Health promoting, safe seafood of high eating quality in a consumer driven fork-to-farm concept

EU Integrated Project no 506359

Report on techniques applicable to verify various traceability information

Iciar Martinez

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A report from RTD area 6

'Seafood traceability to ensure consumer confidence'

Project 6.3 VALID

Report on techniques applicable to verify various traceability information

Deliverable D6

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Deliverable 6

Report on techniques applicable to verify information regarding:

- Discrimination of farmed from wild fish
- Identification of geographic origin
- Verification of the content in some bioactive components and
- Verification of some processing conditions

By

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SINTEF Fisheries and Aquaculture Ltd

April, 2005

Deliverable 6

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Summary

This is a report on techniques applicable to verify information regarding:

- Discrimination of farmed from wild fish
- Identification of geographic origin
- Verification of the content in some bioactive components and
- Verification of some processing conditions

The report is divided in two parts: Part A is a "State of the Art" review on the different types of information to verify, and Part B describes some of the most relevant techniques, equipment necessary and their advantages and drawbacks.

Farmed from wild Atlantic salmon have been discriminated based on the fact that the fatty profile of their storage lipids reflects the composition of the feed and that artificial diets have a higher amount of total fat and of typical vegetable oils than natural diets, which are small fish and pelagic fish. Techniques used to obtain information about the fat content and composition are gas chromatography and nuclear magnetic resonance analyses. No systematic studies have been carried out yet on other cultivated species or on lean species. There are not officially recognized standard methods to ensure the production method.

Research into developing methods to identify the geographic origin of fish lags behind even more. No officially recognized methods exist. Among the methods examined, isotope signatures analyzed by isotope ratio mass spectrometry seem to be the most promising.

The content of some bioactive components - marine oils, taurine, betaine and anserine - may be examined by the classical chromatographic methods (gas chromatography and high performance liquid chromatography respectively) and by ^{1}H and ^{13}C nuclear magnetic resonance spectrometry. The former are cheaper and standard in many laboratories while the latter are newer and more expensive but also require simpler sample preparation and render more additional and relevant information: for example the origin of the oils and presence of additional compounds. The versatility and amount of information that it is possible to obtain from the nuclear magnetic resonance techniques is starting to find application in many aspects of food manufacture (composition, control safety etc.) and it is likely that they will eventually become standard analyses.

Regarding the verification of some processing conditions, there are no methods developed or under examination to determine how a product has been salted or dried. Several methods can be used to differentiate frozen-thawed from fresh unfrozen fish, but the distinction is more difficult when one has to differentiate thawed from old-not frozen fish. The easiest to discriminate are species that form DMA upon freezing, *i.e.*, gadoids. Methods to detect DMA are high performance liquid chromatography and nuclear magnetic resonance spectrometry. Methods used to identify the heating temperature are based on the fact that proteins suffer conformational changes and loose their solubility and enzymatic activity when the temperature increases. Methods that detect these alterations are: differential scanning calorimetry (indication that the transition temperature has been reached), protein solubility and enzymatic activity. However, protein denaturation and loss of enzymatic activity may occur as a result of many treatments: salting, loss of freshness, freezing, etc. and therefore the history of the fish should be known to estimate the heating temperature by the mentioned methods. Also, the type of species (cold or warm adapted, resiliency, etc.) has to be taken into account to select an optimal method to determine the heating temperature. No standard method exists yet.

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Abbreviations

2DE	Two-dimensional electrophoresis
DESIR	Dry extract spectroscopy by infrared reflection
DHA	Docosahexaenoic acid: C226n-3 (an ω-3 fatty acid)
DMA	dimethylamine
DNA	Deoxirobonucleic acid
DSC	Differential scanning calorimetry
EPA	Eicosapentaenoic acid,: C205n-3 an ω-3 fatty acid)
FA	Fatty acid
FAME	Fatty acid methyl ester
FID	Free induction decay
FT	Fourier transform
GC	Gas chromatography
HPLC	High performance liquid chromotography
ICP-MS	Induction coupled plasma mass spectrometry
IEF	Isoelectric focusing
IRMS	Isotope ratio mass spectrometry
MRI	Magnetic resonance imaging
NIR	Near infrared spectroscopy
NMR	Nuclear magnetic resonance
PAGE	Polyacrylamide gel electrophoresis
PCBs	Polychlorinated biphenyls
PCR	Polymerase chain reaction
PUFA	Polyunsaturated fatty acid
RAPD	Random amplification of polymorphic DNA
RFLP	Restriction fragment length polymorphism
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SNIF	Site-specific natural isotope fractionation
SSCP	Single strand conformation polymorphism
TCA	Trichloracetic acid
TMA	Trimethylamine
TMAO	Trimethylamine oxide
TMS	Tetramethylsilane
TSP	Trimethylsilypropionate
uv	Ultra violet

Part A. State of the art

A-1. Discrimination of farmed from wild fish

The EU Commission regulation No 2065/2001 of 22 October 2001 has laid down detailed rules for the application of Council Regulation (EC) No 104/2000 as regards informing consumers about fishery and aquaculture products. The information includes specification of the commercial designation and scientific name, method of production of a species ("caught" or "caught in freshwater" or "farmed" or "cultivated") and the area in which it was caught. In the case of cultivated species, article 5 of the regulation indicates that a reference should be made to the country in which the product undergoes the final developmental stage.

Within the last 10-15 years, aquaculture has become a major commercial activity in the world. European countries farming Atlantic salmon (*Salmo salar*) include Norway, Ireland, Scotland, but Atlantic salmon is also farmed at least in Chile, Canada and Tasmania. Farmed and wild Atlantic salmon have different prices, wild salmon being most expensive, followed by organically farmed salmon and then traditionally farmed fish, the cheapest. The location where the fish is fished or farmed is also important since some areas are considered to be "clean" and others "polluted", specially regarding the content of environmental pollutants in the fish [Foran *et al.*, 2004; Hites *et al.*, 2004; Jacobs *et al.*, 2002; Madeniian *et al.*, 2002] and because some consumer favour and are willing to pay higher prices, for fish from certain regions.

Correct identification and labelling of fish as farmed or wild is also of relevance for the authorities in order to protect wild and endangered stocks. For example in the case of Atlantic salmon, farmed salmon may escape and be captured by fishermen that would consider it wild, and price it accordingly with the consequent misinformation to customers. Mapping of the extent of escaped salmon is important, in addition to the issue of consumer protection, to protect wild populations: escaped salmon is considered at present a high risk regarding the contamination of the genetic stocks of wild populations that may then disappear as such. Also, many populations of wild Atlantic salmon are severely endangered and therefore they must not be found available for consumption.

In the case of cod (*Gadus morhua*) the situation is similar: some wild populations are protected due to overexploitation and therefore, unless illegal captures have occurred, they must not be found as available for human consumption. On the other hand, farming of cod is relatively new and as of now not as successful as the farming of salmon: deformations, a greyish flesh color [Luten *et al.*, 2002] and abnormal features in the fillet (such as what looks like black small veins) [Cooper and Midling, 2004] are still common, although it is to be expected that technical improvements and a deeper knowledge of the fish biology and physiology will help to overcome these problems in the near future. There is however an "intermediate" type of cod: wild caught cod that is kept alive, *i.e.* farmed for a period of time until it is convenient to slaughter it, for example due to free slaughtering and processing capacity. Luten *et al.* [2002] found that consumers gave similar quality profiles to wild cod and to wild caught farmed cod, although the composition of the wild caught farmed specimens is affected by the composition and amount of feed received.

Genetic contamination of wild stocks with escaped farmed cod is not yet considered a risk, but it will when farming takes over, specially if the wild stocks are still reduced to low numbers.

1.1 Morphological analyses

There are no official guidelines to differentiate farmed from wild cod or salmon based on morphological characters, although there are some general aspects that may be used to differentiate farmed from wild salmon and cod.

Morphological abnormalities, such as spinal deformities, are known to occur frequently in many species of intensively reared fish [see Doulas *et al.*, 1991; Fraser *et al.*, 2004 and references therein], including salmonids [Dabrowski *et al.*, 1990; Toften and Jobling, 1996 and references therein]. Farmed cod specimens also show morphological deformities.

Color is also a variable that may be used to differentiate farmed from wild fish. The diets of farmed Atlantic salmon are formulated to yield a nice bright pinkish-redish color, while wild specimens may vary in color according to their diet. Wild salmon is usually caught when migrating back to the rivers for spawning and may display in higher or lower degree the morphological characters associated to sexual maturation: deformation of the jaw, stronger red pigmentation of the skin, and muscle depletion (low fat content, due to the effort of migration). Farmed salmon on the hand is selected to reach a commercially interesting size (4-5 kg) in the shortest possible time and with no display of signs of sexual maturation. Individuals that display such characters associated to fast growth are eliminated from the production and certainly from the line of breeders.

Farming of cod is relatively new compared to farming of other species and therefore the farming conditions have not been optimized yet, which permits at present to differentiate farmed from wild cod. Farmed Atlantic cod has a body morphology different from wild-captured cod. The most prominent differences are the higher condition factor, larger liver and smaller head [Gildberg, 2004 and references therein] as well as backbone malformations in farmed specimens. Farmed, but not wild, cod often present as well unattractive black lines in the fillet due to deposition of melanin in muscle and blood vessels. Cooper and Midling [2004] have examined the role of tyrosinase on these melanin depositions without finding a clear answer. The flesh of farmed cod presents a translucent greyish aspect, in contrast to the white opaque color of the wild. Farmed cod flesh also has a higher water content and is less firm than the flesh of wild cod. Finally the liver in farmed cod is much bigger than the liver of wild fish [Jobling, 1988].

Part of the farming success consists in reaching a high survival rate from fertilization of eggs until the fish reaches a commercially interesting size. In nature, however, most of the eggs would not reach maturity. Farming is therefore permitting the survival of fish that would otherwise be selected for destruction, for example if they had abnormal development. It is not known yet whether some of the malformations noticed in farmed cod are because the fish is a carrier of genetic malformations or because the farming conditions are not optimized, for example by selecting the correct photoperiod, temperature, exercise regime and physical characteristics of the feed and chemical composition of the diet.

1.2 Individual tagging of fish

Individual tagging of fish would be optimal to differentiate farmed from wild fish and to identify the farm where the fish were bred. There are several types of tags commercially available manufactured using different technologies that may carry variable amounts of

information. Unfortunately, the process of tagging itself implies extra costs to the breeder, and when escaped fish is identified, the farmers received very high penalty fees. Therefore implement of this type of technology is not extremely popular. The situation would be reversed in fish from some particular farm or region could achieve higher market prices, which may be the case in some European regions, such as Scotland and France.

1.3 Genetic analyses

Genetic analysis have traditionally been based in the analyses of proteins and more recently of the DNA [Hoelzel, 1992]. Doyle *et al.* [1991] proposed that the genetic diversity of aquacultured stocks of fish should be maintained and their genetic impact on wild stocks minimized by using breeding programs designed to generate genetic diversity. If this policy had been followed it would be relatively difficult to find markers for wild and farmed fish, since diversity would be one of the selected traits in the farmed fish. However, in most breeding programs the fish are indeed selected based on commercially interesting traits such as growth performance [Friars et al, 1995; Herbinger *et al.*, 1999] and resistance to diseases or to stress [Fevolden *et al.*, 2003]. Moreover, in the later years research has been carried out to find genetic markers, usually DNA markers, for traits of interest. Indeed, effort is being invested in mapping the whole genome of salmon and cod with the intention of optimizing the farming of these species.

Analysis of protein isoforms also has the potential to generate genetic markers. In addition to some of the very polymorphic enzymes commonly used for population genetic analyses [Hoelzel, 1992; Manchenko, 1994], many proteins, including most or all myofibrillar proteins are present as isoforms. In particular the isoforms of the myosin light chains are very polymorphic and they can be used to identify the species and tissue [Martinez *et al.*, 1991;1990a, 1990b] as well as the breeding stock [Martinez and Christiansen, 1994; Martinez *et al.*, 1990c] and the developmental-stage [Martinez *et al.*, 1991].

It is theoretically possible to differentiate wild from farmed fish based on these markers with a relatively high probability of making correct classifications. However, this requires that the markers should be identified and collected in a database to which the public or at least some laboratories should have access. Such databases are not currently available and need to be constructed.

1.4 Analyses of the fat content

The proportion of body fat in storage compartments in fish correlates with the total amount of fat in the feed and the fatty acid profile of the storage fat (triacylglycerols) reflects the fatty acid composition of the diet [Henderson, 1996; Jobling, 1994, 2004; Jobling *et al.*, 2002; Tocher *et al.*, 2000; Watabe, 1982]. Conventionally farmed Atlantic salmon has a fat content of up to 20%, while in wild specimens it varies from 4-10%.

Diets used for intensive aquaculture have been mainly based on the use of fish meal and oils. This imposes a serious risk to the world fisheries specially that of pelagic fisheries [Naylor *et al.*, 2000] most of which have already reached sustainable limits or are already overexploited and endangered [Sargent and Tacon, 1999]. Therefore, there is great interest in the development of synthetic diets where fish oils and meal are substituted by vegetable oils [Bell *et al.*, 2002,2003; Kaushik *et al.*, 2004; Mooney *et al.* 2002; Nichols *et al.*, 2002; Watanabe *et al.*,

1999] and proteins [Burel *et al.*, 2000; Carter and Hauler, 2000; Gomes *et al.*, 1995a,b; Kaushik *et al.*, 1995]. An additional reason to use vegetable oils to substitute fish oils is the possibility to reduce the load of toxic dioxins (is a generic term given to polychlorinated dibenzo-p-dioxins and dibenzofurans) and PCBs (polychlorinated biphenyls) and PCB-like compounds [Bell el *et al.*, 2005]. PCBs and dioxins are biomagnified as they progress through the food chain, concentrating in the fatty tissues of land animals and fish, thus fish oils should be submitted to an additional purification step to eliminate these compounds and make them safer.

Changes in the amount and composition of the feeds are reflected in the amount of storage fat and its fatty acid profile [Mooney *et al.*, 2002; Nichols *et al.*, 2002]. Thus, while the fatty acid profile in wild fish will be dictated by the composition of the natural prey [Jobling, 2004 and references therein], farmed fish will reflect the vegetable and fish oils contained in the feed. Accordingly, the fatty acid profile in the storage tissue, which is muscle in salmonids but liver in cod [Jobling, 1988] may be used as a marker to distinguish between farmed and wild fish [Aursand and Axelson, 2001; Aursand *et al.*, 1994a,2000,2004; Igarashi *et al.*, 2002; Bell *et al.*, 2002; Villarreal *et al.*, 1994]. Fatty acid profiling has also been proposed as a forensic tool for conservation biologists and law enforcement officials to distinguish cultured red drums from illegally marketed wild red drums [Villarreal *et al.*, 1994].

The fatty acid profile of the polar lipids (phospholipids), which are the main constituents of membranes, does not resemble so closely the composition of the feed [Lie *et al.*, 1986; dos Santos *et al.*, 1993] and it has been successfully used to identify the species in canned tuna [Medina et al, 1997]. Fatty acid profiling of several tissues has also proved useful to differentiate among several species of *Sebastes* [Joensen and Grahl-Nielsen, 2000,2001].

Robin *et al.* [2003] and Jobling [2004] have shown that a dilution model could be used to describe the changes in the fatty acid profiles of Atlantic salmon muscle following a change in the dietary fatty acid source and composition. Studies in cod [Morais et al., 2001] indicate that the fatty acid composition of the feed is mainly reflected in the fatty acid profile of the liver in this species, although the fatty acid profile of muscle is also altered. The practical application of the model is the prediction of fatty acid profiles within the fillet in salmon and the liver in cod following a dietary shift.

The fatty acid profile will also indicate the production method (wild, farmed, organic) and give an indication of the natural-to-artificial feed ratio used. Artificial feeds usually have a higher content of typically vegetable oils (C18:2n6, C18:1n9), at the expense of the typical fish oils (C22:6n3, C20:5n3, C20:1n9 and C22:1) which should be the main constituent for wild and organically farmed fish. Analyses of the fat content and composition are therefore necessary to verify claims of ecologically produced fish.

There are several methods suitable to estimate total fat and fatty acid profiles, including GC, NMR and non-destructive methods such as low field NMR and NIR, but the correct classification of samples demands the construction of databases containing information from authentic samples from wild, conventionally and ecologically farmed specimens for each fish species.

Recently, the total amount of omega-3 fatty acids and the content of DHA has been estimated by ^{1}H NMR spectroscopy and this method gave values comparable to those obtained by GC or ^{13}C MR [Aursand *et al.*, 1994b Sacchi *et al.*, 1993]. The analysis can be carried out with a high degree of automation and with short acquisition times (2-5 min). Additional relevant information, however, such as the identification and quantification of individual fatty acids, total saturated, mono- and polyunsaturated fatty acids, omega-3 and omega-6 and the preferential positional distribution of 20:5, 22:5 and 22:6 in triacylglycerols does require ^{13}C NMR analyses [Aursand *et al.*, 1994b]. ^{2}H and ^{13}C NMR isotope ratios have also been used to differentiate wild from farmed Atlantic salmon [Aursand *et al.*, 2000; Aursand and Axelson, 2000] and ^{1}H and ^{2}H NMR spectroscopy to discriminate between essential fatty acids of plant and animal origin [Aursand *et al.*, 1997].

Finally, an estimation of whether a fish has been farmed or wild may also be made by measuring its total fat content. This estimation may be invalid in the case the fish was originally farmed but escaped (it will show a low fat content) or in the case of organically farmed fish, that should resemble the fat content and profile of wild individuals.

