0.2	2.1 ± 0.1

[a)] Determined by using polar solvents.

Table 2

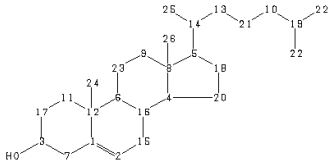
	Roe	Milt
	% of total fatty acid	
12:0	0.0 ± 0.0	0.0 ± 0.0
14:0	2.4 ± 0.2	1.7 ± 0.4
16:0	17.9 ± 2.0	0.1 ± 0.1
16:1n7	3.7 ± 0.1	16.8 ± 0.7
18:0	1.3 ± 0.2	2.0 ± 0.9
18:1n7	13.0 ± 1.0	2.3 ± 0.0
18:1n9	2.7 ± 0.0	18.4 ± 3.1
18:2n6	0.8 ± 0.1	2.7 ± 0.0
18:3n6	0.1 ± 0.0	0.7 ± 0.1
18:3n3	0.5 ± 0.1	0.1 ± 0.0
18:4n3	0.5 ± 0.0	0.3 ± 0.1
20:0	0.0 ± 0.0	0.2 ± 0.1
20:1n9	0.6 ± 0.0	0.0 ± 0.0
20:1n7	4.2 ± 0.5	6.5 ± 3.4
20:2	0.1 ± 0.0	0.1 ± 2.4
20:3n6	0.0 ± 0.0	0.2 ± 0.0
20:4n6	1.4 ± 0.2	0.1 ± 0.0
20:3n3	0.2 ± 0.0	2.0 ± 0.3
20:4n3	0.4 ± 0.0	0.1 ± 0.0
20:5n3	14.6 ± 0.5	13.2 ± 0.8
22:0	0.0 ± 0.0	0.0 ± 0.0
22:1n11	1.8 ± 0.2	2.0 ± 0.1
22:1n9	0.1 ± 0.0	0.2 ± 0.0
22:5n3	1.3 ± 0.1	1.3 ± 0.2
24:0	0.0 ± 0.0	0.0 ± 0.0
22:6n3	29.5 ± 2.0	24.0 ± 3.2
24:1n9	0.9 ± 0.0	0.3 ± 0.3
SFA	21.9	4.2
MUFA	26.8	46.5
PUFA	49.2	44. 8
n-3	46.9	41.1
n-6	2.3	3.6
n-3/n-6	20.1	11.3

Table 3

Assignment	SDPC	SDPE	AAPE	SAPC	DDPC	AAPC	DDD (TAG)
Methyl/methylene region							
CH_3 – all FA	14.07	14.13	14.05	14.05-09	-	14.03	-
CH_3 – all n-3 fa	14.23	14.28	-	-	14.24	-	14.25
CH_2 all n-3	20.49	20.55	-	-	20.51	-	20.52
	-	-	-	-	22.50	-	-
	22.54	22.55	22.55	22.54	22.54	22.52	22.57
	22.63	22.69	-	-		24.69	22.58
	24.82	24.86	24.72	24.72		24.79	-
	24.88	-	24.79	24.81		25.31	-
	25.31	25.35	25.31	25.32	25.49	25.55	-
	25.46	25.52	-	25.57	25.57	25.68	-
	25.55	25.57	25.58	25.59	25.70	-	-
	-	25.60	26.48	26.47	26.06	26.45	25.56
	-	-	26.51	-	26.15	26.47	25.60
	29.11	29.23	27.18	27.18	-	27.16	-
	29.27	-	29.29	29.18	-	-	-
	29.30	-	31.48	29.29	-	29.26	-
	-	29.37	33.47	29.34	-	-	-
	29.48	29.40	-	29.56	-	-	-
	29.61	29.60	-	29.64	-	-	-
	29.66	29.67	-	29.71	-	-	-
	-	29.73	-	-	-	-	-
	-	29.75	-	31.48	-	31.45	-
	31.86	31.92	-	33.47	-	33.47	-
	33.86	33.86	33.66	33.66	33.87	33.67	33.83
	-	-	-	34.08	34.03	-	-
Glycerol region							
CH_2N PE	-	40.44	40.50	-	-	-	-
$N(CH_3)_3$ PC	54.40	-	-	54.30	54.38	54.32	-
CH_2N PC	59.44	-	-	59.31	59.30	59.35	-
α(sn1,3) TAG	-	-	-	-	-	-	62.14
(sn1) PE	-	62.60	62.73	-	-	-	-
(sn1) PC	62.76	-	-	62.93	62.96	62.97	-
(sn3) PC	63.52	-	-	63.34	63.42	63.38	-
(sn3) PE	-	63.70	63.67	-	-	-	-
PE (CH_2O)	-	64.40	64.31	-	-	-	-
PC (CH_2O)	66.25	-	-	6625	66.30	66.25	-
β(sn2) TAG	-	-	-	-	-	-	68.61
β(sn2) PL	-	70.31	70.47	70.50	70.52	70.45	-
β(sn2) PL	70.55	-	-	70.55	70.57	70.49	-
Olefinic region							
	126.95	127.02	127.49	127.47	126.95	127.45	126.96
	127.70	127.74	127.78	127.77	127.73	127.75	127.59
	128.00	128.08	128.04	128.05	127.80	128.02	128.02
	128.23	-	-	-	127.94	-	128.20
	128.24	-	-	-	128.00	128.24	128.28
	128.29	128.28	128.27	128.27	128.24	128.27	129.29
	128.31	128.36	128.30	-	128.31	128.56	128.52
	128.52	128.39	128.59	128.58	128.52	128.76	129.42
	127.80	128.57	128.79	128.81	128.30	128.86	127.82
	127.93	128.86	128.88	128.86	-	128.87	129.93
	127.99	127.99	128.90	-	-	-	128.04

	-	128.06	130.45	130.45	131.97	130.43	-
	129.31	129.40	-	-	-	-	129.44
	131.99	132.15	-	-	-	-	131.97
Carbonyl region							
DHA PL (sn2)	172.43	172.39	-	-	172.46	-	-
DHA PL (sn1)	-	-	-	-	172.80	-	-
AA PL (sn2)	-	-	172.93	172.94	-	172.90	-
AA PL (sn1)	-	-	173.20	-	-	173.25	-
18:0 PL (sn1)	173.52	173.41	-	173.56	-	-	-
DHA TAG (sn2)	-	-	-	-	-	-	172.09
DHA TAG (sn1,3)	-	-	-	-	-	-	172.47

Table 4



Carbon number	Cholesterol Reference standard (ppm)	Roe	Milt	Cross peaks ^{13}C:^{1}H
1	140.80	140.83	140.80	-
2	121.69	121.50	121.55	5.34
3	71.81	71.73	71.57	3.51
4	56.80	56.69	56.70	0.98
5	56.21	56.06	56.08	1.08
6	50.19	50.05	50.06	0.92
7	42.35	42.24	42.26	2.27
8				
9	39.83	39.70	39.71	1.15, 2.00
10	39.56	39.44	39.46	1.12
11	37.31	37.21	37.21	1.07, 3.14
12	36.56	36.44	36.45	-
13	36.24	36.11	36.12	1.34, 1.01
14	35.81	35.72	35.74	1.37
15	31.94	31.85	31.86	1.96, 1.46
16				
17	31.71	31.61	31.60	1.81
18	28.24	28.18	28.19	1.81, 1.20
19	28.02	27.95	27.96	1.51
20	24.32	24.23	24.24	1.57, 0.98
21	23.89	23.76	23.77	1.18 [m]
22	22.81	22.76	22.77	0.86
22	22.60	22.50	22.51	0.86
23	21.15	21.01	21.03	1.49
24	19.43	19.35	19.36	1.00
25	18.74	18.64	18.66	0.91
26	11.89	11.79	11.80	0.67

[m] multiplet

Table 5

Peak No	Assignment	Resonances – chemical shift values (ppm)	
		Roe	Milt
Glycerol region			
1	$N(CH_3)_3$ PC	54.31	54.35
2	CH_2N PC	59.20	59.27
3	TAG (sn1,3)	62.02	62.03
4	(sn1) PE/PC	62.80	62.84
5	(sn3) PC	63.35	63.42
6	(sn3) PE	63.63	63.66
7	PE (CH_2O)	64.44[s]	64.44[s]
8	PC (CH_2O)	66.27	66.33
9	β(sn2) TAG	68.89	68.78[s]
10	β(sn2) PL[m]	70.5[m]	70.4[m]

[m] multiplet

[s] small

Table 6.