1.5 Analyses of the protein/enzyme profiles in some tissues

As already mentioned, fish feeds are being developed that contain protein of vegetable origin to substitute fish meal proteins [Burel *et al.*, 2000; Carter and Hauler, 2000; Gomes *et al.*, 1995a,b; Kaushik *et al.*, 1995]. The source of protein is very important because most teleost fish species are adapted to use protein as a preferred energy source over carbohydrate, and thus require high levels of dietary protein (30–60%) [Cowey, 1995]. The essential amino acid requirements of fish correlate well with the amino acid composition of the whole animal and to a certain extent that of the muscle tissue alone [Mambrini and Kaushik, 1995; Wilson and Cowey, 1985]. The use of plant proteins in feed diet formulations requires the supplementation with synthetic amino acids, because the amino acid profiles of plant proteins do not meet the essential amino acid requirements of fish [Krogdahl *et al.*, 1994]. Also, plant ingredients contain different antinutritional factors of different nature and at different concentrations having adverse effects in fish [Francis *et al.*, 2001; Krogdahl *et al.*, 1994; Moyano *et al.*, 1991; Vielma *et al.*, 2002,2002].

Martin *et al.*, [2003] used a proteomics approach to study the protein profiles of livers of rainbow trout that have been fed diets containing different proportions of plant -soy- ingredients and found that growth rates of fish were not altered by the dietary treatments, although protein consumption was greater for fish fed diets with higher amounts of soy protein. Their work led to the identification of 33 proteins including heat shock proteins, enzymes, fatty acid binding proteins and structural proteins that were differentially expressed between the livers of fish fed diets with lower and higher amount of soy protein. Two structural proteins, keratin II and tubulin, were down-regulated in fish fed the diet higher in soy protein; reflecting the animals' increased requirement for energy metabolism and thus, less energy available to synthesis of structural proteins, when fed the diet high in soy protein. Several heat shock proteins (HSPs) were down-regulated in fish fed the diet high in soy protein which may impair the ability to grow efficiently in these fish. Also several enzymes involved in anabolic metabolism, such as phosphogluconate dehydrogenase, pyruvate kinase, adenosylhomocysteinase and hypoxanthine–guanine phosphoribosyltransferase were down-regulated in fish fed the diet rich in soybean meal, which reflects the increased emphases on catabolism relative to anabolism in

the fish fed this diet. Aldolase B protein was increased in abundance in the fish fed the diet rich in soy, which is most likely a reflection of increased metabolism and general turnover of proteins that is occurring in the fish fed this diet and energy demand in these fish. The most likely reason for altered metabolism was considered to be the co-purification of soy protein with vegetal antinutritional factors such as phytoestrogens, antigenic agents and many other compounds such as phorbol esters. Similar diverse alterations in gene expression have been shown in rats fed soy protein extracts [Iqbal *et al.*, 2002].

The conclusion of this work is that a proteomics analysis of certain tissues of the fish can be used to estimate the ingestion of artificial feeds, an indication that the fish had been farmed and are not wild. This approach has therefore the potential to discriminate as well between organically and intensively farmed fish. In order to apply it systematically to discriminate farmed from wild fish, one would have to map first the changes provoked by the actual diets used, and identify the differentially expressed proteins. Once the proteins are identified, it is possible to simplify the analysis by targeting only those proteins, so that it would be possible to perform immunological tests, which are easier to perform, and many of them can be made semi-quantitative and in a portable format.

A-2. Identification of the geographic origin

For certain foodstuffs (cheese, wines, ham) it is common to be described, or named, according to the particular region where they come from, and very often this description goes hand in hand with an expected quality and price. European legislation has been developed for the protection of geographical indications and designations of origin for agricultural produces and foodstuffs (EC No 2081/92). Analytical tests to determine the region of origin have been established for wines: ^{2}H SNIF[1] NMR is the official EU method to identify the origin of wines and detect falsifications (Official Journal of the European Communities (1990) Vol. 33, L272.). An integrated project financed by the EU is currently aiming at developing and standardizing analytical methods to confirm the geographical origin of several foodstuffs but not fish. Salmon was the subject of the recently finished research project COFAWS: Confirmation of the origin of farmed and wild salmon and other fish. Project GRD2-2000-31813.

The European legislation establishes that the FAO area (Table 1) in which wild fish was caught should be part of the information available to consumers [EC regulation No 2065/2001 of 22 October 2001]. The FAO areas are wide regions and very often marketing is made based on a narrower and even local area where the fish comes from, because consumers usually appreciate more and are willing to pay higher prices for their local products.

Table 1. Catch area and identification of the area. [Commission Regulation (EC) No 2065/2001 of 22 October 2001 Laying down detailed rules for the application of Council Regulation (EC) No 104/2000 as regards informing consumers about fishery and aquaculture products. Published in the Official Journal of the European Communities. Date: 23.10.2001. Pages: L278/6 to L278/8)]

Catch area	Identification of area ([1])
North-West Atlantic	FAO area 21
North-East Atlantic([2])	FAO area 27
Baltic Sea	FAO area 27.IIId
Central-Western Atlantic	FAO area 31
Central-Eastern Atlantic	FAO area 34
South-West Atlantic	FAO area 41
South-East Atlantic	FAO area 47
Mediterranean Sea	FAO area 37.1, 37.2 and 37.3
Black Sea	FAO area 37.4
Indian Ocean	FAO area 51 and 57
Pacific Ocean	FAO area 61, 67, 71, 77, 81 and 87
Antartic	FAO area 48,58 and 88

([1]) FAO yearbook. Fishery Statistics. Catches. Vol. 86/1. 2000
([2]) Excluding the Baltic Sea

Another reason to establish methods to confirm the geographic origin lays in the quality, or toxicity of the fish. Fish may be marketed according to how pristine and unpolluted the farming region is perceived by the customers. Reports indicating high levels of environmental pollutants (lead, cadmium, dioxins or PCBs) in certain areas and lack of toxic compounds in others [Hites *et al.*, 2004] may make the customers even more aware and demand more accurate information about the geographic origin of fish. It is evident that as long as there are no methods to

[1] SNIF: Site-specific natural isotope fractionation

demonstrate the geographic origin, false labels will be found in the markets offering fish from less attractive areas labelled as their more expensive counterpart.

2.1 Genetic analyses

Genetic analyses are useful to discriminate geographic origin of fish as long as different populations may be discriminated by these analyses and also segregate in space. As already mentioned, traditional genetic studies are based on the analysis of isoenzymes and more recently on the DNA [Hoelzel, 1992]. In the case of cod it is important to discriminate fish from protected endangered regions (Northwest Atlantic, the Baltic) and that from Barents and the Norwegian coastal fisheries. But it is the fish from Lofoten the one with the highest reputation and price. Fevolden and Pogson [Fevolden and Pogson, 1997; Pogson and Fevolden, 1998] showed profound allele frequency differences at the pantophysin (PanI) between coastal and north-east Arctic populations of cod ("skrei") in northern Norway. However, whereas the divergence between coastal and north-east Arctic populations is indisputable there may be less variation between Norwegian and Icelandic cod at this locus. Other markers like microsatellites are presently being used to discriminate those stocks.

Using RAPD fingerprinting, we have been able to find genetic markers to discriminate minke whales captured in the Atlantic from those captured in the Pacific [Martinez and Pastene 1999], but not between different areas in the Northeast and central Atlantic [Martinez *et al.*, 1999], although probably the use of more primers would eventually have produced population-specific primers. However, in migratory species such as the minke whale, it is possible that different populations can be found in a given area at different periods of the year [Pastene *et al.*, 2003], so the genetic stock would not indicate the geographic origin unless also the date of capture was known.

In any case, the usually high gene-flow between populations in highly abundant and widely distributed marine fish species [Waples 1998; Ward *et al.*, 1994] makes it difficult to find genetic markers to unequivocally identify the breeding stock as well as the geographic origin.

2.2 Analyses of the protein/enzyme profiles in some organs

As is the case for DNA analysis, proteins can be used as markers for the geographic origin when the protein expression is genetically determined and different allozymes are expressed in different stocks and different locations which is the classical approach used in population genetic studies [Drengstig *et al.*, 2000; Galand and Fevolden, 2000; May, 1992, Mork and Giæver, 1999]. In addition, we found that the structural myofibrilar proteins called myosin light chains have the potential to serve as genetic markers in the herring [Martinez *et al.*, 1990c] and the Arctic charr [Martinez and Christiansen, 1994].

Protein expression can also be used as indicator of geographic origin in those cases where certain compounds in the environment impose a stress on the organism that forces the expression of certain proteins. Novel applications of proteomics such as the characterization of changes induced in alive organisms by environmental pollutants add new possibilities to using this set of techniques to ensuring correctly labelled and safe seafood. Thus, several works [Rodriguez-Ortega, 2003; Rodriguez-Ortega *et al.*, 2003; Shepard and Bradley 2000; Shepard et al, 2000; Olsson *et al.*, [2004] have identified protein markers in molluscs that can be used as indicators of the type and level of pollutant in the environment in which these organisms are

reared. These findings may help to identify the origin, if it is a polluted region, and to ensure that only specimens coming from clean areas reach the consumers.

There are yet no validated techniques in this group that can be systematically applied to identify the geographic origin, or the pollutants in the environment. These works are at the research stadium.

2.3 Analyses of fat and oils

The composition of fatty acids in the lipids of selected fish tissues appears to be population dependent in some species. Thus as for genetic analysis, they can also be of value to identify the geographic origin when the populations discriminated by these analyses also segregate in space. Fatty acid profiling of heart tissue by gas chromatography permitted the differentiation among populations of herring [Grahl-Nielsen and Ulvund, 1990] and red fish [Joensen and Grahl-Nielsen, 2004] in the Atlantic, striped bass stocks in American rivers [Grahl-Nielsen and Mjaavatten, 1992] and to between two stocks of cod reared under identical conditions on the Faroe Islands [Joensen *et al.*, 2000]. The method has been included in the ICES Stock Identification Methodology [Grahl-Nielsen, 1997]. As previously mentioned, fatty acid profiling of several tissues has also proved useful to differentiate among several species of *Sebastes* [Joensen and Grahl-Nielsen, 2000,2001] for which protein [Nedreaas and Nævdal, 1991a,b; Nedreaas *et al.*, 1994; Rehbein 1983] and DNA analyses [Roques *et al.*, 1999a,b; Sundt and Johansen, 1998; Danielsdottir, 1998] did not render clear cut results.

The reports by Mooney *et al.* [2002] and Nichols *et al.* [2002] give the fatty acid composition of several Australian seafood, including that of Atlantic salmon, a species native of the Atlantic introduced in Tasmania some years ago. Comparison of the fatty acid profiles of oils from Atlantic salmon from different locations in the Atlantic and the Pacific permitted the identification of the origin, in our opinion reflecting that of the feed available at different locations [Aursand *et al.*, 2004].

^{1}H and ^{13}C NMR spectroscopic studies of lipid extracts of muscles and industrial fish oils have proved effective at providing a fingerprint of the specific lipids and oils, including fatty acid composition, lipid classess and the positional distribution of mono-, di- and polyunsaturated fatty acids in the triacylglycerides. The latter parameter seems to be unique for each species and therefore of potential value for species identification [Aursand *et al.*, 1995a,b]. In addition, the water contained in the muscle tissue of fish reflects the aqueous environment in which the fish has been harvested. The two isotopic parameters that can be determined ^{2}H and ^{18}O NMR, seem to be strong candidates as indicators of the geographical origin of harvest. There are however, no standardized official methods to verify the geographic origin of fish, which has been one of the aims of the European project COFAWS (EC, DG RTD, Measurements and Testing, contract number G6RTD-CT-2001-00512), namely to develop methods for the authentication of the origin of salmon and salmon based products and oils. The five partners in the project tested the suitability of a series of analytical techniques including ^{1}H-NMR, ^{2}H-NMR; ^{13}C-NMR; ^{13}C-IRMS; ^{18}O-IRMS, GC and GC-IRMS. Rezzi *et al.* [2003] were able to discriminate the geographic origin (Scotland., Iceland, Norway) of oils obtained from farmed Atlantic salmon using ^{1}H-NMR. The results of this project have not been published yet.

2.4 Stable isotope analysis: NMR

Stable isotope analyses is considered to be an excellent tool for origin assesment: the ratio $^{13}C/^{12}C$ gives straightforward responses concerning the primary photosynthetic metabolism of plant products [O'Leary, 1981], and the ratios of the stable isotopes of oxygen ($^{16}O/^{18}O$) and hydrogen ($^{2}H/^{1}H$) are good indicators of environmental conditions [Ziegler, *et al.*, 1976]. The two main techniques used to determine the isotope ratios of natural products are isotope ratio mass spectrometry (IRMS) and site-specific natural isotope fractionation studied by nuclear magnetic resonance (SNIF-NMR). IRMS has the advantage over NMR that all except 12 elements can analyzed by the technique.

Regarding the analyses of hydrogen isotopes, NMR has the advantage over IRMS that the natural abundance of ^{2}H isotopomers may be precisely and accurately quantified by SNIF-NMR [Martin and Martin, 1991], whereas IRMS only gives a mean value of the deuterium content of a given chemical species. In the 1980´s, Site-specific Natural Isotope Fractionation (SNIF) NMR was applied to detect adulteration of wine [Martin and Martin 1988,1991; Martin *et al.* 1988] and today this method has been adopted as an official European method for authentication of wine [Official Journal of the European Communities, 1990] and an AOAC approved technique for control of sugar addition in fruit juices [Martin *et al.*, 1996].

Significant differences have been found in the non-statistical distribution of ^{2}H analysed by NMR, that can be used to detect Atlantic salmon from different sources [Aparicio *et al.*, 1998, Aursand and Axelson, 2001; Aursand *et al.*, 1995a,2000]. Also the "fingerprint" that results from the chemical shift position and peak height of ^{13}C NMR spectra of lipids has been used to identify the species and origin of purified marine oils [Aursand *et al.* 1995a,b]. Using GC, IRMS, and high-resolution ^{2}H SNIF-NMR spectroscopy to study different kinds of fish oils and lipids extracted from muscle of wild (Norway) and farmed (Norway and Scotland) salmon it was possible to correctly classify all samples [Aursand *et al.*, 2000]. Interestingly, while Norwegian and Scottish farmed salmon were clearly differentiated, there was no significant difference between the $(^{2}H/^{1}H)tot$ isotope ratios of feeds from Norway and Scotland although the feeds were correctly assigned to their corresponding fish group, *i.e.*, Norwegian or Scottish. Therefore the differentiation between farmed salmon from Norway and Scotland does not seem to be directly related to the composition of the feed-stuffs, it may mainly depends on the ^{2}H distribution in the fatty acids. On the other hand, the authors were not able to attribute the differences observed between farmed and wild salmon to the diet (the wild diet being unknown) and concluded that differences in the biological activity of farmed and wild fish might be called upon and considered as a factor with discriminator value between the two groups [Aursand *et al.* 2000].

2.5 Stable isotope analysis by IRMS or trace element signature analyses

As mentioned above, IRMS has the advantage over NMR that all except 12 elements can be analyzed by the technique. Trace element signatures can then be used to identify the geographical provenance of a sample because organisms accumulate in their tissues, from the water, food and air, the elements available from the environment where they live. Differences in the isotope distributions of the trace element among different geographical locations gives a different "signature" of isotopes in the organism. This has been used to distinguish between Atlantic and Mediterranean tuna [Secor *et al.*, 2002], different spawning aggregations of cod

[Campana *et al.*,2002] and salmon parr from 14 rivers feeding into the Trondheimsfjord in Norway [Lierhagen, unpublished results, Norwegian Institute for Nature Research, Trondheim].

Otolith microconstituent analysis has been applied recently to study stock structure [Campana *et al.*, 1994,1995; Edmonds *et al.*, 1989; Proctor *et al.*, 1995] and migration rates [Secor, 1992; Secor and Piccoli, 1996] in a variety of fish species. The premise of this approach is that trace elements are incorporated into otoliths in direct proportion to their availability in surrounding water or food. Few laboratory experiments have been conducted to verify the assumption that otoliths can record environmental histories, but such studies have supported this assumption for uptake of strontium [Farrell and Campana, 1996; Fowler *et al.*, 1995; Limburg, 1995; Secor *et al.*, 1995;]. Physiological factors, temperature, and genetics may also affect the uptake of specific elements into otoliths [Kalish, 1989; Thresher *et al.*, 1994].

In a study of bluefin tuna to differentiate fish from the Atlantic and the Mediterranean, larvae or young-of-the-year exposed to either the Gulf of Mexico or Mediterranean waters were expected to incorporate different mixtures of elements into their otoliths [Secor and Zdanowicz, 1998]. The authors found that the analyses of microconstituents measured in whole otoliths of juvenile northern bluefin tuna (Thunnus thynnus) from the Mediterranean Sea and western Pacific Ocean analyzed by inductively coupled plasma mass spectrometry and multivariate statistical analysis showed clear separation between the two groups and that sodium (Na), magnesium (Mg), manganese (Mn), and zinc (Zn) were most useful in differentiating Mediterranean and Pacific samples [Secor and Zdanowicz, 1998]. Most of these works are based on the analyses of otholites and/or scales [Campana, 1999; Campana and Thorrold, 2001], but Born *et al.*, [2003] have recently shown the approach is also valid when applied to soft tissues: their study determined regional variations in long-term elemental diagnostics of stock differences among 159 minke whales harvested in West Greenland, the Northeast Atlantic Ocean and the North Sea in 1998 and the diagnostics tested included mercury (Hg), selenium (Se) and cadmium (Cd), ^{15}N and ^{13}C and stable lead (Pb) isotope ratios in muscle liver and kidney and the trace and major element composition of baleen.

Hobson has recently published a review illustrating the use of stable isotopes, primarily $\delta^{13}C$; $\delta^{15}N$; $\delta^{34}S$; $\delta^{2}H$ and $\delta^{87}S$; to trace nutritional origin and migration in terrestrial and aquatic animals and in bats.

There are yet no officially recognized, standard or validated methods to identify the geographic origin of fish.