Peak No	Assignment	Resonances – chemical shift values (ppm)	
		Roe	Milt
Carbonyl region			
1	DHA (sn2) TAG	172.09	-
2	DHA (sn2) PE	172.33	172.32
3	DHA (sn2) PC	172.40	172.41
4	DHA (sn1,3) TAG	172.47	-
5	DHA (sn1) PE	-	172.69
6	DHA (sn1) PC	172.73	172.73
7	EPA (sn2) TAG	172.79	-
8	EPA (sn2) PE	-	172.78
9	Other FA (sn1,3) TAG	172.82	-
10	EPA (sn2) PC	172.87	172.88
11	MUFA/SFA (sn2) PL	173.09	173.10
12	MUFA/SFA (sn2) PL	173.13	173.13
13	Other FA (sn1,3) TAG	173.21	-
14	MUFA/SFA (sn1) PL	173.44	173.42
15	MUFA/SFA (sn1) PL	173.48	173.45
Up –field region			
16	DHA (sn2) PL	33.82	33.85
17	EPA (sn 2) PC	33.66	33.67
18	EPA (sn2) PE	33.64	33.65
19	EPA (sn1) PL	-	-

Figure legends

Fig. 1. ^{13}C-NMR spectra (180 – 10 ppm) of lipids extracted from cod roe (A) and milt (B) illustrating the different regions

Fig. 2. ^{13}C-NMR glycerol region of lipids extracted from cod roe (A) and milt (B) with interpretation of the main resonances.

Fig. 3. ^{13}C-NMR olefinic region of lipids extracted from cod roe (A) and milt (B).

Fig. 4. ^{13}C-NMR carbonyl region of lipids extracted from cod roe (A) and milt (B) with assignments based on results from analysis of reference standards. Enlargement of parts of the up-field region of the spectra.

Fig. 5. Contour plot of C,H correlated HETCOR analysis of $CDCl_3$ lipid extracts of cod roe providing assignments of the main peaks of the glycerol region.

Fig 6. Enlargement of the aliphatic region of the ^{1}H NMR spectra of lipids extracted from cod roe and milt showing resonances from cholesterol and n-3 fatty acid.

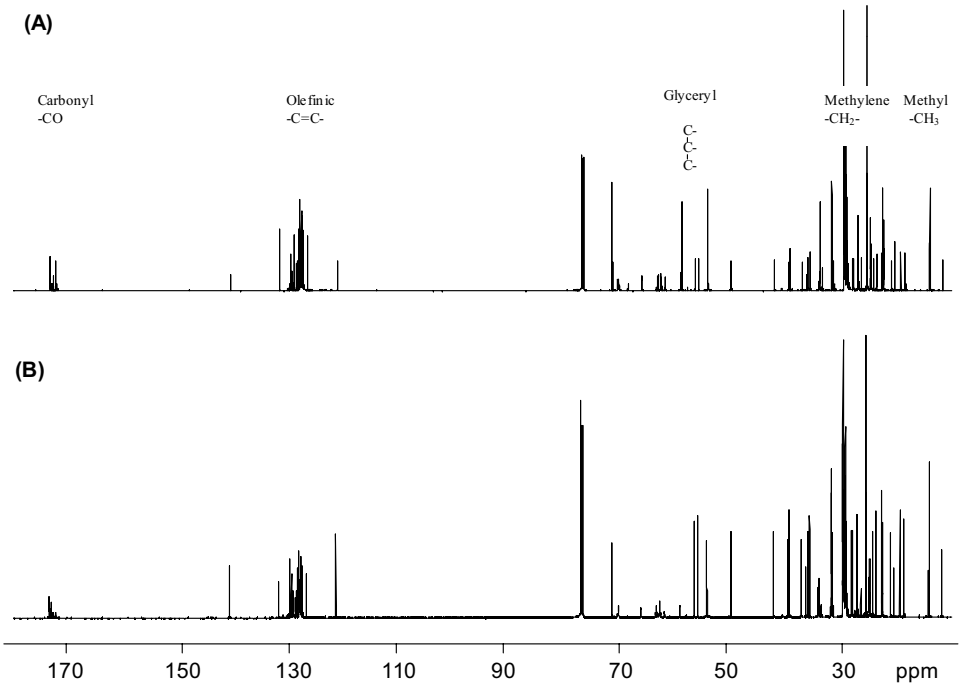

(A)
Carbonyl
-CO
Olefinic
-C=C-
Glyceryl
C-
C-
C-
Methylene
-CH2-
Methyl
-CH3
(B)
170
150
130
110
90
70
50
30
ppm

Fig. 1.

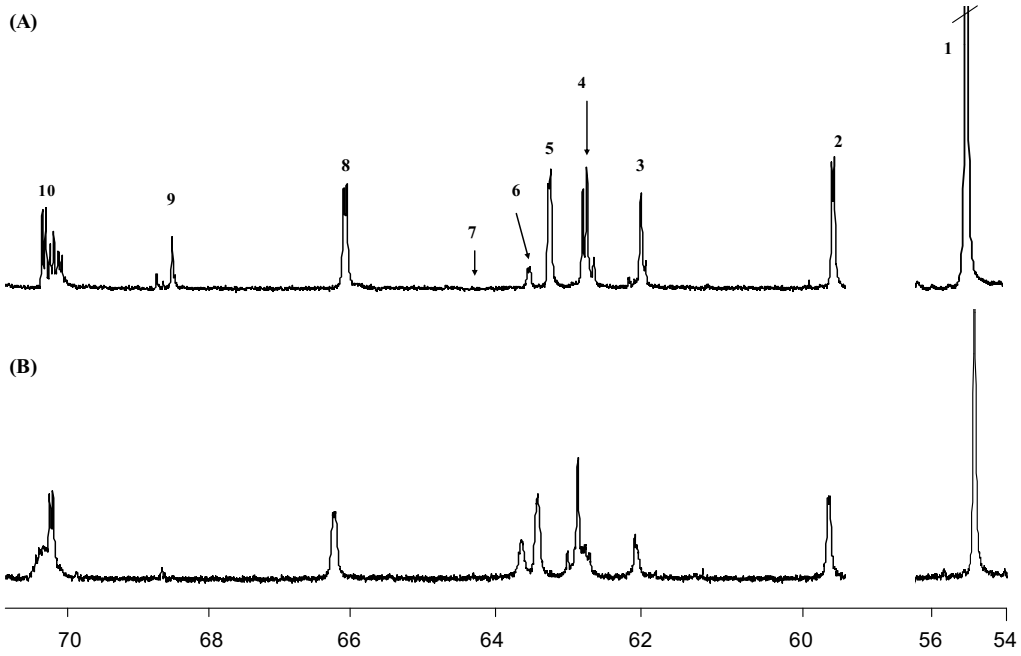

(A)
(B)
1
2
3
4
5
6
7
8
9
10
70
68
66
64
62
60
56
54
* lower chemical shifts in PE

Fig. 2.