A-3. Content of some bioactive components

The reason to include the content of some selected bioactive "nutritious" components is because seafood may be marketed based on these components. In particular the content in marine polyunsaturated fatty acids (PUFAs), specially docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), are the basis for certain classical products such as cod liver oil and the more modern "nutraceuticals", such as capsules containing fish oils. Frauds regarding inconsistencies between the species declared and the content as well as the quality of the oils in the capsules have been noted by several research works, although none have been published. The authorities need standard procedures suitable for this type of analysis that can be used to detect frauds and stop fraudulent and health-damaging compounds (oxidized and polymerized fats) form reaching the consumers.

3.1 Polyunsaturated fatty acids

The nutritional benefits of fish and fish oils were first suspected and later confirmed in the early 70's by Bang and Dyerberg as a result of their comparative studies on the morbidity of cardiovascular diseases among Greenland Eskimos and Danish inhabitants in Greenland whose diets were very different: the former with a high intake of marine lipids from fish, seal and whale and the later with a more western diet with higher proportion of saturated fats [Bang and Dyerberg, 1973; Dyerberg *et al.*, 1975, 1978; Dyerberg and Bang, 1979]. Similar results came from studies on Japan where the consumption of fish and whale products are also high [Yamori *et al.*, 1985]. Many studies have followed, generally focused on the level of omega-3 (n-3) fatty acids, primarily EPA (20:5n-3) and DHA (22:6n-3). They have shown that consumption of marine oils diminishes risk factors implicated in the pathogenesis of atherosclerotic and thrombotic diseases [Connor 2000; Dewailly *et al.*, 2001; Harris, 1989; Nordoy *et al.*, 1993,2001; Phillipson *et al.*, 1985; Simopoulos *et al.*, 1991]. It has also been shown an inverse relationship between fish consumption and death from ischemic heart disease [Bang *et al.*, 1976; Burr *et al.*, 1989; Kromhout *et al.*, 1985; Oomen *et al.*, 2000; Schmidt *et al.*, 2000] and there is general consensus that consumers should increase their intakes of these PUFAs [Anon 1994; Simopoulos *et al.*, 1999].

As a consequence many nutraceuticals have been commercialized in the last few years claiming to contain marine oils, or omega-3 fatty acids, occasionally also the species from which the oil is produced is given on the label. However both our and other researcher's works (personal comunications) have shown that the oil contained in this capsules does not always come from the claimed species [Aursand *et al.*, 1999] and that it may contain additional products derived from the production of the oil as well as a results of the oxidation and autoxidation: marine oils are namely highly susceptible to oxidation because of the very same fatty acids that make them healthy when they are intact, EPA and DHA.

The standard analysis of fatty acids is by gas chromatography, although more recently the use of NMR-based techniques seems to be increasing. GC does not seem to be able to identify the species from which the oils were produced, while the distribution of ^{2}H analysed by NMR can be used to detect Atlantic salmon from different sources [Aparicio *et al.*, 1998, Aursand and Axelson, 2001; Aursand *et al.*, 1995a,2000] and ^{13}C NMR profiling of lipids produces a “fingerprint” useful to identify the species and origin of purified marine oils [Aursand *et al.*,

1995a,b]. ^{1}H NMR has also been recently tested as a new method to identify oxidation products derived from DHA [Falch *et al.*, 2005].

3.2 Taurine, betaine and anserine

In addition to the lipids, there are other molecules in seafood that are also bioactive components: one such group is made up of osmolytes, small water-soluble molecules that may be methylamines, polyalcohols, sugars and amino acids and amino acid derivatives [Arakawa and Timasheff, 1985; Burg *et al.*, 1997; Konosu and Yamaguchi, 1982], and whose main function is to stabilise the structure of proteins [Arakawa and Timasheff, 1985], protect cells against osmotic stresses [Burg *et al.*, 1997] and protect lipids from oxidation [Undeland et al, 2002].

Being water- (and saliva-) soluble, organic osmolytes are the substances that give the characteristic flavour to the foods. Each species and tissue seem to have a particular qualitative and quantitative osmolyte-profile that contributes to the species-specific taste and has the potential to be used as a reference for product authentication [Konosu and Yamaguchi, 1982, Fan *et al.*, 1993]. However, the osmolyte profile varies according to the season of the year, physiological and environmental conditions, the diet and the post-mortem elapsed time [Konosu and Yamaguchi, 1982], and it has not been fully examined the effect that different processing conditions may have on these desirable compounds. We have decided to include them in this review because they may be of relevance when considering seafood as healthy food. The chosen osmolytes, taurine, betaine and anserine, are known to have positive physiological effects on humans and that is the reason to include these compounds in the present work.

Taurine is widespread in the animal kingdom and the major source of taurine for maintenance of the large intracellular taurine pool is the diet. Taurine is found in very high concentration in excitable tissue, such as muscle and nerve. It regulates an unusual number of biological phenomena, including heart rhythm, contractile function, blood pressure, platelet aggregation, neuronal excitability, body temperature, learning, motor behaviour, food consumption, eye sight, sperm motility, cell proliferation and viability, energy metabolism and bile acid synthesis [Schaffer *et al.*, 2000a and references therein]. Correspondingly, cellular depletion of taurine has been linked to developmental defects, retinal damage, immunodeficiency, impaired cellular growth and the development of cardiomyopathy. Taurine has been used as a therapeutic agent in cardiovascular diseases, including heart failure and arrythmia, for over 40 years [Read and Welty, 1963, Schaffer *et al.*, 2000a] and in the treatment of liver diseases such as acute hepatitis for over 20 [Nakashima *et al.*, 1983, Schaffer *et al.*, 2000a]. Taurine is an effective inhibitor of certain actions of angiotensin II [Azuma *et al.*, 2000; Schaffer *et al.*, 1998, 2000a], in particular on Ca^{2+} transport, protein synthesis and angiotensin II signalling. These findings have encouraged the use of taurine in infant formula, nutritional supplements and energy promoting drinks [Schaffer *et al.*, 2000a and references therein]. It would therefore be very interesting to document the amount and fate of taurine in seafoods from different origin and/or submitted to different processing conditions.

Betaine (trimethylglycine, also called glycinbetaine) is abundant in crustaceans and mollusks, especially in octopus [Konosu and Yamaguchi, 1982] and it functions as a methyl donor together with choline, folic acid, vitamin B12 and the methionine derivative S-adenosylmethionine (SAMe) [Selhub, 1999; Barak and Tuma, 1983]. Betaine is important for

proper liver function, cellular replication, and detoxification reactions and, in addition, plays a role in the synthesis of carnitine and serves to protect the kidneys from damage [Chambers, 1995; Chambers *et al.*, 1999; Peddie and Chambers, 1993; Soderling *et al.*, 1998]. It has been shown to be helpful in certain rare genetic disorders involving cysteine metabolism [Barak *et al.*, 1996; Gahl *et al.*, 1988; Selhub, 1999; Urbon Artero et al, 2002; Wendel and Bremer, 1984]. Betaine is often referred to as a "lipotropic factor" because of its ability to help the liver process lipids. In animal studies, betaine supplementation has been shown to protect against chemical damage to the liver [Junnila *et al.*, 1998; Kim *et al.*, 1998; Barack *et al.*, 1993, Murakami *et al.*, 1998]. Its primary use as a nutritional supplement is in supporting proper liver function and possibly reducing the risk for urinary tract infections.

Anserine, together with carnosine, are the most abundant dipeptides in the skeletal muscle of vertebrates [Chan and Decker, 1994; Crush, 1970; Wu and Shiau, 2002] and its levels are specially high in salmonids [Aursand *et al.*, 1995c; Shirai *et al.*, 1983]. These dipeptides play an important role in physiological functions, they are potent intracellular pH-buffer agents [Abe, 1985; Abe and Okuma, 1991], antioxidants [Abe, 1995; Bolyrev *et al.*, 1988; Chan and Decker, 1994] and they also can activate of enzyme activity [Ikeda *et al.*, 1980; Johnson *et al.*, 1989], act as neurotransmitters [Boldyrev, 2001] and display angiotensin converting enzyme inhibitory (ACEI) activities [Hou et al, 2003].

Classical methods to detect and measure these compounds are by aminoacid analysis using amino acid analyzers or by HPLC [Schönherr, 2002; Shirai *et al.*, 1983; Sotelo *et al.*, 1995; see also Masson, 2002 for a review]. The measured values of some of these small compounds may vary depending on the method used: standard procedures using a classical extraction method and hydrolysis render lower values than those noted by less destructive and newer approaches as ^{13}C or ^{1}H NMR [Aursand *et al.*, 1995c; Gribbestad *et al.*, 2005]. We have recently published the interpretation of the spectra from high resolution ^{1}H NMR spectroscopy of extracts and muscle of whole Atlantic salmon [Gribbestad *et al.*, 2005]: it was possible to identify single chemical components, such as hypoxanthine, amino acids, anserine, lactate and some fatty acids, in extracts, whole muscle and whole fish. Our research group has also recently interpreted the ^{1}H NMR spectrum for cod and where we could identify, among other compounds, taurine, betaine, anserine, lactate, ATP, ADP, AMP, hypoxanthine, trimethylamine oxide (TMAO), trimethylamine (TMA) and dimethylamine (DMA) (Standal *et al.*, manuscript in preparation).

In a recent work [Martinez *et al.*, 2005] we have mapped the changes in the bioactive components found in several cod products by ^{1}H NMR spectroscopy of perchloric acid extracts and multivariate data analysis: the drip-loss resulting from thawing had a high content in these molecules, which indicates their loss from the thawed fillet and should be a reason to demand labelling of all products that had been frozen during their manufacture. In addition we found a great variability in the spectra of the newly-killed unprocessed samples and that rehydrated klippfish had the lowest amount of osmolytes.

A-4. Processing conditions

4.1 Salting and drying

It has been claimed that dried cod (tørrfisk in Norwegian) is like wine and that some years the products can be considered as excellent vintages. The same may perhaps be claimed of salted dried cod. And this is easy to understand: the quality and functional properties of these products depend on the raw material, including availability of food in the wild (what species and how much), prevalence of parasites, water temperature, how the catch has been treated, whether it has or not been frozen, type of salt, etc. [Gallart-Jornet, 2004; Lauritzsen, 2004]. But it must also be kept in mind that the manner in which the drying and salting processes occur influence these properties. Thus, one should not expect that drying in industry scale within drying cabinets with regulated conditions produces the same results than drying in open air under suitable environmental conditions. The latter will give more variable results: some years the conditions of temperature, relative humidity, wind and insects will be such that excellent "vintages" may be produced, others it may be destroyed by high humidity and abundance of insects. As it is the case for wines. The former will produce dried fish of standard quality with little variations assuming that the raw material quality and processing conditions are kept under control.

Similarly, salted and dried fish produced in the traditional fashion, *i.e.*, from newly catch fish opened, salted and dried to such a low water content that permits its storage at room temperature both in Nordic and tropical countries is a safer product, and has different functional properties, than salted-dried fish industrially produced from frozen-thawed raw material salted by brine immersion or by injection [Barat *et al.*, 2003; Lauritzsen, 2004] to give a product with a final higher water content. When of high quality the former produces the most desirable foods, with longer shelf lives and also gives the highest quality dishes according to traditional southern European recipes, as shown in a study by AZTI [2001]. Newer processes have been developed usually trying to render a standard quality, decrease production time and increase profits, including higher yields and water content [Lauritzsen, 2004], which have as a consequence an increased safety risk (specially if salting is performed by injecting the fillets with needles), lower shelf lives, need of chill storage, shorter production times and a standard acceptable quality [Barat *et al.*, 2003; Lauritzsen, 2004]. When compared to the authentic traditionally produced salted dried cod, the latter rate lower among consumers after they are cooked [AZTI, 2001]. It has also been shown that production of salted cod from frozen thawed cod, which is becoming a rather common practice [Gallart-Jornet *et al*., 2004] and not mentioned on the label of the finished products, renders products with a lower liquid holding capacity (and therefore less juicy to the palate) after rehydrating [Erikson *et al.*, 2004].

Salting is also an important step in the manufacture of smoked salmon. Cardinal *et al.* [2001] showed that the both the salting method and previous freezing storage of Altantic salmon influenced both the yield of the process and properties of the final product.

In the same manner as customers demand, and receive, information about the different vintages and production methods of wines, they should be entitled to information about the production method of the dried and salted fish they purchase. That information is usually not available and sometimes falsified in order to achieve higher prices. In addition, modifications in the

production method that lead to the manufacture of products with a higher water content needs to be reported to the consumers and health control authorities because often microorganisms that were eliminated by the traditional methods may survive the newer and milder conditions, thus diminishing the safety of the product and constituting a potential health hazard. These products should be submitted to more stringent microbiological quality criteria than the traditional ones.

There are standard analytical methods to measure the water and salt content in food products, but there are none to verify the drying and salting procedures to which these products have been submitted, which are variables that keep a relationship with their organoleptic and functional properties, and their safety and should be part of the information given to consumers.

^{1}H MRI, a non-destructive technique, may have the potential to assess the drying method, our working hypothesis being that fish industrially dried using fast methods will probably have bigger structural pockets, since the water may evaporated too fast for the muscle components (proteins, membranes, etc) to shrink and fill the holes left by the water. We also think that ^{23}Na MRI, equally non-destructive, may be a candidate to estimate some of the salting methods because it permits to visualize the distribution of salt within the fillet [Erikson *et al.*, 2004]. The hypothesis in this case is that salt-dried cod should show an uneven salt distribution within the fillet, with higher salt content in the outside part, while brine salted fillets should have a more even distribution within the meat and muscle salted by injecting salt should present internal pockets with high salt content, in addition to showing structural damage where the needles have penetrated the flesh.

No studies have yet been performed to validate or reject any these hypotheses or to find methods suitable to verify the salting and drying procedures.

4.3 Freezin and thawing

Freezing is a very efficient manner to increase the shelf life of fish, but fresh and frozen thawed products, hardly discernible just by how they look, do have different organoleptic and functional properties, frozen thawed products being usually drier due to their liquid loss and lower liquid holding properties [Erikson *et al.*, 2004; Foucat *et al.*, 2001 and references therein]. Frozen-thawed products are also poorer in bioactive components [Martinez *et al.*, 2005]. Since customers are willing to pay higher prices for fresh fish, the sale of thawed fish mislabelled as it were fresh seems to be an increasing practice.

Duflos *et al.* [2000] compared the suitability of three different methods, namely a physical determination (torrymeter), a physiological examination (eye lens) and three enzymatic assays (α-glucosidase, β-N-acetylglucosaminidase and β-hydroxyacyl-CoA-dehydrogenase) to differentiate fresh from thawed plaice (*Pleuronectes platessa*), whiting (*Merlangus merlangus*) and mackerel (*Scomber scombrus*). None of the methods was suitable for all possible products. The torrymeter was considered to be a reliable indicator but only for whole fish with an intact skin, for fillets they recommended the enzymatic method using the α-glucosidase assay but the fact that the analysis should be accompanied by measurement of the freshness - to avoid confusing a frozen-thawed fish and a fish in an advanced stage of spoilage - makes doubtful the suitability of these approach for real-life cases. Older works [Kim *et al.*, 1987] have also proposed the use of skin conductivity using the Torrymeter to differentiate fresh from frozen-

thawed yellowtail (*Seriola quinqueradiata*) but, as Duflos *et al.* [2000] together with the K-value, to give an indication of freshness.

The potential of near-infrared (NIR) spectroscopy to distinguish fresh from frozen-thawed fish has been examined by Pink *et al.*, [1998, 1999]. Discrimination in these cases was based on the prediction of the levels of dimethylamine (DMA), which is formed from trimethylamine oxide (TMAO) during frozen storage of gadoids. The traditional analyses for DMA are by GC [Keay and Hardy, 1972] or HPLC [Gill and Thompson, 1984] and estimation of the DMA contentby NIR has to be done after calibration and by using chemometric techniques. DMA can be estimated also by ^{1}H NMR from PCA extracts [Martinez *et al.*, 2004,2005]. In any case, this approach is only valid for gadoids, since other species do not form DMA during freezing storage.

Uddin and Okazaki [2004] used the spectral changes that take place between fresh and frozen-thawed samples of horse mackerel in the 1920- to 2350-nm region measured by "dry extract spectroscopy by infrared reflection" (DESIR). In DESIR spectra, the overall absorbance level was found to decrease in frozen-thawed samples, and after application of principal component analysis (PCA) and multiple linear regressions (MLR) the approach was able to classify correctly all the samples analyzed.

Based on the fact that quality deterioration during frozen storage of lean fish like cod is largely due to denaturation and aggregation of muscle proteins, the profile obtained during thermal denaturation of muscle samples is likely to change depending on whether the muscle had been frozen or not. This has led several authors to test the use of differential scanning calorimetry (DSC) as a mean of detecting protein changes due to freezing and frozen storage in fish muscle [see Jensen and Jorgensen, 2003 and references therein]. This approach was not completely successful because the results were influenced by the length of time the fish were chill stored prior to freezing for samples stored at -30^{o}C, and by the length of frozen storage for samples stored at -20^{o}C.

MRI has been satisfactorily used by Nott *et al.* [1999a] and Foucat *et al.* [2001] to distinguish between freshly killed and frozen thawed trout muscle and to reveal the method of freezing and the duration of the storage period. Nott *et al.* [1999b] showed that the approach is also valid to differentiate fresh and frozen-thawed products of cod and mackerel. This is, as previously mentioned a non-destructive technique that permits the examination on whole intact fish.