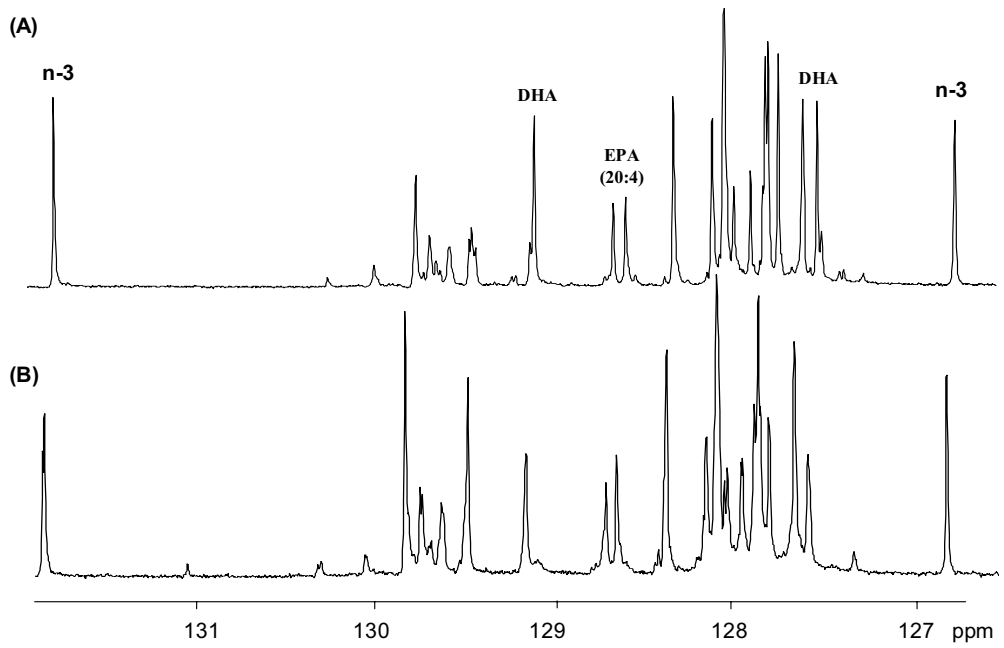


Fig. 3.

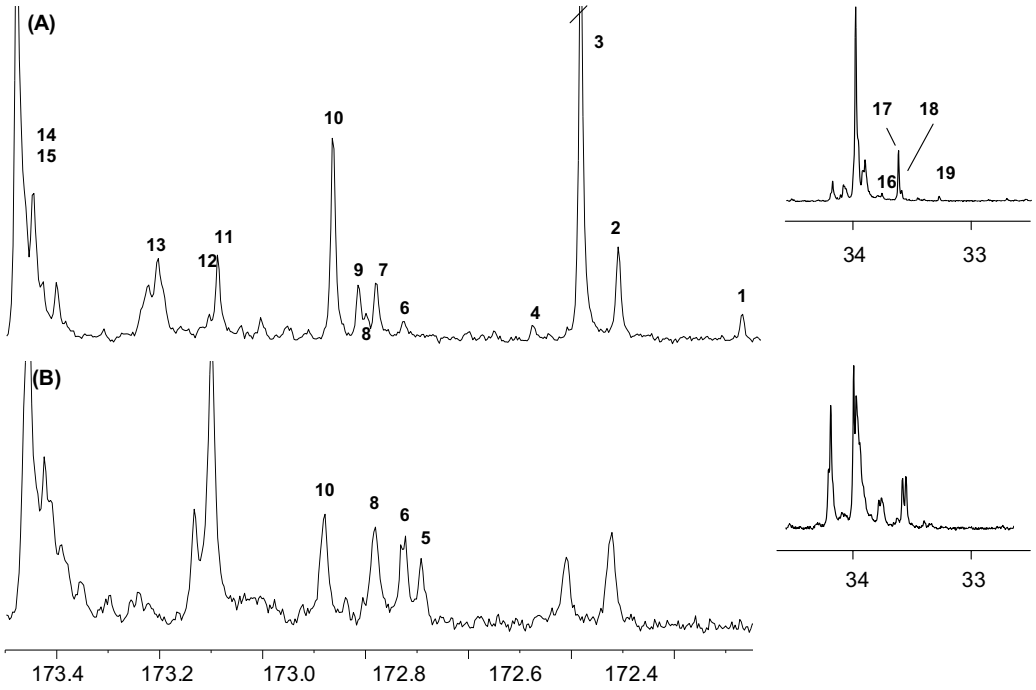

(A)
14
15
13
12
11
10
9
7
8
6
4
3
2
1
17
18
16
19
34
33
(B)
10
8
6
5
34
33
173.4
173.2
173.0
172.8
172.6
172.4

Fig. 4.

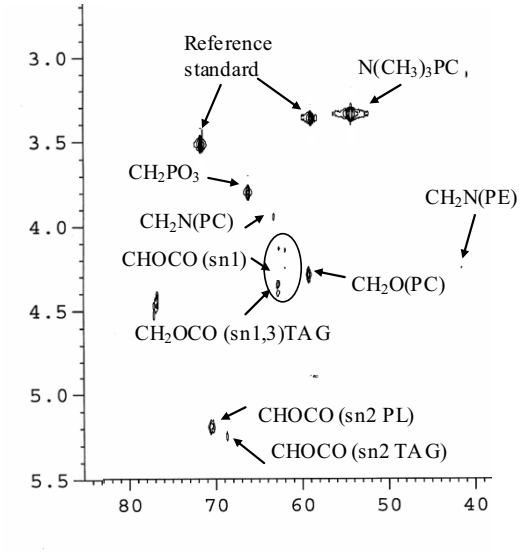

Reference
standard
N(CH3)3PC
CH2PO3
CH2N(PE)
CH2N(PC)
CHOCO (sn1)
CH2O(PC)
CH2OCO (sn1,3)TAG
CHOCO (sn2 PL)
CHOCO (sn2 TAG)
3.0
3.5
4.0
4.5
5.0
5.5
80
70
60
50
40

Fig. 5.

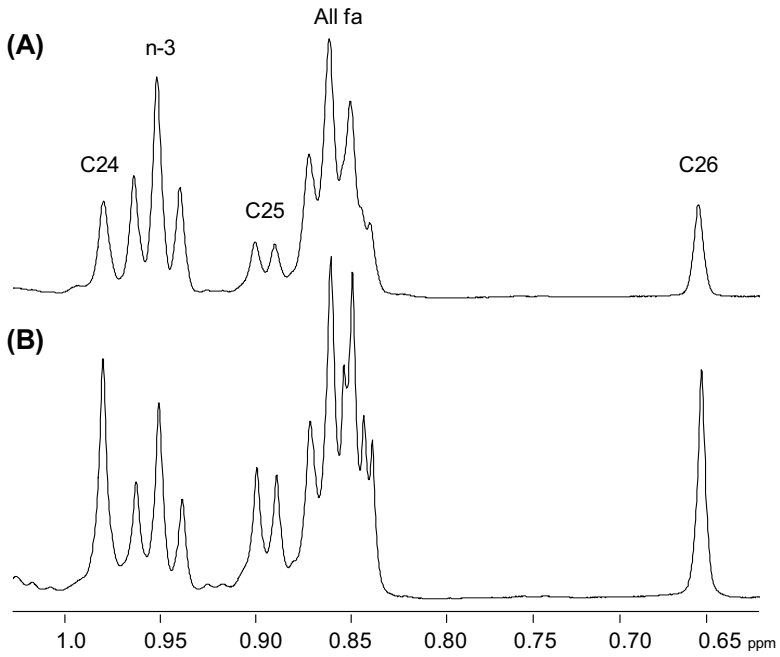


Fig 6.