Because freeze-thawing destroys membranes (thus liberating lysosomal enzymes) and also affects protein structure and activity (freezing denatures proteins) measurement of the enzymatic activity of some lysosomal enzymes in salmonids has been tested as a method to identify frozen fish by Rehbein and Cakli [2000]. The authors found that lysosomal enzyme activities of the press juice of frozen-thawed salmon fish fillets were higher than those of fresh fillets and concluded that the lisosomal enzyme α-glucosidase can be used to differentiate between processed, nonprocessed, fresh and frozen-thawed fish and fillets. One drawback with this kind of approach is that it requires a screening of potential candidate enzymes for each species and analogous enzymes from different species (for example those acclimated to temperate and to very cold waters) can behave very differently and enzymes suitable for one species may not be so for another.

4.5 Heating temperature

Determination of the heating temperature is important, specially in products intended to be consumed without a final cooking step (for example pre-prepared food that only needs to be heated, but not properly cooked, prior to consumption), to ensure that potentially pathogenic bacteria, parasites etc have been killed or rendered uninfective. Methods aiming at determining the heating temperature are based on the fact that proteins denature and/or loose activity and solubility after a critical temperature is reached.

The simplest of these methods is the coagulation test [Townsend *et al.*, 1984]. Samples to be examined are extracted with water or a non-denaturing buffer and the extracts are heated at increasing temperatures: a coagulum will be formed if the sample has not been previously heated at that temperature.

Following the same principle, the loss of protein solubility due to heating can be assessed by examining the proteins extracted by water or non-denaturing buffers by SDSD-PAGE [Uddin, 2001] and, more often, by IEF [King, 1978, our unpublished results]: heated samples will display less proteins than raw unheated samples, the difference being the proteins that have lost their solubility due to heating.

Differential scanning calorimetry (DSC) is also a suitable technique to give information: for example, let us say that the transition temperature (Tmax) for myosin, actin and sarcoplsmic proteins are 43.5 °C, 73.6 °C and 59.3 °C respectively. If an unknown sample is analyzed and it is found out that the peak at 43.5°C has disappeared while the other two remain, it may be concluded that the sample has been heated to a temperature higher than 43.5 °C (since the myosin peak is gone) but lower than 59.3 °C (since the peak corresponding to sarcoplasmic proteins remains).

Uddin [2001] found it possible to detect the maximum internal temperature to which Pacific saury mince had been submitted if it was up to 65°C by the coagulation method and up to 70 °C by SDS-PAGE and DSC analyses. As expected, raw meat showed a wide variety of protein bands in SDS-PAGE with molecular mass between 19 and 53 kDa. However, only a single protein band with molecular weight of 33 kDa was detected after heating at 65°C, probably responsible for producing the coagulum observed at the same temperature. All protein bands disappeared after heating at 70°C. Saury raw meat had four endothermic DSC peaks. As the temperature of heat treatment increased, peaks gradually disappeared and the thermogram of meat previously heated at 70°C was featureless.

Elimination of the enzymatic activity due to denaturation induced by heating has also been tested as a potential indicator of heating temperature [unpublished results of the project *Advanced methods for species identification in seafoods*, partially financed by the European Commission: EU-FAIR project no. PL95-1227]. Of several enzymes tested, we found that for Arctic shrimp (*Pandalus borealis*) only the determination of the β-N-acetylglucosaminidase activity had the potential to be used in the temperature range 50-70°C. In the range 70-80°C the slope was too flat to permit an accurate estimation.

A general problem with all these approaches is that the appearance of coaguli, transition temperature peaks, loss of protein solubility and enzymatic activity are dependant on the whole

history of the product of which prior heating is only one variable. Thus, freshness, storage conditions, freezing, salting [Schubring, 2004; Thorarinsdottir *et al.*, 2002], the environmental temperature to which the animal is acclimated, [Watabe *et al.*, 1997, our unpublished results] and physiological condition [Beas *et al.*, 1991] all of them variables that will affect or modify the degree of protein denaturation and/or protein thermostability and will therefore also interfere with the estimation of any previous heating temperature.

No standard official method exist, several research groups are still trying to develop reliable methods to verify this kind of information.

Part B. Analytical techniques

B-1. Morphological analyses

Fields of application

Sometimes, morphological analyses may be used to differentiate wild for farmed fish, as well as the location of fish when the breeding conditions or population isolation have morphological differentiation as a consequence.

Current morphological tests

As already mentioned, there are currently no official morphological manuals suitable to differentiate farmed from wild fish.

Technical basis

It would be based on comparison of the specimen with a description in a book or manual.

Equipment and materials required

Dissection equipment and visual observation is required.

Strong and weak aspects of the methodology

Such a method would be convenient and relatively easy to use provided the whole fish is available for examination. However, the fact that such test would have to be based on observation, the individual morphological variability - specially within wild individuals - the possibility to modify the characteristics in the farmed ones and the fact that not all farmed specimens present all the characteristics (for example not all farmed fish present deformities, not all salmon have high fat content for example if farmed ecologically, etc), limit the usefulness of such a test, *i.e.*, morphological examination is indeed used to discriminate suspected wild or farmed fish, but there is a need for tests to objectively confirm the production method.

Adequacy for each fish link

It might be used in any fish link where one can examine whole fish, fillets (salmon and cod) or livers (cod).

Bibliographic standards of reference

Currently, none.

B-2. Individual tagging of fish

Fields of application

To differentiate individual fish or groups of fish, depending on the amount of information one wishes the tag should carry.

Current tags in use

Some species are being tagged for research purposes and for limited periods of time, depending on the amount of funding the research receives. In farms, they are currently not implemented.

Technical basis

There are different types of labels. The most primitive consists in implanting a label inside the fish (usually the nose), or clap it on fins or parts of the shell (crabs) in which a code is written. After capture of the fish, the label has to be retrieved by a person who knows where to look for it and how to extract it (their size may be very small). Then it has to be sent by mail to the research group who tagged it or to the producer of the label to be read. Sometimes reading requires the aid of a microscope and a trained person. The information may be sent back to the person who collected the label.

More modern methodologies, based in the use of new materials are more likely to be implemented, as they have already been implemented in pet monitoring. Information of one such company, retrieved form internet says: "*Applications for the Company's products include identification and monitoring of pets, fish and livestock through its patented implantable microchips; location tracking and message monitoring of vehicles and aircraft in remote locations through systems that integrate GPS and geosynchronous satellite communications; and monitoring of asset conditions such as temperature and movement, through advanced miniature sensors. Digital Angel Corporation is majority-owned by Applied Digital Solutions, Inc. (Nasdaq:ADSX). For more information about Digital Angel, please visit http://www.DigitalAngelCorp.com.*"

The so-called RFID; chips as small as dust specks may be placed into products for customer control, although not yet on animals. The chip contains a unique serial number which becomes associated with the purchaser. The chips are sewn into clothing, moulded into the plastic of shaving razors for example, or installed in a myriade of different ways. A scanner can read the serial number as the carrier of the chip walks past. In the case of control of customers, the scanner that reads the serial number of the chip brings up the account which the customer used to purchase the item carrying the chip and all the information linked to it (http://www.spychips.com/).

The Norwegian company VivID AS is involved in the manufacture of ID-chips made of silicon using ultrasound detection. The chips are designed in MicroSystems technology and made by SINTEF (Norwegian Patent NO 315396, granted in July 2003). The chips are implanted in the fatty ventral tissue or in the intestinal cavity with a syringe. The fish must then rest at least 1 day prior to recording of measurements. Additional equipment necessary is: PC, a signal generator, two 250 Khz transducers one amplifier and one oscilloscope. The measurements are

performed by placing the anaesthetized fish in the field of the sender-transducer, which sends a pulse with a given frequency. The reading itself takes 2 milliseconds.

Equipment and materials required

Equipment required includes: the chips or labels, equipment to insert them inside the fish, equipment to read the chip (which varies according to the technology used) or extract the label, and trained personal to read it with a microscope, if the latter.

Strong and weak aspects of the methodology

The procedure is expensive and labour intensive, since each juvenile fish needs to suffer the implant (this is done manually at present). It can only be used as long as the part of the fish with the implant is not separated from the rest, *i.e.*, not useful for fillets if the implant is in the head or in the intestinal cavity. An additional negative aspect of the labels is that they need first to be extracted by a trained person who must somehow keep track of the label and the fish; then they have to be sent by mail to the producer of the label, who reads it and send the information back. The cost of tagging, reading and receive the information back and link it to the fish, after a certain period of time, makes this method time consuming, expensive and impractical for systematic use in large scale.

Positive aspects of both electronic chips and labels are that the implanting procedure does not seem to affect survival of the fish, and the chip and label last for the whole life of the fish. Further positive aspects of the electronic chips are that it is theoretically possible to include a large amount of information in the chip (the limit being the size of the chip), that the information may be read externally without any special training and that that information, being electronic can be automatically linked to any traceability record of the batch.

Adequacy for each fish link

Useful for any link in which the part of the fish being examined contains the implant. If the implant is by an electronic chip it can be performed anywhere, *i.e.* it is a portable analyses.

Bibliographic standards of reference

Currently, none.

B-3. Techniques for genetic DNA-based analyses

Fields of application

Optimally, but not always, for species and breeding stock identification. Its application requires the finding of diagnostic genetic markers: in the case of farmed specimens if they belong to a special or selected breeding stock and in the case of wild fish, when the geographical origin corresponds to selected populations for which it is possible to find genetic markers.

Techniques in use

Hoelzel [1992] has a good description of many of the techniques used in population genetics that can be used to identify the breeding stock, which in turn may be the key to identify the geographic origin of fish. The reader should refer to specialized literature for a detailed description of each methodology.

Technically, most analyses of genetic material will involve first a extraction of the DNA from the sample. The selected method will depend on several factors: number of analyses to be performed, cost, expected yield and type of sample. Methods to extract DNA include commercial kits (NucleoSpin Food from Macherey-Nagel GmB and Co, Germany; Genomic Prep Cells and tissue DNA Isolation kit from Amersham Pharmacia Biotecg, Sweden; Wizard™ DNA Clean-up system from Promega; Dynabeads® DNA DIRECTTM Universal, from Dynal Biotech etc.); robotic stations (BioRobot M96 workstation from Quiagen; RoboSeq® from MWG; MagNA Pure LC from Roche, etc) or in house standarized procedures some of which are developed to avoid the use of toxic components, for example CTAB-extraction [Quinteiro et al, 1998] or salt-extraction procedures [Miller *et al.*, 1988; Martinez *et al.*, 2001] and others use the classic phenol-chloroform extraction method [Sambrook *et al.*, 1989].

If the laboratory can afford it and the samples are not very degraded, the use of robotic stations is to be recommended not only because it permits a larger number of samples to be analyzed simultaneously but also because it should minimize inter-laboratory variations and the risk of human error. Batch to batch variations in the quality of the products (specially if they contain enzymes) should always be kept in mind.

Once the DNA is extracted, the technique to be used should be suitable for the type of tests the diagnostic marker is designed for: hybrization procedures in the case of DNA-probes or amplification and sequencing if confirmation of internal sequences. The latest trends include the use of chips to which diagnostic DNA probes are covalently linked: the extract under examination (with or without prior amplification of the targeted sequence) is hybridized to the chip and the result is read in a special station.

The most important element to use this approach is the availability of probes, specific for the intended target and that do not cross-hybridize with related sequences. The probes would have to be developed to target each of the markers that, as mentioned above, are not currently available. Alternatively, one can fingerprint the unknown sample and compare it to those of reference samples [Martinez and Daniélsdóttir, 2000; Martinez and Malmheden Yman, 1999; Martinez *et al.*, 2001]. For the latter approach there is no need to know any sequence a priori, but one needs to have a good database of reference samples.

Current genetic tests

The principle of these tests is universal. One would need to have available probes or sequences specific for each farmed and wild stock, and there are data on only a few of them, *i.e.*, the species valued by Western countries, while there are thousands of edible species worldwide. Genetic data on wild populations are being provided by research institutions, mostly universities, while databases for farmed fish will have to be constructed and updated by breeders and farmers.

Technical basis

Common to all genetic analysis, the techniques include at least extraction, usually electrophoresis, amplification, hybridization and/or sequencing of the genetic material.

Equipment and materials required

Standard for molecular biology laboratories. More advanced robot and hybridization (chips) equipment might be required, specially if a large amount of samples is to be screened. Efforts are being invested in the development of "Portable laboratories" or "Lab in a chip" mostly using micro fluidic systems that permit the in situ analysis of biological material. Such systems are currently being developed to detect virus or bacteria that impose a serious risk to human health and to fight bioterrorism, although it is to be expected that once the main frame is developed for one application it will soon be adapted to other ones, such as the detection of production methods for foods.

Strong and weak aspects of the methodology

A strong aspect is that methodologies based on the analysis of DNA are easy to perform, robust, permit high throughput of samples are their costs are quite reasonable especially if the reagents needed are prepared in house. Molecular biology equipment is standard is many laboratories and there is no lack of trained personnel in the work market to perform these analyses. Use of kits and robotic stations may increase the price unless a reasonable high volume of samples is processed, which will then decrease the price per analysis and the likelihood of human errors.

Weak aspects include the fact that examination of the genetic material will indicate the pedigree of the donor, not its rearing conditions. Thus, analysis of the genetic material of a wild caught farmed cod will show a match with the wild population from which it originates, and not necessarily give information about where it was caught. Similarly, genetic analysis of Atlantic salmon from Tasmania or Chile might show a close relationship match with the Atlantic populations from which the original breeders came. Also this methodology requires that specific probes and/or sequences and/or authentic reference samples be made available for each farmed and wild stock of fish, which is lacking at present.

Adequacy for each fish link

It might be used in any fish link, only a small sample is necessary, provided it contains sufficient DNA for the required analysis. If portable laboratories are developed it could also be applied in situ in any place of the link (boat, port, processing line, retailer shop, restaurant, etc).

Bibliographic standards of reference

Some references have been given in the above paragraph *"Techniques for genetic analysis*". Suitable methods to be applied will depend on the information available (probes, sequences, commercial kits, etc.) and each of these methods will have its own standard of reference that should be provided by the researchers who made the method available, and the suppliers of equipment and products. A universal manual for molecular biology workers is Maniatis *et al.* [1988].

B-4. Protein analysis for identification of the production method and geographic origin

Fields of application

Usually the same as DNA-analyses: identification of species and breeding stock. Protein (isoenzyme) analyses where the first analyses used in population genetics, although their discriminatory power is smaller than that of DNA analyses due to the less variability found in proteins than in DNA. On the other hand, protein, but not DNA analyses, can be used to identify the tissues included in a product and sometimes the age of the individuals, as well as the species and breeding stock. It may also be useful to identify farmed fish or the geographic origin when these two variables affect the protein expression, for example because different breeding stocks may display different polymorphic proteins and also because the presence of certain compounds in the environment can induce the expression of certain proteins. A typical example of the latter is fish or clam reared in areas polluted by toxic wastes that display the expression of stress proteins [Rodriguez-Ortega, 2003; Rodriguez-Ortega *et al.*, 2003].

Technical basis

Analysis of proteins require first an extraction suitable for the tissue and protein or enzymes under study. Isoenzyme analyses usually require simple equipment: electrophoretic chambers to separate the isoenzymes in their native form and for incubation with suitable substrates. The enzymes are separated on the basis of their electrophoretic mobility, which under these conditions is influenced both by the molecular mass and charge the protein. The composition of the buffers and substrates depends on the activities ones wishes to analyze [Hoelzel, 1992; Manchenko, 1994]. Usually one has to make first a general screening of many enzymes known to be polymorphic in order to select the enzymes that are polymorphic for the given population under study.

Protein analysis can also be used to discriminate breeding stocks if some protein isoforms can be used as markers, which requires a previous screening to find the polymorphic markers. Proteins can be separated according to their isoelectric point by isoelectric focusing, according to their molecular mass by SDS-PAGE and by both criteria by two-dimensional electrophoresis (2DE).

Isoelectric focusing can be performed on water and urea extracts as described by Rehbein *et al.*, [1995] and Mackie *et al.*, [2000] respectively. Extraction with urea and/or some detergents compatible with IEF may be necessary if the samples have been submitted to heat treatment.

Application of SDS-PAGE to species identification is described by Piñeiro *et al.*, [1999] and the same principle and technique are used to analyze stock-specific markers. The more stringent conditions permitted in the extraction of samples that are going to be analyzed by SDS-PAGE makes it possible to analyze samples submitted to a wide variety of processing conditions including boiling [Rehbein *et al.*, 1999], smoking [Mackie *et al.*, 2000] and high pressure processing [Etienne *et al.*, 2001].

2DE is a combination of IEF and SDS-PAGE that permits the use of strong extractants and therefore the analysis of heat treated and processed samples as described by Martinez *et al.*,

[1992]. It is has been useful to detect potential stock-specific markers in herring [Martinez *et al.*, 1990c] and Arctic charr [Martinez and Christiansen 1994] and for determination environmental pollution that can in turn be used to determined the geographical origin [Rodriguez-Ortega, 2003; Rodriguez-Ortega *et al.*, 2003].

Equipment and materials required

Standard for molecular biology laboratories: centrifuges, mixers, electrophoresis chambers, power supplies, stains to visualize the proteins and equipment to preserve the results, either drying cabinets for the gels or photographing equipment. Software suitable for gel analysis is also required, specially if many samples are to be compared.

Strong and weak aspects of the methodology

Weak aspects include that it is a very hands-on technique and require - 2DE in particular - well trained personnel. It also needs of a previous screening of the samples of interest to find the markers that give the information one is looking for. Application of this type of techniques to the issues covered in this review, *i.e.*, cultivation method and geographic origin, is still in the research stadium and therefore there are no fully developed protocols ready to be used: they require first the development of suitable databanks containing data from reference specimens wild and cultivated and from the different regions of interest.

An advantage is that these techniques are very robust if performed correctly, and once suitable markers are found it is very easy to raise antibodies that will allow later to detect the markers using easier methods, such as ELISA-based analysis, or immunochomatographic rapid test such as Lateral flow tests already being used to detect pathogenic bacteria in foods and toxins in water and shellfish, that permit the detection of targets in a few minutes with very few sample handling steps.

Adequacy for each fish link

In the present state of development these techniques can be performed at any link but samples need to be transported to a specialized lab for extraction and analysis.

Bibliographic standards of reference

For IEF: Rehbein *et al.*, [1995] and Mackie *et al.*, [2000]. For SDS-PAGE Piñeiro *et al.*, [1999]. For for two dimensional electrophoresis using ampholines Martinez *et al.*, [1992] and Rodriguez-Ortega *et al.* [2003]. For 2DE using immobilines Molloy [2000] and Ge *et al.*, [2004].

B-5. Gas chromatography

Fields of application

GC has many fields of application but this chapter is limited to the analysis of fatty acids. FA analysis has application in discrimination of wild and farmed fish and in the analysis of marine oils in nutraceuticals.

Technical basis

Gas liquid chromatography separates solutes by partitioning them between a mobile gas phase and a stationary solid phase held on a solid support. Gas-solid chromatography employs a solid adsorbent as the stationary phase.

The first step in the analysis consist in extracting the lipids from the fish, which is usually done by the Bligh and Dyer [1959] method after which the fatty acids need to be methylated to fatty acid methyl esters (FAME) by reaction with BF_3/methanol at 100^{o}C [British Standard Methods of Analysis of Fats and Oils, (1980) BD 684:section 2.34]. An internal standard needs to be added to the extract prior to methylation, so that both the sample and the internal standard are subject to the same treatment.

The sequence of GC separation is as follows: the sample is injected into a heating block where it is immediately vaporized and swept by the carrier gas into the separation column. The solutes are adsorbed at the head of the column by the stationary phase and then desorbed by fresh carrier gas. The sorption-desorption process occurs repeatedly as the sample is moved towards the outlet by the carrier gas. Each solute travels at a different rate through the column and their bands will separate according to the individual partition ratios and the extent of band spreading. The solutes are eluted sequentially in the increasing order of their partition ratios and enter a detector attached to the column exit. In a printer, the signals appear on the chart as a plot of time versus the composition of the carrier gas stream. The time of emergence (retention time) is characteristic for each component and the area under the peak is proportional to the concentration of the component in the mixture. Identification of the components is done by comparison to the retention times of a series of known compounds. This reference or standard fatty acids mix, is prepared by mixing in suitable ratios fatty acids that one may expect to have in the sample.

The conditions for injection, column, elution and detection have to be optimized for each lab and sample. The analysis of salmon lipids by GC has been standardized in during the course of the project COFAWS but they have not been published yet. Similar works however, have been published earlier, see Aursand *et al.* [2000] for a description of this technique applied also to the analysis of lipids extracted from salmon muscle.

Equipment and materials required

A lab equipped to handle organic compounds and waste, such as chloroform and methanol, rotavapor and gas chromatograph. One also needs a suitable standard mix of fatty acids.

Strong and weak aspects of the methodology

It is a classical method that has been satisfactorily used for decades and there are many works published using the technique. One major drawback is that it requires the use of toxic compounds such as methanol and chloroform for the extraction of lipids and the extra care necessary in the handling and elimination of the toxic waste.

If one wishes to confirm that the oils contained in the product indeed come from marine species, or to discriminate wild form farmed fish or identification of the origin, or confirm organic production, one needs to have built up a suitable databases containing the results of FA composition of authentic reference samples. Classification of the unknown is then done by multivariate data analysis.

Adequacy for each fish link

It may be applied to any link of the traceability chain, but the sample needs to be taken to a suitable laboratory for extraction and analysis.

Bibliographic standards of reference

For application t analysis of salmon fatty acid composition see Aursand *et al.*, [2000]. Dobson [2002] presents a review of methods applicable to diverse food matrices, including fish oils.

B-6. NMR spectroscopy applied to the analyses bioactive compounds

Fields of application

As described in part A, NMR methods in combination with multivariate data analysis of the spectra have been applied to the characterization of complex mixtures, identification of the positional distribution of fatty acids in phospholipids, lipid profiling and characterization of metabolite composition and profiling. Of these methods lipid profiling has successfully used to identify the species from which the lipids derive and to discriminate of wild from farmed fish due to the different fatty acid composition of the diets that appears reflected in the storage lipid fraction.

Claims of healthy products, regarding the content in bioactive components such as marine lipids and some small metabolites (taurine, anserine, betaine) can be confirmed also by NMR, since the spectra reflects the total composition of the sample.

Technical basis

For a complete description of the technique, the reader should refer to suitable in depth literature [Friebolin, 1998; Keeler, 2002; Kemp, 1986]. A succinct and clear description can be found in biochemistry books such that authored by Nelson and Cox [2000] on which part of the description given here is based.

Nuclear magnetic resonance is a manifestation of nuclear spin angular momentum, a quantum mechanical property of atomic nuclei. Only certain atoms posses the type of nuclear spin that generates a magnetic dipole, such as ^{1}H, ^{13}C, ^{17}O, ^{15}N, ^{31}P and ^{19}F. Application of a strong, static magnetic field to a solution containing the mixture under examination forces the magnetic dipoles to align in the field in one of two orientations: in parallel (low energy) or antiparallel (high energy). A short pulse of electromagnetic energy of suitable frequency (the resonant frequency, which is the radio frequency range) is applied at right angles to the nuclei aligned in the magnetic field. Some energy is absorbed as nuclei switch to the high energy state and the absorption spectrum that results contains information about the identity of the nuclei and their immediate chemical environment.

^{1}H is particularly important in NMR experiments because of its high sensitivity and natural abundance. ^{1}H NMR spectra can be quite complicated because even a relatively simple mixture may have many different types of ^{1}H atoms, resulting in one-dimensional spectra that may be difficult to interpret in the case of complex biological mixtures that may contains hundreds for different ^{1}H nuclei.

The chemical environment of nuclei tends to modify the magnetic field “seen” by them. It often shifts their Larmor frequency by an amount that is small but detectable. Such “chemical shifts” are usually quoted in parts per million, ppm (multiplication by the operating frequency, n, yields the Hz value). The chemical shift is the difference between the resonance frequency of the moiety (e.g., -CH vs. -OH) and a standard, given by δ. In NMR spectroscopy, this standard is often trimethylsilypropionate (TSP) or tetramethylsilane (TMS).

A convenient way of visualizing the time domain NMR signal, which is known as the free induction decay (FID), is in the frequency domain by Fourier transform (FT). The FT of an FID leads to a "spectrum" with peaks (with different chemical shifts) each of which has a line width that is partially dependent on the T_2[2] and B_o[3] homogeneity (shim).

A major difference between ^{13}C and ^{1}H spectra is that the latter is much less abundant in nature than the former, which makes the spectra clearer and easier to interpret (because there are less peaks) but also increases the necessary acquisition times.

Equipment and materials required

A schematic description if the equipment required is given in Keeler [2002]. In short, a spectrometer consists of:

- an intense homogeneous magnetic field generated by a magnet, which consists of a coil wire through which a current passes, thereby generating a magnetic field. The wire is immersed in a bath of liquid helium to keep its superconducting state;
- a "probe", which enables the coils used to excite and detect the signal to be placed close to the sample. It is a cylindrical metal tube which is inserted into the bore of the magnet. The key part of the probe is the small coil used to excite and detect the magnetization that is held in the top of the assembly in such a way that the sample can come down from the top of the magnet and drop into the coil;
- a high-power radio frequency transmitter capable of delivering short pulses;
- a sensitivity receiver to amplify the NMR signals;
- a digitizer to convert the NMR signals into a form that can be stored in computer memory;
- a pulse programmer to produce precisely timed pulses and delays and
- a computer to control everything and to process the data.

Strong and weak aspects of the methodology

Advantages of NMR techniques over the more traditional high performance liquid chromatography and gas chromatography are that i) the detection is not limited by different properties of the molecules such as polarity or presence of chromophores in the molecule, ii) it renders information about the structure of the compounds from extracts with less sample preparation than HPLC or GC, iii) unexpected and novel molecules will also be detected, iv) the detection of the components is specific and v) all the components are detected simultaneously.

The procedures of sample preparation and extraction are practically identical for NMR spectroscopy and for traditional GC and HPLC techniques, but the targeted molecules do not need to be modified afterwards. The acquisition times required for ^{1}H NMR spectroscopy can

[2] After excitation by a pulse, the net magnetization, which is the vector sum of the magnetic moments of many nuclei, begins to dephase, or spread out by interacting with other spins in a process called spin-spin relaxation. Because of intrinsic molecular differences or inhomogeneities in the magnetic field, the magnetic-moment components precess at slightly different rates. The rate at which they dephase is characterized by a time constant, T_2, which is reflected in the observed linewidths of that moiety.

[3] The magnetic field strength, designated by B_o, reflects the strength of the instrument system's magnet. The field strength is most properly given in units of Gauss (G) or Tesla (T = 104G), although NMR spectroscopists commonly refer to the ^{1}H resonant frequency instead.

be much shorter than those of GC or HPLC but those of ^{13}C may also be longer (about 30 min per sample) in order to obtain good quality spectra. Shorter times may be used at the expense of resolution, which is affordable if the sample is known.

Weak aspects of NMR analyses are: the very high price of the equipment which requires in addition special facilities for its placement and highly specialized and qualified technicians to perform the sample extractions and the NMR analysis. Once the spectra are obtained, interpretation of the results is not straightforward and it needs personnel highly qualified both in spectroscopy to interpret the spectra and in statistics for the classification of unknown samples. Regarding choice of method between ^{1}H and ^{13}C NMR spectroscopy (and provided that both of them are suitable to obtain the desired information) the former are more complicated to interpret due to the larger number of peaks, but the latter require longer acquisition times. Since the instrument-time is expensive, one should consider carefully which of the two approaches is more convenient, *i.e.*, provides sufficient information with a cost as low as possible.

Adequacy for each fish link

It can be applied to samples in any link of the chain, but they need to be extracted and analyzed under strictly standardized conditions, in a suitable laboratory with access to NMR equipment and personnel trained in the interpretation of the results.

Bibliographic standards of reference

There is yet no standardized officially accepted method, although it is possible that some standards may result from the EU-funded research projects COFAWS and TRACE. For suitable references to works applicable to different types of samples the reader may refer to the particular applications cited in the section A of this review, to the recently held congress on application of NMR to food science [Belton *et al.*, 2003] and to suitable textbooks such as those authored by Friebolin [1998], Keeler [2002] and Kemp [1986].

Additional relevant information and didactic descriptions of NMR techniques can be found at http://spincore.com/nmrinfo/ and http://www.spectroscopynow.com.

B-7. Isotope analysis for geographic origin

Fields of application

Determination of geographic origin and discrimination of farmed and wild fish.

Technical basis

The source of information about the technical basis of this technique mentioned here has been taken from http://www.Iso-analytical.com as quoted in "The Forensic Isotope Ratio Mass Spectrometry (FIRMS) Network. Developing forensic applications of stable isotope mass spectrometry", University of Reading, (http://www.forensic-isotopes.rdg.ac.uk/techniq/ techniq.htm).

The stable isotope compositions of elements which are part of a substance, are a function of the origin and history of that substance. That is, two substances which are chemically the same may have different stable isotope compositions if either their origin and/or history differ. Stable isotopes are defined as atoms whose nuclei contain the same number of protons, but a different number of neutrons. All but 12 elements exist as mixtures of isotopes. The proportions of these isotopes can vary greatly.

The technique to analyze stable isotopes is mass spectrometry. A mass spectrometer is an instrument which separates charged molecules by mass. One type of mass spectrometer, called isotope ratio mass spectrometer (IRMS), has been specifically designed to measure the proportions of particular isotopes. An IRMS will be much more precise, but much less sensitive than other mass spectrometers.

The mass spectrometers used for isotopic analysis generally comprise three basic sections; an ion source, a mass analyser and an ion collection assembly. Gaseous molecules are introduced into the ionisation chamber where interaction with a focused electron beam causes electrons to be stripped from the molecules, resulting in the formation of positive ions. The ions are then accelerated out of the chamber, down a flight tube which is placed between the poles of an electromagnet. Here, they are separated according to their mass-to-charge ratio (m/z). The ions are typically collected by a simple collector array consisting of three Faraday cup collectors. In order to carry out IRMS analysis, only pure gases, e.g. N_2, CO_2, or pure gas contained within a carrier gas, can be used.

Two main types of preparation techniques are employed to convert solid, liquid and gaseous samples into pure gases for analysis: Continuous flow elemental analyser (CF-EA) and Dual inlet.

In continuous flow elemental analyser (CF-EA) sample preparation, and subsequent analysis, is carried out on-line in a continuous flow of helium. An elemental analyser is an automated sample preparation instrument in which samples are converted into pure gases via combustion, reduction, and pyrolysis reactions in the presence of catalysts. In dual inlet the samples are prepared off-line. The pure gas is admitted into the IRMS by a variable volume, *i.e.* bellows. A reference gas is also admitted into the spectrometer via a bellows system.

There are other methods of introducing samples to an IRMS exist. One of these involves the attachment of a gas chromatograph. This technique is ideal for analysing samples which are complex mixtures of organic materials held in a solvent. The separation of the compound which is to be analysed occurs during gas chromatography before being converted to a pure gas by a high temperature reactor.

Equipment and materials required

Highly advanced chemical laboratory for sample preparation and IRMS equipment for analysis of samples. For interpretation of the results well developed databases and chemometrics software. It requires databases with the fingerprints of authentic samples and different geographic locations.

Strong and weak aspects of the methodology

It requires a very advanced and well equipped laboratory with expert personnel both for the chemical analyses and for the statistical treatment necessary to interpret the results as well as suitable databases with data from authentic samples. An advantage is that there are commercial companies, such as Iso-analytical.com, from which the technical description has been taken, that perform these analyses commercially.

Adequacy for each fish link

Suitable for all links but the samples need to be taken to the laboratory for analysis and interpretation of the results.

Bibliographic standards of reference

There are no standards of reference to apply this technique to aquatic organisms and there is a need to develop suitable databases of authentic reference samples. Works published than can be used as references for certain species and locations are Secor *et al.* [2002] for tuna; Campana *et al.* [2002] for cod; and Edmonds *et al.* [1989], Secor, [1992], Campana *et al.*, [1994, 1995], Proctor *et al.* [1995] and Secor and Piccoli, [1996] for a variety of fish species and Born *et al.*, [2003] for the minke whale. Aursand *et al.* [2000] used the $^{2}H/^{1}H$ and $^{13}C/^{12}C$ ratios to discriminate wild form farmed salmon.

B-8. HPLC analyses

Field of application

To assess the content of some small molecules, including amino acids and the three addressed bioactive compounds: taurine, betaine and anserine in seafoods.

Technical basis

The first step in all procedures is to deproteinize the sample, which can de done by using trichloracetic acid (TCA) [Konosu *et al.*, 1974; Sotelo *et al.*, 1995; Wu *et al.*, 2002], perchloric acid [Ruiz-Capillas and Moral, 2001; Wolff *et al.*, 1989; Fetterer *et al.*, 2003], ethanol [Shirai *et al.*, 1983], HCl [Massom, 2002] or sulphosalicilic acid [Paccioretty *et al.*, 2001; Schönherr, 2002]. The supernatants containing free amino acids and dipeptides are recovered after centrifugation, neutralized and analyzed by HPLC [Ruiz-Capillas and Moral, 2001; Schönherr, 2002] or by using an automated amino acid analysator [Shirai *et al.*, 1983; Wu and Shiau, 2002]. An internal standard with physicochemical properties as close as possible to those of the sample, with a similar relative retention time and that must not coelute with the amino acids in the sample, should be added as early as possible to the sample in order to map the loss of analyte during the procedure: if no loss occurs then 100% of the internal standard should be recovered at completion of the analysis; if lower, the calculated concentration of amino acids analyzed should be adjusted accordingly. If the recovery of internal standard is too low the whole procedure should be re-examined. Commonly used internal standards are amino acids that are not usually found in nature or that are not present in the food matrices analyzed: nor-leu and β-ala are common for acid hydrolysis and 5-methyl-trp for alkaline digest. In addition to the internal standard, one must also have control samples: samples similar to the one under examination. So if one has different types of food matrices to analyze (raw fish muscle, crustaceans, cooked shrimps, surimi-based products, ready-to-eat fish dishes, etc.) one must have a known control sample for each one of these matrices reflecting the composition of the unknowns. For each batch of similar samples, one should include one control matrix. If one has to analyze different food matrices, then one control for each type of food matrix should be included in order to obtain reliable results.

For detection of amino acids only tyr, trp and phe have significant uv absorption at useful wavelengths and only trp and tyr are fluorescent. Accordingly, in order to detect them all the usual procedure is to label them by derivatization with a substance that absorbs in the visible/uv or that fluoresces. The derivatization can be done before (precolumn derivatization) or after (postcolumn derivatization) HPLC elution.

The mobile phase in HPLC is the solvent being continuously applied to the column and it acts as a carrier for the sample solution while the column is the stationary phase. The sample is injected into the mobile phase and flows through the column with the mobile phase, the components in the sample migrate according to the non-covalent interactions of the compound with the column. The chemical interactions of the mobile phase and sample with the column determine the degree of migration and separation of components contained in the sample. Of the three types of elution (isocratic, gradient and polytyptic mobile phase) gradient is the most common for amino acid analysis. In gradient elution different compounds are eluted by

increasing the strength of the organic solvent and this can be done in a stepwise or linear fashion.