Paper VIII

High resolution NMR for studying lipid deterioration in cod (*Gadus Morhua*) gonads

E. Falch[1,2] T. R. Størseth[1], and M. Aursand[1]

[1] SINTEF Fisheries and Aquaculture, Trondheim, Norway
[2] The Norwegian University of Science and Technology, Department of Biotechnology, Trondheim, Norway

Corresponding author:

Eva Falch

SINTEF Fisheries and Aquaculture Ltd., 7465 Trondheim, Norway

Tel: + 4740 0053 50, Fax: + 47 932 70 701

Eva.Falch@sintef.no

ABSTRACT

High resolution NMR was applied to study biochemical changes of lipids in cod (*Gadus morhua*) gonads during 7 days storage at 4°C. Changes were observed in the ^{13}C and ^{1}H resonances of cholesterol which were due to esterification of fatty acids at the hydroxyl position in roe and milt. Furthermore, the ^{13}C NMR spectra showed that the lipolytic changes in milt and roe where different. In milt, new resonances appeared during storage, due to formation of specific free fatty acids, with the corresponding changes in resonances of the esterified carbonyls and glycerols. Minor changes were found in the spectra of roe. No detectable levels of free fatty acids were found in these spectra. However, other compounds were detected in the same chemical shift regions. The current data demonstrate that high resolution NMR may be a suitable method to study heterogeneous lipid mixtures and the deterioration of lipids non-destructively in a one step procedure. However some more effort is needed to assign the new resonances and utilize the potential of the method.

Key Words: NMR, fish oil, n-3, PUFA, cholesterol esterification, lipases

1 INTRODUCTION

Phospholipids and triacylglycerols from fish are important sources of health beneficial n-3 polyunsaturated fatty acids (PUFA) with applications in food, feed and pharmaceutical products. Fish raw materials are highly susceptible towards biochemical deterioration that might lead to reduced nutritional value and negative consumer acceptance due to formation of off-taste. Biochemical processes in marine lipids may be catalysed by a variety of endogenous lipases (Søvik and Rustad, 2005; Lopez-Amaya and Marangoni, 2000). Lipolytic enzymes in fish attack different positions of the phospholipids; the phospholipase A_1 and A_2 produce free fatty acids and lysophospholipids, the phospholipase C and D cleaves in the polar head groups of phospholipids, and the lysophospholipases attack the lysophospholipids (Lopez-Amaya and Marangoni, 2000). The activity of these enzymes along with the activity of other endogenous and bacterial enzymes will necessarily form a wide range of reaction products.

Cod roe is a commercial food product used as an ingredient in spreads (Bledsoe et al, 2003), and fish gonads, in general, may be a basis for production of pure phospholipids with applications in health promoting products and speciality feed (Coutteau et al, 1997; Schneider, 2001; Takahashi, 2004; Guo et al, 2005). The interest in using phospholipids as carriers of n-3 PUFAs is increasing and PLs with DHA inserted in the *sn-2* position have been reported to receive attention because of their beneficial effects in metabolism (Takahashi, 2004). Growing awareness of human health results in a demand for trustworthy methods for mapping lipid molecular species and controlling the biochemical processes that occurs preferably in a non-destructive way.

The compositional changes due to biochemical processes are generally determined by different chromatographic methods that are selective towards specific reactions products. These techniques are generally destructive due to the derivatization during the pre-treatment and have a potential risk of incomplete extraction of desired components (Kuksis et al, 2003). Thus, one single analytical method to non-destructively determine chemical composition and compositional changes in marine lipids would be valuable for elucidating unknown changes in lipids during storage and processing, shelf life determination, and for finding optimal stabilisation technologies for preventing unwanted changes.

Previous work on marine lipids has shown that ^{13}C-NMR provides objective information of positional distribution of fatty acids in acylglycerols (Aursand et al., 1993; Gunstone and Seth, 1994; Medina and Sacchi, 1994), phospholipids (Falch et al, 2005a),

lipid class distribution (Siddiqui et al, 2003, Falch et al, 2005ab) and other specific lipid components such as alcohol and esters (Gunstone, 1991, 1993). The polar head groups of different phospholipids, has previously been assigned by ^{13}C NMR (Everts and Davies, 2000; Jimeno et al, 2002; Falch et al, 2005a). ^{1}H-NMR has been shown to provide quantitative data of n-3 fatty acids and DHA (Igarashi et al, 2000), cholesterol (Pollesello et al, 1992; Tosi et al, 2003; Yoshicoka et al, 2000) and semi-quantitative data of reaction products from lipid oxidation (Falch et al, 2004).

We have previously applied ^{1}H and ^{13}C NMR to study the chemical composition of lipids extracted from fresh cod roe and milt (Falch et al, 2005a). In these studies, ^{13}C NMR spectra was used to obtain positional distribution of polyunsaturated fatty acids in phospholipids and the spectra obtained from gonads showed that most of DHA was esterified in the *sn-2* position of the phospholipids. Moreover, these spectra showed that roe contained much higher levels of phosphatidyl choline (PC) compared to phosphatidyl ethanolamine (PE) while these two phospholipids were found in equal amounts in milt.

In the present work, we have used the same samples for compositional analysis before and after experimental storage at refrigerated temperature in order to study the lipid deterioration by high resolution NMR.

2 EXPERIMENTAL

2.1 Sample preparation

Roe and milt were withdrawn from spawning cod (*Gadus morhua*), less than 2 hours (<4^{o}C) after catch. Intact tissue consisted of roe and milt. The outer membranes of roe were not used in order to get homogeneous samples. The control samples (at day 0) were immediately frozen at -80^{o}C until analysis. Samples were stored in triplicates (10g wrapped in plastic films and aluminium foil) in the dark at 4^{o}C. Samples were withdrawn daily for 7 days. Lipids were extracted by the method of Bligh and Dyer (1959).

The lipid extracts were introduced (50 mg in 0.6 ml $CDCl_3$) into 5 mm NMR sample tubes (Wilmad lab-glass, Buena, USA). Ethylene glycol dimethyl ether (EGDM, 99.5%, Igarashi et al, 2000) purchased from Fluka (Buchs, Switzerland) was used as an internal standard. A reference sample of lysoPC (L-α-Lysophosphatidylcholine (egg-chicken)) was obtained from Avanti Polar Lipids (Alabama, US) and recorded by HR NMR.

2.2 NMR analysis

The NMR experiments were carried out on a Bruker DRX 600 spectrometer (Bruker Biospin GmBH, Germany), resonating at 600.13 MHz for ^{1}H and 150.90 for ^{13}C, using a 5 mm BBO probe at 298 K. For each sample a ^{1}H and ^{13}C spectra were obtained with standard pulse programs from the Bruker pulse-library. ^{1}H experiments were recorded with a center frequency of 4.5 ppm; spectral width 11.97 ppm; acquisition time 4.56 sec.; 64 K time domain data points; 90^{o} pulse angle relaxation delay 3 sec. 256 free induction decays (FIDs) were collected for each sample. Zero-filling using an exponential window function of 0.3 Hz was applied before Fourier transform. ^{13}C spectra were recorded with a center frequency of 100 ppm, spectral width 200.79 ppm, acquisition time 1.08 sec., 128 K time domain data points, relaxation delay 3 sec. Proton decoupling were used. 64 K FIDs were collected for each sample. Zero filling and 0.3 Hz exponential line broadening was applied before Fourier transform. All samples were analysed by 2D HETCOR (^{13}C:^{1}H) NMR with following experimental conditions: data points: 4096 (t_2) x 512 (t_1), 356 scans were averaged for each FID with a relaxation delay of 3 sec. The FIDs were zero filled to 1024 for t_1 and were apodized by exponential (F1) and squared sine bell (F2) function.

NMR spectra were recorded using an internal standard enabling comparison of relative intensities between spectra. Processing of NMR spectra was performed by Topspin v 1.3 software (Bruker Biospin GmBH, Germany). NUTS - NMR Utility Transform Software (Acorn NMR Inc., Livermore, USA) was used for line fitting to enable quantification of n-3 fatty acids.

2.3 Lipid classes by TLC

Composition of lipid classes was determined by thin-layer chromatography using an Iatroscan TLC Flame ionisation detector (TH-10 MK-IV, Iatron Laboratories, Tokyo, Japan) according to procedures described in Falch et al (2005a).

2.4 Fatty acid composition by GC

Fatty acid composition was analysed by gas-liquid chromatography after derivatization to fatty acid methyl esters according to Metcalfe et al. (1966). Fatty acid methyl esters were analysed on a Fison 8160 capillary chromatograph (Fisons Instruments S.pA., Milano Italy) according to method described in Falch et al (2005a). These results are presented in Falch et al (2005a).

3 RESULTS AND DISCUSSION

3.1 Changes in lipid class distribution by TLC

Both roe and milt contained initially high levels of polar lipids and especially the milt contained high levels of cholesterol (Table 1). After one week of refrigerated storage, the initial levels of cholesterol and phospholipids decreased while the levels of free fatty acids and cholesterol esters increased. Cholesterol decreased form 8.7 to 5.0% in roe and from 20.6 to 1.4% in milt.

3.2 NMR spectroscopy

^{13}C NMR spectra of the lipid extracted from cod roe and milt (**Fig. 1**) presented a wide range of resonances representing specific fatty acids, lipid classes and cholesterol (Falch et al, 2005b). ^{1}H NMR provided additional data on n-3 fatty acids and cholesterol compounds.

3.2.1 Changes in cholesterol during storage

Among the resonances in the ^{13}C NMR spectra of roe and milt (**Fig. 1**) 24 were assigned to cholesterol (Falch et al, 2005a). During one week of refrigerated storage evident differences were observed both in the intensity and chemical shift value of several of the resonances associated with cholesterol. Particularly, changes in the olefinic region (140-120 ppm) were found. The intensity of resonances originating from the olefinic carbons of cholesterol (121.7 ppm and 140.8 ppm) decreased, while new resonances appeared at 139.57, 139.62, 122.52 and 122.57 ppm (**Fig. 2**). These new peaks have previously been assigned to cholesteryl esters (Tosi et al., 2004). Cholesterol esters also gave rise to other new resonances in these materials at 73.5-74.0, 49.9, 56.0 and 57.3 ppm according to Tosi et al. (2004). The resonance originating from the carbon attached to the hydroxyl group of cholesterol is assigned at 71.7 ppm (**Fig 3**) and the three new resonances at 73.5 – 74.0 ppm are assigned as cholesteryl esters (Nouri-Sorkhabi et al, 1995; Tosi et al, 2004), probably differentiation between different fatty acids esterified to this position. The intensity of resonances from cholesteryl esters were higher in stored milt than in stored roe, which is in agreement with the TLC results.

The formation of cholesteryl esters was also observed in the ^{1}H NMR spectra assigned at 1.0 ppm, while the free cholesterol signal were assigned at 0.99 (**Fig. 4**). These resonances

are previously used for quantification of cholesterol and cholesteryl esters (Pollsello et al, 1992; Tosi et al, 2003; Yoshicoka et al, 2000) in human biological materials. The ratios between cholesterol and cholesteryl esters in the spectra of milt in the present study were 1.0 before storage and 0.3 after storage. The corresponding ratios in roe were 0.9 before storage and 0.7 after storage, and were comparable to the TLC results.

The esterification of cholesterol might be due to a glycoprotein enzyme called lecithin:cholesterol acyltransferase (LCAT) that is reported to be responsible for synthesis of most cholesteryl esters in human plasma (Glomseth, 1968). In human, the LCAT enzyme is reported to play a role in the maturation of HDL secreted by liver and intestines (Parks et al, 2000). LCAT is reported to directly transfer a fatty acid moiety from PC to cholesterol and this has been proposed to be the main pathway, also during formation of cholesteryl esters in fish roe (Kaitaranta and Ackman, 1981).

3.2.2 Hydrolysis of phospholipids and acyl glycerols

In the carbonyl region of the ^{13}C spectra of milt new peaks appeared which were assigned to free fatty acids (DHA, EPA and other fatty acids) (**Fig 5**). These are reaction products from lipolytic activity. Carbonyl resonances from 174 – 172 ppm also revealed changes and showed that the following signals were shifting or disappeared during storage of milt: DHA in *sn-1* and *sn-2* of PE, DHA in *sn-1* of PC and EPA in *sn-2* of PE according to assignments by Falch et al. (2005a). I addition, new signals were formed particularly in the 173.5 – 173.0 ppm region.

In roe, new peaks appeared between 176.4- 175.3 ppm (**Fig 5**). These did not have the same chemical shift values as those from free fatty acids in the milt spectrum. These peaks were not assigned. The carbonyl region at 174 – 172 ppm of the roe spectra indicated that the DHA levels in TAG were unchanged during storage. Furthermore, by studying the relative intensity of the carbonyl peaks originating from phospholipids these molecules appeared to be unchanged, or alternatively equally changed (non specific changes). This was also seen in other regions of the roe spectra. No noticeable reduction of specific resonances was found in this region; however, new peaks were formed at 173.8, 172.5, and 173.0 ppm. The minor changes in the composition of lipid classes (TAG, PE, PC) in roe during storage were in agreement with the TLC results (Table 1).

In the glycerol region of milt, new resonances appeared at 64.4, 65.3, 66.5, and 67.7 ppm with HETCOR cross peaks to ^{1}H resonances at 4.35, 3.92, 5.11, 4.29 and 3.89 ppm respectively (Fig 6). The glycerol carbons of the 2-acyl lysoPC reference standard appeared at 65.0, 67.4 and 68.5 ppm (Fig. 7.) while the ^{1}H resonances were located at 3.9 – 4.1 ppm. This may indicate formation of lysoPC during storage. Changes in the chemical shift regions of resonances previously assigned to glycerols from the *sn-1* position of PE and PC (62.8 ppm) (Falch et al, 2005a) were observed which indicate that deterioration in these molecules have occurred. Additionally reduction of the signal intensity of resonances from glycerols in the *sn-3* position of PE was observed (63.6 ppm).

In the glycerol region of roe, minor changes were found which were in accordance with that was found in the carbonyl region of the same spectrum. New resonances appeared at 64.8, 66.1, 67.5, and 68.5 ppm which were correlated to ^{1}H resonances at 4.06, 3.71, 4.62, and 3.94 ppm respectively. These signals may originate from glycerols of lysoPLs. When the signal intensities between resonances from glycerols at the *sn-2* position of PL were compared with the *sn-2* glycerols of TAG a decrease in the intensity of signals from PLs were found during storage. Similar to what was found in the stored milt, the chemical shift values of resonances assigned as glycerols from the *sn-1* position of PE and PC shifted downfield, indicating deteriorations in these molecules also in the roe samples.

The spectra presented in this paper were not completely assigned and more reference standards should be analysed in order to identify all new compounds that are formed during storage of this heterogeneous material. Due to the use of other solvent systems in other comparable trials (Medina et al, 1994) the chemical shift values are not easily obtained for specific reaction products from hydrolysis of phospholipids. 2D NMR techniques were valuable for finding the changes occurring.

3.3 Conclusions

The analytical methods currently available for determining lipid composition and lipid deterioration are selective and generally require pre-treatment steps that may lead to loss of information. HR NMR has in the present work shown that it is possible to non-selectively and non-destructively study lipid deterioration in the heterogeneous total lipids from fish. It is thereby possible by using NMR techniques, to study a broad range of chemical compounds that would otherwise demand the use of numerous conventional analyses to

obtain. Analysing extracts from samples using NMR will provide information on all compounds that are observable by NMR and soluble in the solvent used for extraction. NMR offers the advantages of being non-destructive and allow for assessment of a wide range of lipid compounds without pre-treatment steps of the total lipids. Cholesterol has gained a growing awareness based of its health influence. Not only cholesterols are assigned in these spectra but also reaction products from deterioration of cholesterols are found. Furthermore, the method can provide knowledge that can be used to find the most suitable conservation and processing of these nutritious lipids.