The stationary phase in HPLC is the solid support contained within the column over which the mobile phase flows. The sample is injected into the mobile phase and as it flows with the mobile phase through the stationary phase, its components will migrate according to the non-covalent interactions of those compounds with the stationary phase. The chemical interactions of the stationary phase and the sample with the mobile phase, determine the degree of migration and separation of the components contained in the sample. The most common stationary phases for amino acid analyses are reverse phase and ion exchange. Reverse phase operates on the basis of hydrophilicity and lipophilicity. The stationary phase consists of silica based packings with n-alkyl chains covalently bound. The more hydrophobic the matrix on each ligand, the greater the tendency of the column to retain hydrophobic groups and hyrophilic compounds elute faster than hydrophobic compounds. Ion exchange, on the other hand, operates on the basis of selective exchange of ions in the sample with counterions in the stationary phase. It is performed with columns containing permanently attached charged functional groups where each ion has a counterion attached. The sample is retained by replacing the counterions of the stationary phase with its own ions, and is subsequently eluted by changing the properties of the mobile phase so that the mobile phase displaces the sample ions from the stationary phase.

Ion exchange chromatography is used mainly with postcolumn derivatization and reversed-phase chromatography with pre-column derivatization. Derivatization (by using ninhydrin or *o*-phthalaldehyde with postcolumn derivatization and *o*-phthalaldehyde, 9-fluorenylmethyl or other methods for precolumn derivatization) is necessary for the detection of the amino acids when they elute from the column. A detailed description of methods for amino acids analysis in foods is given by Massom [2002]. Amino acids are identified by their retention times and comparison with internal standards.

Equipment and materials required

Equipment necessary to perform extractions, centrifuges, HPLC or automated amino acid analysator.

Strong and weak aspects of the methodology

Both are standard methods for analysis of amino acids and dipeptides in many laboratories, but implementation in each laboratory demands the optimization of the steps of hydrolysis, chromatography (and selection of the column), mobile phase, gradient and derivatization for the given sample to analyze. General problems associated to these type of techniques is that the sample may be modified due to the extraction procedure itself and the values measured may be lower than the real ones [Aursand *et al.*, 1995c] and that, in order to avoid detection of interfering substances, the amino acids need to be derivatizated in order to be detected by either uv/visible or by fluorescent detection. In particular the harsh conditions required for sample preparation may induce loss of sample, which makes the use of internal standards very important. The internal standards need to be added as soon as possible to the extract (so that the standard suffers the same processes and/or losses than the sample and always before hydrolysis or oxidation) and its physicochemical properties should be as close to those of the sample under study as possible.

Adequacy for each fish link

It can be applied to any link, but the extraction and analyses have to be performed in a specialized laboratory.

Bibliographic standards of reference

For betaine Wolff *et al.*, [1989], for taurine and anserine Konosu *et al.* [1983] and Shirai *et al.* [1983]. A general reference with a review of the technique is Massom [2002].

B-9. Low field NMR and MRI

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Fields of application

Low field NMR is a rapid and non-destructive analytical technique. It is an excellent tool to study changes in the fish muscle and to perform measurements of fat and water contents and of the diffusion constants. Typical applications of the technique include studies of the drip-loss and firmness of the muscle, structural muscle changes as a function of storage time, temperature (freezing and thawing) and follow up of bacterial growth [Bertram *et al.*, 2001a,b; Lambelet *et al.*, 1995]. Methods of rapid quantification of the water and fat content in the fatty fish species have been developed recently [Lambelet, 1995; Pedersen *et al.*, 2001; Sørland *et al.*, 2004] and have the potential of industrial implementation for at-line quality control of the product. A mobile version of the low field NMR analyzer (so called NMR mouse) can be used for fat measurement in live salmon [Veliyulin *et al.*, 2005] [see Figs 1 and 2].

Magnetic Resonance Imaging (MRI) can be used to study different commercial fish salting and de-salting processes. By using ^{23}Na or ^{1}H MRI, sodium or water diffusion in tissues can be studied in vitro. Moreover, the method makes it possible to study salt and water distributions in selected slices of the sample. The images of the distributions may be quantified. By acquiring anatomical images of the same slices, it is for instance possible to evaluate how fat content and distribution affects salt and water diffusion rates.

Technical basis

The NMR instruments make use of the fact that hydrogen nuclei have a magnetic moment which makes them behave like tiny compass needles and at the same time rotate like a spinning top. The hydrogen nuclei are thus said to have a nuclear spin. If these spinning magnetic moments are introduced into a magnetic field they precess around this field direction. The precession frequency is proportional to the magnetic field strength. A short, intensive radio frequency pulse is applied to align all of these tiny magnetic moments so that they become synchronized. The nuclear spins are now in the excited state, and the combined precession of all these spins generate a small but detectable oscillating magnetic field with a certain resonance frequency which induces an alternating voltage in a detection coil. Since the detected NMR response is arising from all the excited nuclei in the sample, the intensity of this signal is a direct measurement of the number of nuclei that are present in the sample. In other words, the NMR signal intensity gives us the content of any particular sort of atoms in the matter. The nuclei are used as sensors of their surroundings yielding a variety of important information about the matter. Both the excitation and detection involve electromagnetic waves in the radio frequency region. The method is very fast, and requires a minimal sample handling, usually only a small sample piece must be cut and placed in the instrument. Some instruments, like the MOUSE are truly non-destructive: The magnet is simply placed on the sample to perform the measurements [Fig. 1]. A typical low field NMR investigation measures the relaxation times of the proton nuclei from the sample. Such relaxation curves may have several exponential components, originating from either a certain type of chemical compound in the sample (for example fat or water) or from different structural phases of the same compound (for example intra- or extracellular water in the muscle). The measured relaxation times can thus give the

semiquantitative information about the distribution of hydrogen atoms in different chemical compounds differentiated by their structural environment in the sample.

MRI relies on the same basic principles where in addition to protons, the sodium nuclei in the sample may be of interest. The images are generated from the acquired NMR data.

Equipment and materials required

Two types of low field NMR instruments exist – a conventional stationary analyzer such as the minispec mq series (Bruker Optik GmbH, Germany) [see Fig. 3] and a mobile analyzer Bruker Professional MOUSE [Fig. 2]. Stationary instruments with different magnetic field strengths and probe sizes, determine the sample sizes, are available. Sample sizes range from small biopsies (10x10x10 mm^3) to larger muscle pieces (50x50x40 mm^3). The mobile NMR MOUSE measures along the surface of samples unrestricted in size: it may be the whole fish.

Unlike the table-top low field NMR instruments, the MRI instruments are large, expensive, require considerable infrastructure and highly skilled personnel and the instruments are usually found in hospitals, universities or research institutions [Fig. 4]. The magnet bore varies considerably. Typically, a fish fillet or a fillet piece can be readily analyzed. However, non-destructive measurements on comparatively large whole fish are possible by using magnets intended for human body examinations. No additional equipment is required for any of the mentioned NMR methods.

Strong and weak aspects of the methodology

Since the NMR methods do not require prior sampling handling or extraction procedures, real-time sample studies can be performed over long time periods looking at development of various processes in the samples. Most of the low field NMR methods are very rapid and have a potential of being implemented at-line. Other advantages of the methodology are that the low field NMR techniques do not demand specially trained staff, need no equipment maintenance and do not require the utilization of toxic solvents or frequent calibration. A weak point is the general low sensitivity of the NMR method which could also require long acquisition times for relatively dry samples. Presence of water (protons) in the samples is essential for using the NMR methods. Interpretation of the relaxation spectra is often non-trivial and can be difficult for complex structures. Restricted sample sizes for the stationary low field NMR equipment can be problematic for inhomogeneous systems and may therefore require many parallel samples to be investigated.

The strong points of MRI are linked to the non-destructive nature of the method. Diffusion and spatial distributions of salt and water can be studied with the 3D-network of the tissues still intact thus avoiding potential sources of error such as sampling and sample preparation. Once the equipment is available, the low sensitivity of the method might be a drawback in some cases.

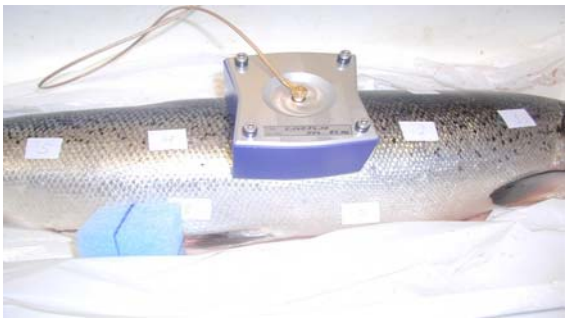

Figure 1. Rapid determination of fat content in whole salmon with NMR mouse

Figure 2. BRUKER Professional MOUSE (Bruker Optik GmbH, Germany)

Figure 3. BRUKER minispec mq series (Bruker Optik GmbH, Germany)

Figure 4. BRUKER Biospec 100MHz NMR imaging system

Adequacy for each fish link

Applicable to any link of production, samples should be taken to be analyzed to a laboratory if the analysis is to be performed with the Bruker minispec. The MOUSE is portable and can be applied on line and in situ. MRI Measurements require that the sample be transported to a laboratory or tank equipped with the required facilities.

Bibliographic standards of reference

The application of low field NMR is still not standard for the fish industry and there are no standarized official procedures. Works relevant to implement the technique are Bertram *et al.* [2001a,b], Lambelet *et al.* [1995] and Veliyulin *et al.* [2005]. For the application of MRI see Erikson *et al.* [2004].

B-10. Determination of the heating temperature

B-10a. Coagulation test

Technical basis

It is based on the fact that proteins loose solubility and form a coagulum when they reach their denaturation temperature. The thermostability of the proteins varies according to the environmental temperature at which they are adapted (or acclimated) and the previous history of the sample.

Equipment and materials required

Equipment to extract proteins, thermostated water bath, centrifuges, spectrophotometer.

Strong and weak aspects of the methodology

It is very easy to perform, but it is not very reliable, since the results are influenced by the previous history of the sample, such as freezing-thawing, salting, and acid treatment, in addition to the prior heating that is the target of the analysis. It is not very easy to quantify the formation of the coagulum, which does not permit a fine estimation of the previous heating temperature.

Adequacy for each fish link

In theory, it may be applied at any stage, as long as it is possible to cook and measure the formation of a coagulum in the extract.

Bibliographic standards of reference

A description of the application of the method can be Towsend *et al.* [1984]. There are no standard methods.

B-10b. Protein electrophoresis

Technical basis

Analysis of proteins require first an extraction suitable for the tissue and protein or enzymes under study. Isoelectric focusing can be performed on water extracts as described by Rehbein *et al.*, [1995]: samples heated at different temperatures are extracted with water and the extracts analyzed by IEF. The number of bands decreases as the temperature increases because denatured proteins loose their solubility. By comparing the IEF pattern of an unknown sample to that of control samples heated up to certain known temperatures it is possible to interpolate the temperature to which the unknown sample had been heated.

Equipment and materials required

Standard for molecular biology laboratories: centrifuges, mixers, electrophoresis chambers, power supplies, stains to visualize the proteins and equipment to preserve the results, either drying cabinets for the gels or photographing equipment and an authentic reference sample that has to be treated under identical conditions to the one under examination.

Strong and weak aspects of the methodology

Weak aspects include that it is a very hands-on technique and require well trained personnel. As the coagulation test, it reflects the total history of the fish, the proteins may have been denatured for other reasons (salting, freezing-thawing, pH conditions, long post-mortem elapsed time, etc.) and the temperature value obtained by this methods is usually a rough estimate.

Bibliographic standards of reference

Manchenko [1994] for a general description of many potential enzymes. For each product there should be a standard. There is no standard for seafood.

B-10c. Enzyme activity

Technical basis

The principle of this analysis is that most enzymes suffer an irreversible denaturation due to heating that is reflected in their loss of activity. Thus, if a given enzyme completely losses its activity at 50^{o}C in a given sample, by testing the activity of an unknown sample it should be possible to determine whether the sample has been heated to a temperature higher than 50^{o}C (if the activity has been lost) or not (if activity remains). In principle any enzyme may be useful and a screening of potential candidates should be done for each sample in order to select the most suitable one. For identification of the temperature, the extract of the sample under examination is compared with a control extract of the same product that is being heating to different temperatures.

There is no one general recipe for this test, since each enzyme requires special substrates, incubation conditions and temperature and the products of the reaction also may require different measuring methods.

Equipment and materials required

Tubes, a thermostated water bather, substrates, controls and a spectrophotometer.

Strong and weak aspects of the methodology

A strong aspect is that it is an easy test and straightforward, if suitable enzymes are found. Drawbacks include the fact that some of these analysis are very rough, so they do no permit an accurate estimation of the heating temperature, only give a wide range: enzymes for cold adapted species (for example *Pandalus borealis*) denature at relatively low temperatures and

therefore estimation of the heating temperature in a range of interest (suitable to kill bacteria, for example over 78°C) is not possible because it has been denatured at lower temperatures.

Another drawback is that, as for the two previous analyses, enzymes may be partial or totally denatured in a product due to many factors, such as loss of freshness, freezing and thawing, addition of salts, changes in the pH, loss of water, proteolysis, etc and not only heat treatment. If activity has been partially lost due to any of these causes, the heating temperature will be overestimated and a product considered for example "safe" (that is has been heated to a sufficiently high core temperature) when perhaps the reason is that the enzyme has been freeze-denatured instead.

Adequacy for each fish link

Suitable for any link but the sample needs to be taken to a laboratory for analysis.

Bibliographic standards of reference

None, the method would need to be developed and standardized for each type of sample and species.

Conclusions

Conclusions

There are currently no standardized analytical methods suitable to verify the production method or geographic origin of seafood. Optimally, farmed fish should be tagged, preferably using micro-devices such as chips, that are easy to read. However that is not enough: the chip may be lost, not inserted or falsified. Therefore, it is imperative to develop analytical methods to verify the information provided by the traceability data, and suitable databases containing the profiles of authentic samples. Parameters that need to be recorded are: lipid, protein and DNA profiles of the fish species from different locations and of their feeds. Suitable techniques are gas chromatography, nuclear magnetic resonance and proteomics. This work has only started for salmonid fish and needs to be done also for other species that have started to be cultivated more recently, such as cod, halibut, shellfish, etc.

Identification of the geographical origin of seafood has been even less investigated than the production method and there is a clear need to develop methods to obtain or verify this information, especially for the protection of fish for certain regions. Stable isotope fingerprinting seems to be one of the most promising parameters and suitable methods are isotope ratio mass spectrometry and ^{2}H site-specific natural isotope fractionation studied by nuclear magnetic resonance.

Analytical methods suitable to verify the content of bioactive components are gas chromatography, high performance liquid chromatography and nuclear magnetic resonance spectroscopy. The latter gives more relevant information that the other two, and also requires less sample preparation which makes it a promising candidate to become one of the dominant techniques for food compositional analyses in the near future. The main problem currently is the high cost of the equipment and need for highly trained personnel.

Freezing and thawing of gadoids may be detected by analyzing the levels of dimethyamine and trimethylamine, but for other species the information is complicated by the fact that all analytical methods used give information on the total history of the product, not only on freezing-thawing. This is one of the data often falsified or omitted in the information given to consumers and that needs confirmation to ensure that the traceability documentation is correct. There are no analytical methods to verify the salting and drying methods or the heating temperature of seafoods. Due to the fact that these variables are of relevance, not only to ensure proper customer information and to verify the traceability data but, more importantly to define how severe a risk these products impose on human health and the stringency of the microbiological criteria to which they should be submitted, it should be relevant for the health control authorities to develop and implement suitable methods as soon as possible. Suitable analytical methods are based on protein analysis and testing of enzymatic activities and on nuclear magnetic resonance imaging and spectroscopic analyses.

Application for all the above methods once they are standardized, demands the building up of suitable databases of authentic products to compare and classify products under examination.

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References

References

Abe, H. (1983) Distribution of free L-anserine and its related compounds in marine fishes. Bull. Jpn. Soc. Sci. Fish., 49: 301-304.

Abe, H. and Okuma, E. (1991) Effect of temperature on the buffering capacities of histidine-related compounds and fish skeletal muscle. Nippon Suisan Gakkaishi, 57: 2101-2107.

Anon (1994) Nutritional aspects of cardiovascular disease: report of the Cardiovascular Review Group, Committee on Medical Aspects of Food Policy. Department of Health London: Her Majesty's Stationery Office, 1994. (Report on Health and Social Subjects no. 46.)

Aparicio, R., MaIntyre, P., Aursand, M., Eveleigh, L., Marighetto, N., Rossell, B., Sacchi, R., Wilson, R. and Woolfe, M. (1998) *Oils and Fats. In: Food Authenticity, Issues and Methodologies. FAIM Concerted Action no. AIR3-CT94-2452.* Ed. Lees, M. Published by Eurofins Scientific, Nantes, France. pp: 211-269.

Arakawa, T. and Timasheff, S.N. (1985) The stabilization of proteins by osmolytes. Biophys. J., 47: 411-414.

AZTI (2001) Carnet de Identidad del Bacalao (*Gadus Morhua*). AZTI, Sukarrieta.

Azuma, M., Takahashi, K., Fukuda, T., Ohyabu, Y., Yamamoto, I., Kim, S., Iwao, H., Schaffer, S.W. and Azuma, J. (2000) Taurine attenuates hypertrophy induced by angiotensin II in cultured neonatal rat cardiac myocytes. Eur. J. Pharmacol., 403: 181-188.

Aursand, M. and Axelson, D. (2001) Origin recognition of wild and farmed salmon (Norway and Scotland) using ^{13}C NMR spectroscopy in combination with pattern recognition techniques. In: *Magnetic Resonance in Food Science: A View to the Future*. Webb, G.A., Belton, P.S., Gil, A.M. and Delgadillo, I. (Eds.). Published by RSC-The Royal Society of Chemistry, pp. 227-231.

Aursand, M., Bleivik, B., Rainuzzo, J.R., Jørgensen, L. and Mohr, V. (1994a) Lipid distribution and composition of commercially farmed Atlantic salmon (*Salmo salar*). J. Sci. Food Agric., 64: 239-248.

Aursand, M., Rainuzzo, J.R. and Grasdalen, H. (1994b) Quantitative High Resolution ^{13}C and ^{1}H Nuclear Magnetic Resonance of omega-3 fatty acids from white muscle of Atlantic salmon (*Salmo salar)*. J. Am. Oil Chem. Soc., 70: 971-981.

Aursand, M., Jørgensen, L. and Grasdalen, H. (1995a) The use of high resolution ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra to obtain information on the composition of fats and oils. LIPIDFORUM, 49: 14-17.

Aursand, M., Jørgensen, L. and Grasdalen, H., (1995b) Information on the composition of fats and oils from their high resolution ^{1}H and ^{13}C Nuclear Magnetic Resonance (NMR) spectra. LIPIDFORUM, Proceedings of the 18th Nordic Lipid Symposium, Reykjavik, Iceland.

Aursand, M., Jørgensen, L. and Grasdalen, H., (1995c) Quantitative high-resolution ^{13}C nuclear magnetic resonance of anserine and lactate in white muscle of Atlantic salmon (*Salmo salar*). Comp. Biochem. Physiol., B112: 315-321.

Aursand, M., Mabon, F., Axelson, D. and Martin, G.J. (1999) Origin testing of fish and fish oil products by using ^{2}H and ^{13}C NMR spectroscopy. 29th WEFTA Conference. 10-14 October, 1999. Thessaloniki, Greece.

Aursand, M., Mabon, F. and Martin, G.J. (2000) Characterization of farmed and wild salmon (*Salmo salar*) by a combined use of compositional and isotopic analyses. JAOCS., 77: 659-666.

Aursand, M., Dauksas, E., Schei, M., Halvorsen, J., Sandbakk, M., Axelson, D. and Martinez, I. (2004) Confirmation of the origin of salmon - Fat analyses reflect the fish diet. 34th WEFTA Conference; September 12-15, 2004; Lübeck, Germany.

Bang, H.O. and Dyerberg, J. (1973) The composition of food consumed by Greenlandic Eskimos. Acta Med Scand, 200: 69-73.

Bang, H.O., Dyerberg, J. and Hjorne, N. (1976) The composition of food consumed by Greenland Eskimos. Acta Med Scand., 200: 69-73.

Barak, A.J. and Tuma, D.J. (1983) Betaine, metabolic by-product or vital methylating agent? Life Sci., 32: 771–774.

Barak, A.J., Beckenhauer, H.C., Matti, J. and Tuma, D.J. (1993) Dietary betaine promotes generation of hepatic S-adenosylmethioine and protects the liver from ethanol-induced fatty infiltration. Alcohol Clin. Exp. Res., 17: 552–555.

Barak, A.J., Beckenhauer, H.C. and Tuma, D.J. (1996a) Betaine, ethanol, and the liver: a review. Alcohol, 13: 395–398.

Barat, J.M., Rodriguez-Barona, S., Andres, A. and Fito, P. (2003) Cod salting manufacturing analysis. Food Res. Int., 36: 447-453.

Beas, V.E., Wagner, J.R., Anon, M.C. and Crupkin, M. (1991) Thermal-denaturation in fish muscle proteins during gelling - effect of spawning condition. J. Food Sci., 56: 281-284.

Bell, J.G., Henderson, R.J., Tocher, D.R., McGhee, F., Dick, J.R., Porter, A., Smullen, R. and Sargent, J.R. (2002) Substituting fish oil with crude palm oil in the diet of Atlantic salmon (*Salmo salar*) affects tissue fatty acid compositions and hepatic fatty acid metabolism. J. Nutr., 132: 222–230.

Bell, J.G., Tocher, D.R., Henderson, R.J., Dick, J.R. and Crampton. V.O. (2003) Altered fatty acid compositions in Atlantic salmon (*Salmo salar*) fed diets containing linseed and rapeseed oils can be partially restored by a subsequent fish oil finishing diet. J. Nutr., 133: 2793–2801.

Bell, J.G., McGhee, F., Dick, J.R. and Tocher, D.R. (2005) Dioxin and dioxin-like polychlorinated biphenyls (PCBs) in Scottish farmed salmon (*Salmo salar*): effects of replacement of dietary marine fish oil with vegetable oils. Aquaculture, 234: 305-314.

Belton, P.S., Gil, A.M., Webb, G.A. and Rutledge, D. (2003) *Magnetic Resonance in Food Science. Latest Developments*. Published by RSC-The Royal Society of Chemistry. Cambridge, UK.

Bertram, H.C., Andersen, H.J. and Karlsson, A.H.(2001a) Comparative study of low-field NMR relaxation measurements and two traditional methods in the determination of water holding capacity of pork. Meat Sci., 57: 125-132.

Bertram, H.C., Karlsson, A.H., Rasmussen, M., Pedersen, O.D., Dønstrup, S. and Andersen, H.J. (2001b). Origin of multiexponential T2 relaxation in muscle myowater. J. Agric. Food Chem., 49: 3092-3100.

Bligh, E.G. and Dyer, W. (1959) A rapid method of total lipid extraction and purification. J. Can. Biochem. Physiol., 37: 911-917.

Boldyrev, A.A. (2001) Carnosine as a modulator of endogenous Zn^{2+} effects. Trends. Pharmacol., Sci., 22: 112-113.

Boldyrev, A.A.; Dupin, A.M., Pindel, E.V. and. Severin S.E (1988) Antioxidative properties of histidine-containing dipeptides from skeletal muscles of vertebrate. Comp. Biochem. Physiol., 89B: 245-250.

Boldyrev, A.A., Stvolinsky, S.L., Tyulina, O.V., Koshelev, V.B., Hori, N. and Carpenter, D.O. (1997) Biochemical and physiological evidence that carnosine is an endogenous neuroprotector against free radicals. Cell. Mol. Neurobiol., 17: 259-271.

Born, E.W., Outridge, P., Riget, F.F., Hobson, K.A., Dietz, R., Oien, N. and Haug, T. (2003) Population substructure of North Atlantic minke whales (*Balaenoptera acutorostrata*) inferred from regional variation of elemental and stable isotopic signatures in tissues. J. Mar Systems, 43: 1-17.

Burel, C. Boujard, T. Kaushik, S.J. Boeuf, G. Van der Geyten, S. Mol, K.A. Kuhn, E.R. Quinsac, A. Krouti, M. and Ribaillier, D. (2000) Potential o f plant-protein sources as fish meal substitutes in diets for turbot (*Psetta maxima*): growth, nutrient utilisation and thyroid status. Aquaculture, 188: 363–382.

Burg, M.B., Kwon, E.D. and Kültz, D. (1997) Regulation of gene expression by hypertonicity. Ann. Rev. Physiol., 59: 437-455.

Burr, M.L., Fehily, A.M., Gilbert, J.F., Rogers, S., Holliday, R.M., Sweetnam, P.M., Elwood, P.C., Deadman, N.M. (1989) Effects of changes in fat, fish, and fibre intakes on death and myocardial reinfarction: diet and reinfarction trial (DART). Lancet., 2(8666): 757-761.

Campana, S.E., Fowler, A.J. and Jones, C.M. (1994) Otolith elemental fingerprinting for stock identification of Atlantic cod (*Gadus morhua*) using laser ablation ICPMS. Can. J. Fish. Aquat. Sci., 51: 1942–1950.

Campana, S.E., Gagne, J.A. and McLaren, J.W. (1995) Elemental fingerprinting of fish otoliths using ID ICPMS. Mar. Ecol. Prog. Ser., 122: 115–120.

Cardinal, M., Knockaert, C., Torrisen, O., Sigurgisladottir, S., Mørkøre, T., Thomassen, M. and Vallet, J.L. (2001) Relation of smoking parameters to the yield, color and sensory quality of smoked Atlantic salmon (*Salmo salar*). Food Res. Int., 34: 537-550.

Carter, C.G. and Hauler, R.C. (2000) Fish meal replacement by plant meals in extruded feeds for Atlantic salmon, *Salmo salar* L. Aquaculture, 185: 299–311.

Chambers, S.T. (1995) Betaines: their significance for bacteria and the renal tract. Clin. Sci., 88: 25-27.

Chambers, S.T., Peddie, B.A., Randall, K. and Lever, M. (1999) Inhibitors of bacterial growth in urine: what is the role of betaines? Int. J. Antimicrob. Agents., 11: 293-296.

Chan, K.M. and Decker, E.A. (1994) Endogenous skeletal muscle antioxidant. CRC Rev Food Sci. Nutr., 34: 403-426.

Connor, W.E. (2000) Importortance of n-3 fatty acids in health and disease. Am. J. Clin. Nutr., 71: 171S-175S.

Cooper, M. and Midling, K. (2004) The use of anti-mouse tyrosinase antibody to investigate mechanisms of melanisation in farmed cod. In: Proceedings of the 34th WEFTA Meeting, Lübeck, Germany, 12-15 Sept 2004. pp: 44.

Cowey, C.B. (1995) Protein and amino acid requirements: a critique of methods. J. Appl. Ichthyol., 11: 199-204.

Crush, K.G. (1970) Carnosine and related substances in animal tissues. Comp. Biochem. Physiol., 34B: 3-30.

Dabrowski, K., El-Fiky, N., Kock, G., Frigg, M. and Wieser, W. (1990) Requirement and utilisation of ascorbic acid and ascorbic sulphate in juvenile rainbow trout. Aquaculture, 91: 317–337.

Danielsdottir, A.K. (1998) Genetic Studies on Redfish species and Stocks. Past-Ongoing-Future. ICES 1998; SGRS:WD3.

Daoulas, C., Economou, A.N. and Bantavas, I. (1991) Osteological abnormalities in laboratory reared seabass (*Dicentrarchus labrax*) fingerlings. Aquaculture, 97: 169–180.

Dewailly, E., Blanchet, C., Lemieux, S., Sauve, L., Gingras, S., Ayotte, P. and Holub, B.J. (2001) n-3 Fatty acids and cardiovascular disease risk factors among the Inuit Nunavik. Am. J. Clin. Nutr., 74: 464-473.

Dincer, B., Spearow, J.L., Cassens, R.G. and Greaser, M.L. (1987) The effects of curing and cooking on the detection of species origin of meat products by competitive and indirect ELISA techniques. Meat Sci., 20: 253-265.

Dobson, G. (2002) Analysis of fatty acids in functional foods with emphasis on ω3 fatty acids and conjugated linoleic acid. In: *Methods of Analysis for Functional Foods and Nutraceuticals.* Hurst, J.W. Ed. CRC Press.

Doyle, R.W., Schackel, N.L., Basiao, Z., Uraiwan, S., Matricia, T. and Talbot, A.J. (1991) Selective diversification of aquaculture stocks: a proposal for economically sustainable genetic stock conservation. Can. J. Fish. Aquat. Sci., 48: 148-154.

Drengstig, A., Fevolden, S.E., Galand, P.E. and Aschan, M.M. (2000) Population structure of the deep-sea shrimp (*Pandalus borealis*) in the north-east Atlantic based on allozyme variation. Aquat. Living Resources., 13: 121-128.

Duflos, G., Le Fur, B., Mulak, V., Becel., P and Malle, P. (2002) Comparison of methods of differentiating between fresh and frozen-thawed fish or fillets. J. Sci. Food Agric., 82: 1341-1345.

Dyerberg, J,. and Bang, H.O. (1979) Haemostatic function and platelet polyunsaturated fatty acids in Eskimos. Lancet, 2: 433-435.

Dyerberg, J., Bang, H.O. and Hjorne, N. (1975) Fatty acid composition of the plasma lipids in Greenland Eskimos. Am. J. Clin. Nutr., 28: 958-966.

Dyerberg, J., Bang, H.O., Stofferson, E., Moncada, S. and Vane, J.R. (1978) Eicosapentaenoic acid and prevention of thrombosis and atherosclerosis. Lancet, 2: 117-119.

Edmonds, J.S., Moran, M.J., Caputi, N. and Morita, M. (1989) Trace element analysis of fish sagittae as an aid to stock identification: Pink snapper (*Chrysophrys auratus*) in Western Australian waters. Can. J. Fish. Aquat. Sci., 46: 50-54.

Erikson, U., Veliyulin, E., Singstad, T.E. and Aursand, M. (2004) Salting and desalting of fresh and frozen-thawed cod (*Gadus morhua*) fillets: a compartive study using ^{23}Na, ^{23}Na MRI, low-field ^{1}H NMR, and physicochemical analytical methods. J. Food Sci., 69: 107-114.

Etienne, M., Jerome, M., Fleurence, J., Rehbein, H., Kündiger, R., Mendes, R., Costa, H. and Martinez, I. (2001). Species identification of formed fishery products and high pressure treated fish by electrophoresis: a collaborative study. Food Chem., 72: 105-112.

Falch, E., Anthonsen, H.W., Axelson, D.E. and Aursand, M. (2005) Correlation between ^{1}H NMR and traditional methods for determining lipid oxidation of ethyl docosahexaenoate. JAOCS, 81: 1105-1110.

Fan, T.W., Colmer, T.D., Lane, A.N. and Higashi, R.M. (1993) Determination of metabolites by ^{1}H NMR and GC: analysis for organic osmolytes in crude tissue extracts. Anal. Biochem., 214: 260-271.

Farrell, J. and Campana, S.E. (1996) Regulation of calcium and strontium deposition on the otoliths of juvenile tilapia, *Oreochromis niloticus*. Comp. Biochem. Physiol., 115A: 103-109.

Fetterer, R.H., Augustine, P.C., Allen, P.C. and Barfield, R.C. (2003) The effect of dietary betaine on intestinal and plasma levels of betaine in uninfected and coccidia-infected broiler chicks. Parasitol. Res., 90: 343-348.

Fevolden, S.E. and Pogson, G.H. (1997) Genetic divergence at the synaptophysin (*Syp I*) locus among Norwegian coastal and north-east Arctic populations of Atlantic cod. J. Fish Biol., 51: 895-908.

Fevolden, S.E., Roed, K.H. and Fjalestad, K. (2003) Title: A combined salt and confinement stress enhances mortality in rainbow trout (*Oncorhynchus mykiss*) selected for high stress responsiveness. Aquaculture, 216: 67-76.

Foran, J.A., Hites, R.A., Carpenter, D.O., Hamilton, M.C., Mathews-Amos, A. and Schwager, S.J. (2004) A survey of metals in tissues of farmed Atlantic and wild Pacific salmon. Environ. Toxicol. Chem., 23: 2108-2110.

Foucat, L., Taylor, R.G., Labas, R. and Renou, J.P. (2001) Characterization of frozen fish by NMR imaging and histology. American Laboratory, 33: 38-43.

Fowler, A.J., Campana, S.E., Jones, C.M. and Thorrold, S.R. (1995) Experimental assessment of the effects of temperature and salinity on elemental composition of otoliths using laser ablation ICPMS. Can. J. Fish. Aquat. Sci., 52:1431-1441.

Francis, G. Makkar H.P.S. and Becker, K. (2001) Antinutritional factors present in plant-derived alternate fish feed ingredients and their effects in fish. Aquaculture, 199: 197–227.

Fraser, M.R., Anderson, T.A. and de Nys, R. (2004) Ontogenic development of the spine and spinal deformities in larval barramundi (*Lates calcarifer*) culture. Aquaculture, 242: 697-711.

Friars, G.W. Bailey J. K. and O'Flynn F.M (1995) Applications of selection for multiple traits in cage-reared Atlantic salmon (*Salmo salar*). Aquaculture, 137: 213-217.

Friebolin, H. (1998) Basic One- and Two Dimensional NMR Spectroscopy. 3rd Revised Edition. Wiley-VCH. Fed. Republic of Germany.

Gahl, W.A., Bernardini, I., Chen, S., Kurtz, D. and Horvath, K. (1988) The effect of oral betaine on vertebral body bone density in pyridoxine-non-responsive homocystinuria. J. Inherit. Metab. Dis., 11: 291–298.

Galand, P.E. and Fevolden, S.E. (2000) Population structure of *Chlamys islandica* in the Northeast Atlantic - northern stocks compared with a southern relict population. Sarsia, 85: 183-188.

Gallart-Jornet, L., Escriche-Roberto, I. and Fito-Maupoey, P- (2004) *La salazón de pescado una tradición en la dieta mediterránea.* Published by La Universidad Politécnica de Valencia.

Ge, Y., Molloy, M.P., Chamberlain, J.S. and Andrews, P.C. (2004) Differential expression of the skeletal muscle proteome in *mdx* mice at different ages. Electrophoresis, 25: 2576-2585.

Gildberg, A. (2004) Digestive enzyme activities in starved pre-slaughter farmed and wild-captured, Atlantic cod (*Gadus morhua*) . Aquaculture, 238: 343-353.

Gill, T.A. and Thompson, J.W. (1984) Rapid, automated analysis of amines in seafood by ion-moderated partition HPLC. J. Food Sci., 49: 603-606.

Gomes, E.F., Rema, P., Gouveia, A. and Teles, A.O. (1995a) Replacement of fish meal by plant-proteins in diets for rainbow trout (*Oncorhynchus mykiss*)—effect of the quality of the fish-meal based control diets on digestibility and nutrient balance. Water Sci. Technol., 31: 205–211.

Gomes, E.F. Rema P. and Kaushik, S.J. (1995b) Replacement of fish-meal by plant-proteins in the diet of rainbow trout (*Oncorhynchus mykiss*)—digestibility and growth performance. Aquaculture, 130: 177–186.

Grahl-Nielsen, O. (1997). Fatty acid profiles as natural marks for stock identification. ICES CM 1997/M:2, Annex 3.

Grahl-Nielsen, O. and Mjaavatten, O. (1992) Discrimination of striped bass stocks: a new method based on chemometry of the fatty acid profile in heart tissue. Trans. Am. Fish. Soc., 121: 307-314.