These preliminary results show that NMR is a unique method for studying lipid composition and deterioration of heterogeneous lipids. However, some more effort is needed to assign the changing resonances and bring out all the potential of the method.

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Tables and figures

Table legends

Table 1. Lipid class composition in roe and milt based on neutral and polar solvent systems before TLC separation.

Figure legends

Fig. 1. 600 MHz ^{13}C-NMR spectra (180 – 10 ppm) of lipids extracted from cod roe (A) and milt (B) before and after experimental storage at 4^{o}C for one week. The arrows are pointing at the regions showing the main changes due to formation cholesteryl ester.

Fig. 2. 600 MHz ^{13}C-NMR of the olefinic region of lipids extracted from cod roe (A) and milt (B) before and after one week of experimental storage at 4^{o}C. Chol: Cholesterol; CholE: Cholesteryl ester. The braces points out the main changes observed in the fatty acid olefins (128.5 – 129.5 ppm).

Fig. 3. 600 MHz ^{13}C-NMR spectra of the regions between 75 and 71 ppm. showing changes due to cholesterol deterioration in (A) roe and (B) milt before and after experimental storage at 4^{o}C for one week. These resonances originate from the carbon at the position of cholesterol (Chol) where the fatty acids are esterified to form cholesteryl esters (CholE).

Fig 4. Enlargement of the low field region of the 600 MHz ^{1}H NMR spectra of lipids extracted from cod roe and milt showing resonances from cholesterol and cholesteryl ester before and after one week of experimental storage at 4^{o}C.

Fig. 5. 600 MHz ^{13}C-NMR carbonyl region of lipids extracted from cod roe (A) and milt (B) before and after one week of experimental storage at 4^{o}C.

Fig. 6. 600 MHz ^{13}C-NMR glycerol region of lipids extracted from cod roe (A) and milt (B) before and after one week storage at 4^{o}C. The * indicate new resonances discussed in the text.

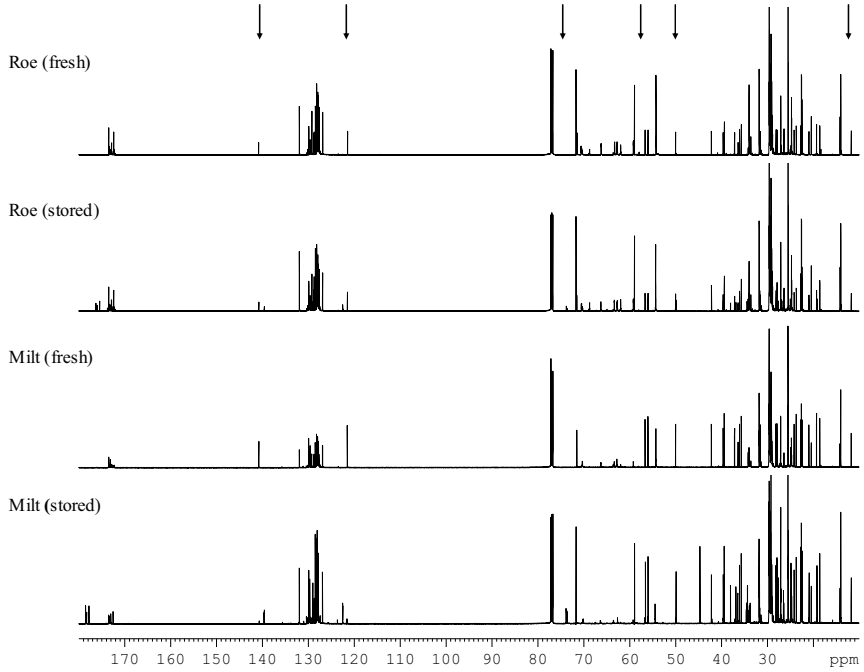
Fig. 7. 600 MHz ^{13}C-NMR spectra of lyso PC (L-α-Lysophosphatidylcholine (egg-chicken)) with enlargement of the glycerol region. The * indicate the glycerol carbons. The resonances from the polar head group of PC are assigned.

Table 1.

	Roe (% of total lipids)		Milt (% of total lipids)	
	Day 0	Day 7	Day 0	Day 7
Neutral solvent system				
Steryl ester	0.4 ± 0.1	4.1 ± 0.4	0.0	30.8 ± 0.9
Triacylglycerols	8.1 ± 0.4	9.5 ± 0.4	0.9 ± 0.00	0.0 ± 0.0
Free fatty acids	0.0	4.4 ± 0.3	0.0	25.0 ± 0.9
Diacylglycerols	0.2 ± 0.42	1.9 ± 1.6	0.0	0.0
Cholesterol	8.7 ± 0.34	5.0 ± 0.6	20.6 ± 0.96	1.4 ± 0.1
Monoacylglycerols	3.6 ± 1.72	n.d.	4.6 ±1.06	0.0 ± 0.0
Polar lipids	79.0 ± 0.98	75.1 ± 2.5	74.0 ± 1.93	41.9 ± 1.7
Polar solvent system				
Neutral lipids	11.7	22.0	21.5	79.4
PE	8.1	4.5	35.2	2.7
PC	69.5	54.3	43.2	0.0
Lyso PL	n.d	3.0		16.5
Total lipids (%)	4.61± 0.15		2.14 ± 0.13	

n.d.) not detected

Fig 1.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Roe (stored)
Milt (fresh)
Milt (stored)
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Fig. 2

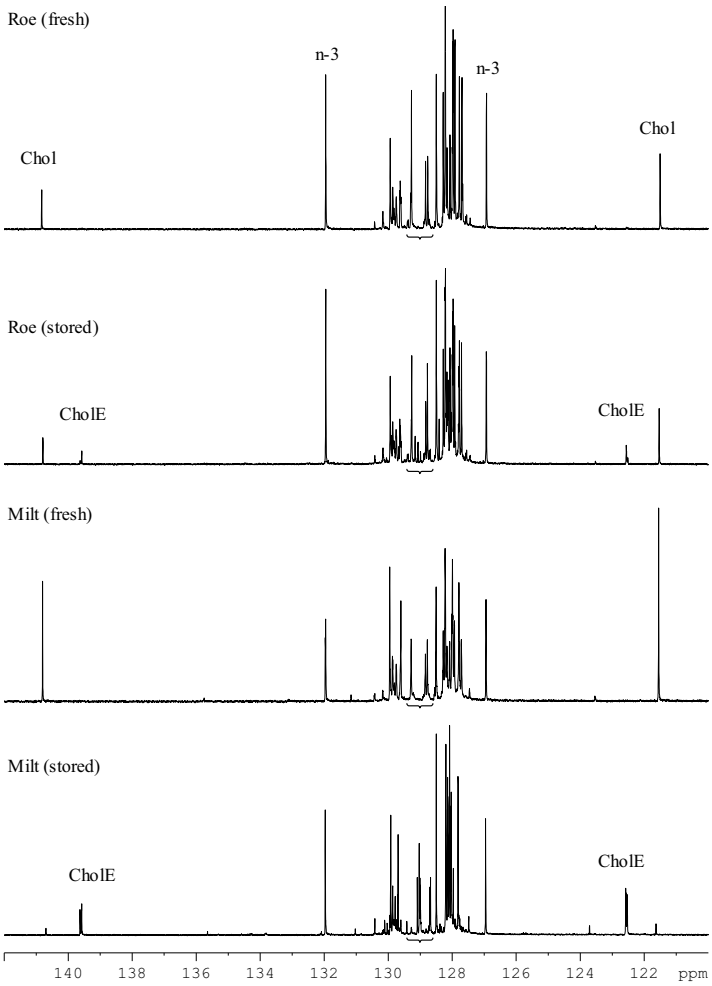

Roe (fresh)
n-3
n-3
Chol
Chol
Roe (stored)
CholE
CholE
Milt (fresh)
Milt (stored)
CholE
CholE
140
138
136
134
132
130
128
126
124
122
ppm

Fig 3.

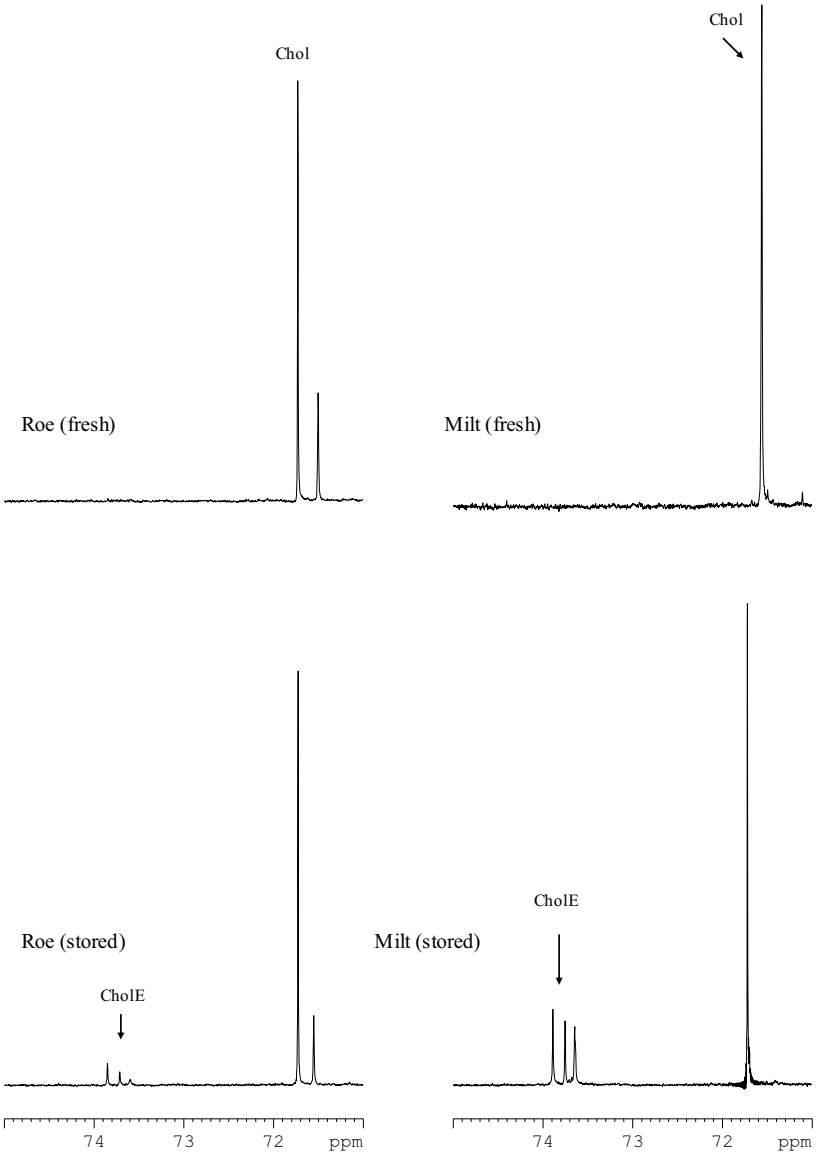

Chol
Chol
Roe (fresh)
Milt (fresh)
Roe (stored)
CholE
CholE
Milt (stored)
74
73
72
ppm
74
73
72
ppm

Fig. 4.

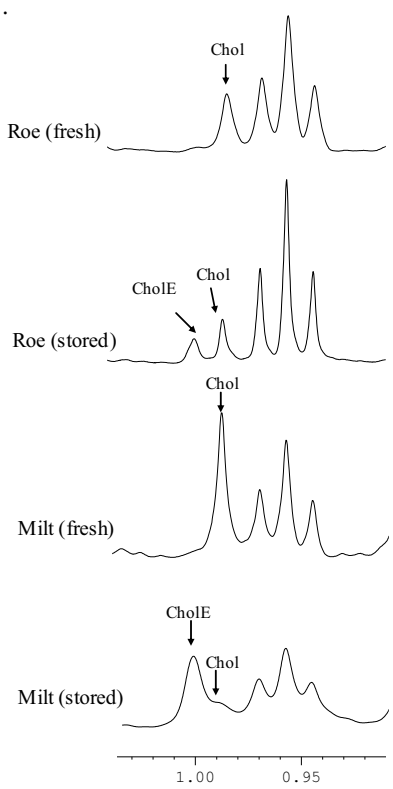

Chol
Roe (fresh)
CholE
Chol
Roe (stored)
Chol
Milt (fresh)
CholE
Chol
Milt (stored)
1.00
0.95

Fig 5

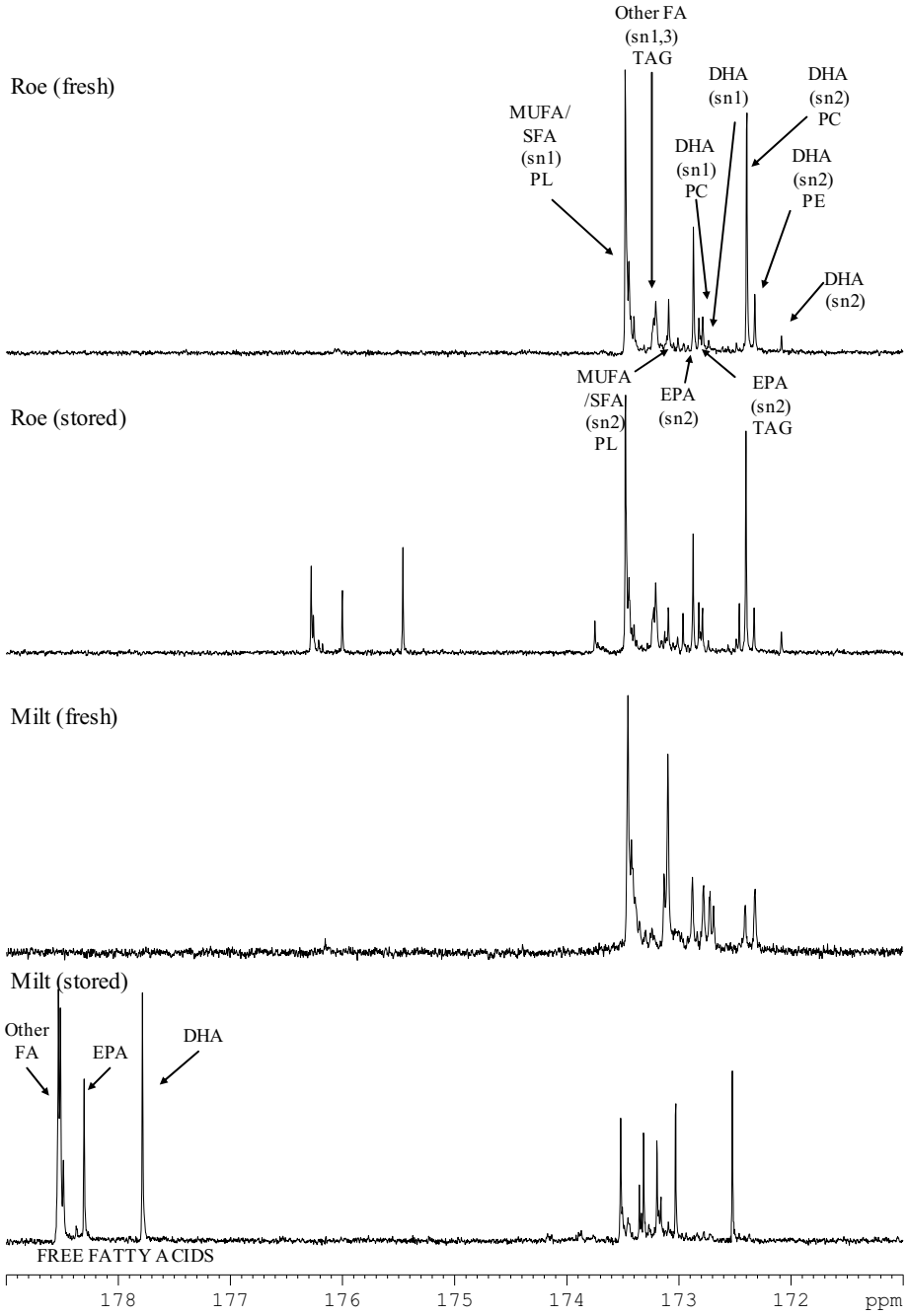

Roe (fresh)
Other FA (sn1,3) TAG
DHA (sn1)
DHA (sn2) PC
MUFA/ SFA (sn1) PL
DHA (sn1) PC
DHA (sn2) PE
DHA (sn2)
MUFA /SFA (sn2) PL
EPA (sn2)
EPA (sn2) TAG
Roe (stored)
Milt (fresh)
Milt (stored)
Other FA
EPA
DHA
FREE FATTY ACIDS
178
177
176
175
174
173
172
ppm

Fig. 6.

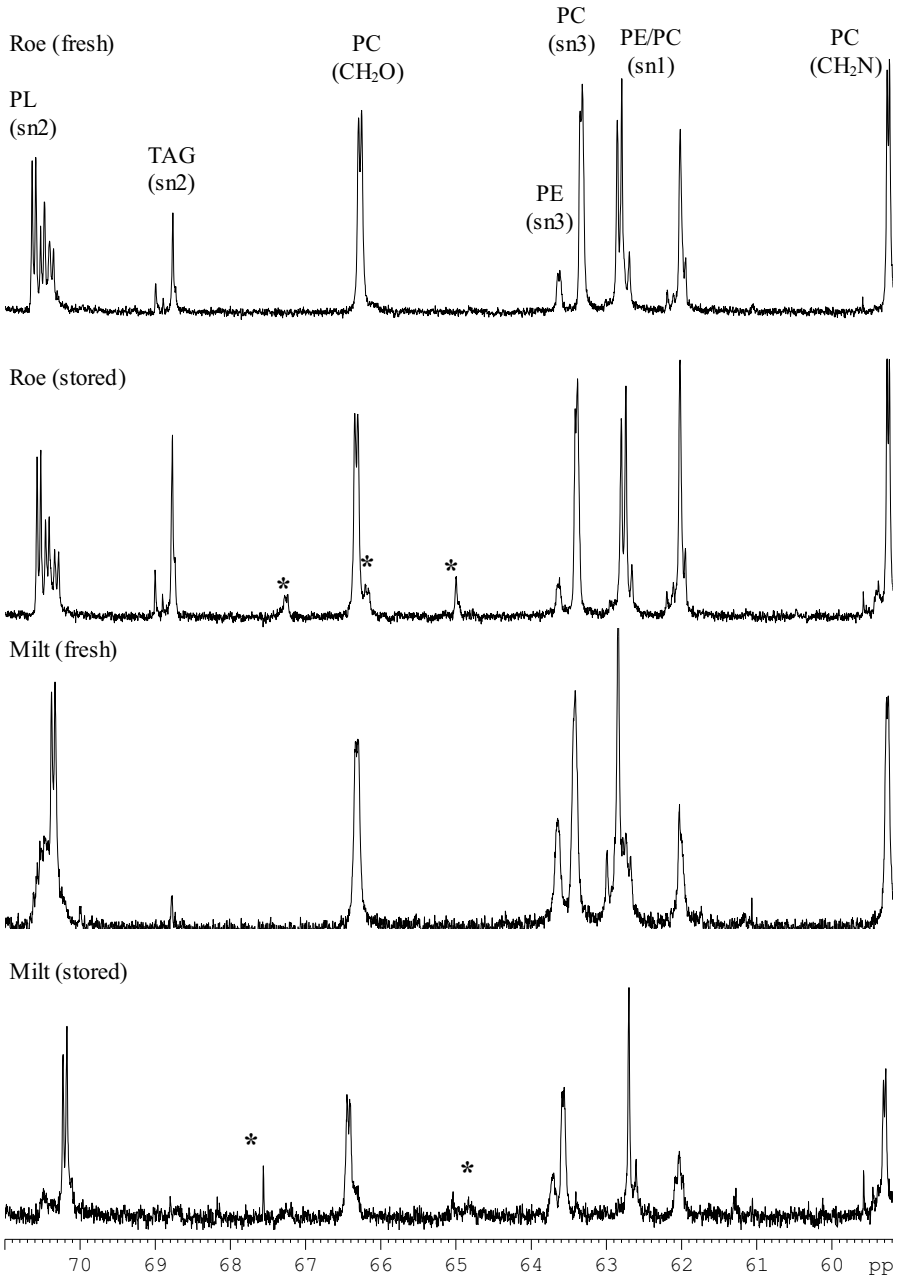
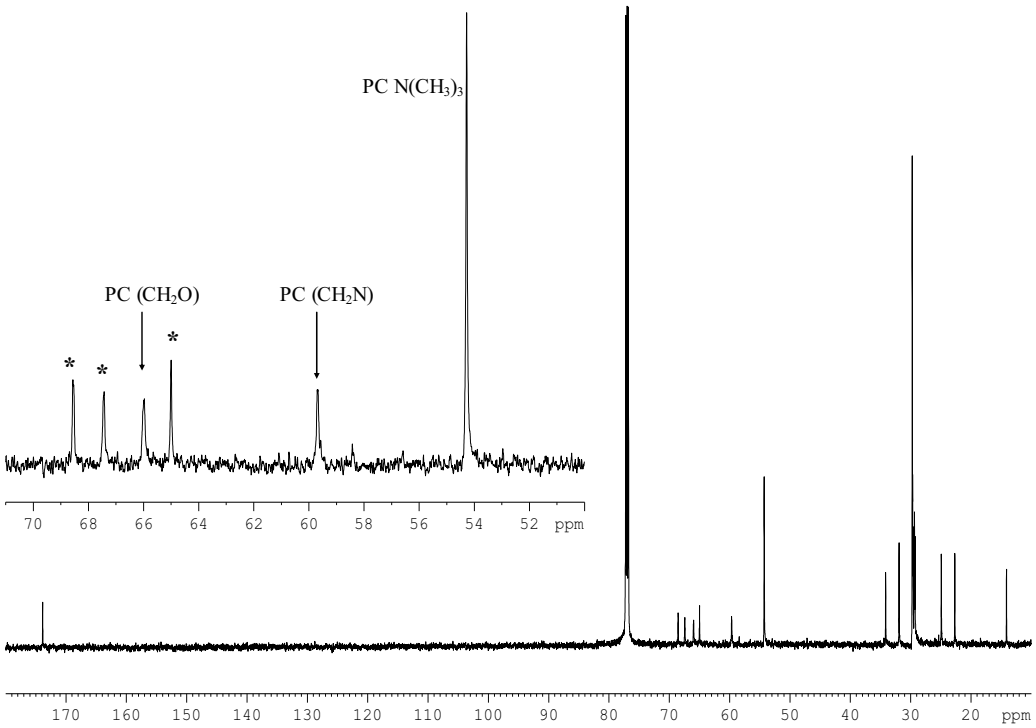

Roe (fresh)
PL
(sn2)
TAG
(sn2)
PC
(CH2O)
PC
(sn3)
PE
(sn3)
PE/PC
(sn1)
PC
(CH2N)
Roe (stored)
Milt (fresh)
Milt (stored)
*
70
69
68
67
66
65
64
63
62
61
60
pp

Fig 7.


PC N(CH3)3
PC (CH2O)
PC (CH2N)
*
*
*
70 68 66 64 62 60 58 56 54 52 ppm
170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 ppm