Grahl-Nielsen, O. and Ulvund, K.A., (1990) Distinguishing populations of herring by chemometry of fatty acids. Am. Fish. Soc. Symp., 7:566-571.

Gribbestad, I.S., Aursand, M. and Martinez, I (2005) High resolution ^{1}H Magnetic Resonance Spectroscopy of whole fish, fillets and extracts of farmed Atlantic salmon (*Salmo salar*) for quality assessment and compositional analyses. Aquaculture, in press.

Harris, W.S. (1989) Fish oils and plasma lipid and lipoprotein metabolism in humans: a critical review. J. Lipid Res., 30: 785-807.

Henderson, R.J. (1996) Fatty acid metabolism in freshwater fish with particular reference to polyunsaturated fatty acids. Archives of Animal Nutrition, 49: 5-22.

Herbinger, C.M., O'Reilly, P.T., Doyle, R.W., Wright, J.M. and O'Flynn, F. (1999) Early growth performance of Atlantic salmon full-sib families reared in single family tanks versus in mixed family tanks. Aquaculture, 173: 105-116.

Hites, R.A., Foran, J.A., Carpenter, D.O., Hamilton, M.C., Knuth, B.A., Schwager, S.J. (2004) Global assessment of organic contaminants in farmed salmon. Science, 303: 226-229.

Hobsen, K.A. (1999) Tracing origins and migration of wildlife using stable isotopes: a review. Oecologia, 120: 314-326.

Hoelzel, A.R. (1992) *Molecular Genetic Analysis of Populations. A Practical Approach.* IRL Press, Cambridge, UK.

Hou, W.C., Chen, H. and Lin, Y.H. (2003) Antioxidant peptides with angiotensin converting enzyme inhibitory activities and applications for angiotensin converting enzyme purification. J. Agric. Food Chem., 51: 1706-1709.

Igarashi, T., Aursand, M., Sacchi, R., Paolillo, L., Nonaka, M. and Wada, S. (2002) Determination of docosahexaenoic acid and n-3 fatty acids in refined fish oils by ^{1}H-NMR spectroscopy: IUPAC interlaboratory study. J. AOAC Int., 85: 1341-1354.

Ikeda, T., Kimura, K., Hama, T. and Tamaki, N. (1980) Activation of rabbit muscle fructose 1,6-biphosphatase by histidine and carnosine. J. Biochem., 87: 179-185.

Iqbal, M.J., Yaegashi, S., Ahsan, R., Lightfoot, D.A. and Banz, W.J. (2002) Differentially abundant mRNAs in rat liver in response to diets containing soy protein isolate. Physiol. Genomics, 11: 219-226.

Jacobs, M.N., Covaci, A. and Schepens, P. (2002) Investigation of selected persistent organic pollutants in farmed Atlantic salmon (*Salmo salar*), salmon aquaculture feed, and fish oil components of the feed. Environ. Sci. Technol., 36: 2797-2805.

Jensen, K.N. and Jorgensen, B.M. (2003) Effect of storage conditions on differential scanning calorimetry profiles from thawed cod muscle. LWT- Food Sci. Technol., 36: 807-812.

Jobling, M. (1988) A review of the physiological and nutritional energetics of cod, *Gadus morhua* L., with particular reference to growth under farmed conditions. Aquaculture, 70: 1-19.

Jobling, M. (1994) *Fish Bioenergetics.* Chapman Hall, London.

Jobling, M. (2004) Are modifications in tissue fatty acid profiles following a change in diet the result of dilution? Test of a simple dilution model. Aquaculture, 232: 551-562.

Jobling, M., Larsen, A.V., Andreassen, B., Sigholt, T. and Olsen, R.L. (2002) Influence of a dietary shift on temporal changes in fat deposition and fatty acid composition of Atlantic salmon post-smolt during the early phase of seawater rearing. Aquaculture Res., 33: 875-889.

Joensen, H. and Grahl-Nielsen, O. (2000) Discrimination of *Sebastes viviparus, S. marinus*, and *S. mentella* from Faroe Islands by chemometry of the fatty acid profile in heart and gill tissues and in the skull oil. Comp. Biochem. Physiol., 126B: 69-79.

Joensen, H. and Grahl-Nielsen, O. (2001) The redfish species *Sebastes viviparus, Sebastes marinus* and *Sebastes mentella* have different composition of their tissue fatty acids. Comp. Biochem. Physiol., 129B: 73-85.

Joensen, H. and Grahl-Nielsen, O. (2004) Stock structure of *Sebastes mentella* in the North Atlantic revealed by chemometry of the fatty acid profile in heart tissue. ICES J. Mar. Sci, 61: 113-126.

Joensen, H., Steingrund, P., Fjallstein, I. and Grahl-Nielsen, O. (2000) Discrimination between two reared stocks of cod (*Gadus morhua*) from the Faroe Islands by chemometry of the fatty acid composition in the heart tissue. Marine Biol., 136: 573-580.

Johnson, P. and Hammer, J.L. (1989) Effects of L-1-methyl-L-histidine and the muscle dipeptides carnosine and anserine on the activities of muscle calpains. Comp. Biochem. Physiol., 94B: 45-48.

Junnila, M., Barak, A.J., Beckenhauer, H.C. and Rahko, T. (1998) Betaine reduces hepatic lipidosis induced by carbon tetrachloride in Sprague-Dawley rats. Vet. Hum. Toxicol., 40: 263-266.

Kalish, J.M. (1989) Otolith microchemistry: Validation of the effects of physiology, age, and environment on otolith composition. J. Exp. Mar. Biol. Ecol., 132: 151-178.

Kaushik, S.J., Cravedi, J.P., Lalles, J.P., Sumpter, J., Fauconneau, B. and. Laroche, M. (1995) Partial or total replacement of fish meal by soybean protein on growth, protein utilization, potential estrogenic or antigenic effects, cholesterolemia and flesh quality in rainbow trout, *Oncorhynchus mykiss*. Aquaculture, 133: 257-274.

Keay, J.N. and Hardy, R. (1972) The separation of aliphatic amines in dilute aqueous solution by gas chromatography and application of this technique to the quantitative analysis of tri- and dimethylamine in fish. J. Sci. Food Agric., 23: 9-19.

Keeler, J. (2002) *Understanting NMR Spectrosscopy.* University of Cambridge, Deptarment of Chemistry. Lecture notes given by Professor James Keeler of the University of Cambridge during his visit to the University of California, Irvine, in 2002. http://www.spectroscopynow.com/Spy /basehtml/SpyH/1,1181,5-14-9-0-0-education_dets-0-1839,00.html

Kemp, W. (1986) *NMR in Chemistry. A Multinuclear Introduction.* Macmillan Educational Ltd., London, UK.

Kim, J.B., Murata, M. and Sakauchi, M. (1987) A method for the differentiation of frozen-thawed from unfrozen fish fillets by a combination of Torrymeter readings and K values. Nippon Suisan Gakkaishi, 53: 159-164.

Kim, S.K., Kim, Y.C. and Kim, Y.C. (1998) Effects of singly administered betaine on hepatotoxicity of chloroform in mice. Food Chem. Toxicol., 36: 655-661.

King, N.L.R. (1978) Isoelectric variants of actin in raw and cooked meat. Meat Sci., 2: 313-321.

Konosu, S. and Yamaguchi, K. (1982) The flavour components in fish and shellfish. In: *Chemistry and Biochemistry of Marine Food Products.* Martin, R.E., Flick, G.J., Hebard, C.E. and Ward, D.E. (Eds.). AVI Publishing Company, Westport, Connecticut. pp: 367-404.

Konosu, S., Watanabe, K. and Shimizu, T. (1974) Distribution of nitrogenous constituents in the muscle extracts of eight species of fish. Bull. Jap. Soc. Sci. Fish., 40: 909-915.

Krogdahl, A., Lea, T.B. and Olli, J.L. (1994) Soybean proteinase inhibitors affect intestinal trypsin activities and amino acid digestibilities in rainbow trout (*Oncorhynchus mykiss*). Comp. Biochem. Physiol., 107A: 215–219.

Kromhout, D., Bosschieter, E.B. and de Lezenne Coulander C. (1985) The inverse relation between fish consumption and 20-year mortality from coronary heart disease. New Engl. J. Med., 312: 1205-1209.

Lambelet, P., Renevey, F., Kaabi, C. and Raemy, A. (1995). Low-field nuclear magnetic resonance study of stored or processed cod. J. Agric. Food Chem., 43: 1462-1466.

Lauritzsen, K. (2004) Quality of salted cod (*Gadus morhua* L.) as influenced by raw material and salt composition. Dr Scient Thesis. Norwegian College of Fishery Science. University of Tromsø.

Lie, Ø., Lied, E. and Lambertsen, G. (1986) Liver retention of fat and of fatty acids in cod (*Gadus morhua*) fed different oils. Aquaculture, 59: 187-196.

Limburg, K.E. (1995) Otolith strontium traces environmental history of subyearling American shad *Alosa sapidissima.* Mar. Ecol. Prog. Ser., 119: 25-35.

Luten, J., Kole, A., Schelvis, R., Veldman, M., Heide, M., Carlehög, M. and Akse, L. (2002) Evaluation of wild cod versus wild caught , farmed raised cod from Norway by Dutch consumers. *økonomisk Fiskeriforsning*, 12: 44-60.

Mackie, I., Craig, A., Etienne, M., Jerome, M., Fleurence, J., Jessen, F., Smelt, A., Krujit, A., Malmheden Yman, I., Ferm, M., Martinez, I., Perez-Martin, R., Piñeiro, C., Rehbein, H. and Kründiger, R. (2000). Species identification of smoked and gravad fish products by sodium dodecylsulphate polyacrylamide gel electrophoresis, urea isoelectric focusing and native isoelectric focusing: a collaborative study. Food Chem., 71: 1-7

Madeniian, C.P., O'Connor, D.V., Stewart, D.J., Miller, M.A. and Masnado, R.G. (2002) Field estimate of net trophic transfer efficiency of PCBs to Lake Michigan chinook salmon from their prey. Environ Sci Technol., 36: 5029-5033.

Mambrini, M. and Kaushik, S.J. (1995) Indispensable amino acid requirements of fish: correspondence between quantitative data and amino acid profiles of tissue proteins. J. Appl. Ichthyol.-Z. Angew. Ichthyol., 11: 240-247.

Manchenko, G.P. (1994) *Handbook of Detection of Enzymes on Electrophoretic Gels*. CRC Press LLC.

Martin, G.J. and Martin, M.L. (1988) The site-specific natural isotope fraction-NMR method applied to the study of wines. In: *Modern methods of plant Analysis, Wine analysis*. Linkens, H.F. and Johnsen, J.F. (Eds.). Springer Verlag, Berlin/Heidelberg, Germany, Vol 6, pp. 258-275.

Martin, G.J. and Martin, M.L. (1991) Deuterium labelling at the natural abundance level as studied by high field quantitative ^{2}H-NMR. Tetrahedron Letters, 22: 3525-3528.

Martin, G.J., Guillou, C., Martin, M.L., Cabanis, M.T., Tep, Y. and Aerny, J. (1988) Natural factors of isotopic fractionation and the characterization of wines. J. Agric. Food Chem., 36: 316-322.

Martin, G.G., Wood, R. and Martin. G.J. (1996) Detection of added beet sugar in concentrated and single strength fruit juice by deuterium NMR (SNIF-NMR method). Collaborative study, J. AOAC Int., 79: 917-928.

Martin, S.A.M., Vilhelmsson, O., Medale, F., Watt, P., Kaushik, S. and Houlihan, D.F. (2003) Proteomic sensitivity to dietary manipulations in rainbow trout. Biochim. Biophys. Acta, 651: 17-29.

Martinez, I. and Christiansen, J.S. (1994) Myofibrillar proteins in developing white muscle of the Arctic charr, *Salvelinus alpinus* (L.). Comp. Biochem. Physiol., 107B: 11-20.

Martinez, I. and Daniélsdóttir, A.K. (2000) Identification of marine mammal species in food products. J. Sci. Food Agric., 80: 527-533.

Martinez, I. and Malmheden Yman, I. (1999) Species identification in meat products by RAPD analysis. Food Res. Int., 31: 459-466.

Martinez, I. and Pastene, L.A. (1999) RAPD typing of Northeast Atlantic and Western North Pacific minke whales (*Balaenoptera acutorostrata*). ICES J. Mar. Sci., 56: 640-651.

Martinez, I., Ofstad, R. and Olsen, R.L. (1990a) Myosin isoforms in red and white muscles of some marine teleost fishes. J. Muscle Res. Cell Motility, 11: 489-495.

Martinez, I., Ofstad, R. and Olsen, R.L. (1990b) Electrophoretic study of myosin isoforms in white muscles of some teleost fishes. Comp. Biochem. Physiol., 96B: 221-227.

Martinez, I., Ofstad, R. and Olsen, R.L. (1990c) Intraspecific myosin light chain polymorphism in the white muscle of herring (*Clupea harengus harengus,* L.). FEBS Lett., 265: 23-26.

Martinez, I., Christiansen, J.S., Ofstad, R. and Olsen, R.L. (1991) Comparison of myosin isoenzymes present in skeletal and cardiac muscles of the Arctic charr *Salvelinus alpinus* (L.). Sequential expression of different myosin heavy chains during development of the fast white skeletal muscle. Eur. J. Biochem., 195: 743-753.

Martinez, I., Solberg, C., Lauritzsen, C. and Ofstad, R. (1992) Two-dimensional electrophoretic analyses of cod (*Gadus morhua,* L.) whole muscle proteins, water soluble fraction and surimi. Effect of the addition of CaCl2 and MgCl2 during the washing procedure. Appl. Theor. Electrophoresis, 2: 201-206.

Martinez, I., Hagavei, H., Øverbø, K., Daniélsdóttir, A.K. and Haug, T. (1999) Study of the population structure of Central and Eastern North Atlantic minke whales (*Balaenoptera acutorostrata*) by RAPD-typing. International Whaling Commission. 51st meeting. 3-15 May, 1999. Grenada.

Martinez, I., Jakobsen Friis, T. and Seppola, M (2001) Requirements for the application of protein SDS-PAGE and RAPD to product speciation. Electrophoresis, 22: 1526-1533.

Martinez, I., Bathen, T., Standal I.B., Halvorsen, J., Aursand, M and Gribbestad, I.S. (2004) Compositional analyses of cod (*Gadus morhua*) and Atlantic salmon (*Salmo salar*) by high resolution 1H MR: application to authentication analyses. 34th WEFTA Conference; September 12-15, 2004; Lübeck, Germany.

Martinez, I., Bathen, T., Standal, I.B., Halvorsen, J., Aursand, M., Gribbestad, I.S. and Axelson, D.E. (2005) Bioactive compounds in cod (*Gadus morhua*) products and suitability of ^{1}H NMR metabolite profiling for classification of the products using multivariate data analyses. Submitted.

Masson, L.R. (2002) Amino acid analysis. In: *Methods of Analysis for Functional Foods and Nutraceuticals.* Hurst, J.W. (Ed.). CRC Press.

May, B. (1992) Starch gel electrophoresis of allozymes. In: *Molecular Genetic Analysis of Populations. A practical Approach.* Hoelzel, A.R. (Ed.). IRL Press, Cambridge, UK., pp: 1-27.

Medina, I., Aubourg, S.P. and Martin, R.P. (1997) Species differentiation by multivariate analysis of phospholipids from canned Atlantic tuna. J. Agric. Food Chem., 45: 2495-2499.

Miller, S.A., Dykes, D.D., Polesky, H.F. (1988) A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Acids Res., 16: 1215.

Molloy, M.P. (2000) Two-dimensional electrophoresis of membrane proteins using immobilized pH gradients. Anal. Biochem., 280: 1-10.

Mooney, B.D., Nichols, P.D. and Elliot, N.G. (2002) *Seafood, the Good Food II.* CSIRO Marine Research and FRDC, Hobart, Tasmania, Australia. 126 pp. ISBN 1 876 996 06 4.

Morais, S., Bell, J.G., Robertson, D.A., Roy, W.J. and Morris, P.C. (2001) Protein/lipid ratios in extruded diets for Atlantic cod (*Gadus morhua* L.): effects on growth, feed utilisation, muscle composition and liver histology. Aquaculture, 203: 101-119.

Mork, J. and Giæver, M. (1999) Genetic structure of cod along the coast of Norway: Results from isozyme studies. Sarsia, 84:157-168.

Moyano, F.J., Cardenete, G. and de la Higuera, M. (1991) Nutritive and metabolic utilization of proteins with high glutamic-acid content by the rainbow-trout (*Oncorhynchus mykiss*). Comp. Biochem. Physiol., 100A: 759-762.

Murakami, T., Nagamura, Y. and Hirano, K. (1998) The recovering effect of betaine on carbon tetrachloride-induced liver injury. J. Nutr. Sci. Vitaminol., 44: 249–255.

Nakashima, T., Sano, A., Nakagawa, Y., Okuno, T., Takino, T. and Kuriyama, K. (1983) Effect of taurine on the course of drug-induced chronic liver diseaes. In: *Sulphur amino acids. Biochemical and Clinical Aspects.* Kuriyama, H., Huxtable, R.J. and Iwata, H. (Eds.). Alan R. Liss, New York, pp: 472-473.

Naylor, R.L., Goldburg, R.J., Primavera, J.H., Kautsky, N., Beveridge, M.C.M., Clay, J., Folke, C., Lubchenco, J., Mooney, H. and Troell, M. (2000) Effect of aquaculture on world fish supplies. Nature 405: 1017-1024.

Nedreaas, K. and Nævdal, G. (1991a) Genetic studies of redfish (*Sebastes* spp.) along the continental slopes from Norway to East Greenland. ICES J. Mar. Sci., 48: 173-186.

Nedreaas, K. and Nævdal, G. (1991b) Identification of 0- and 1-group redfish (genus *Sebastes*) using electrophoresis. ICES J. Mar. Sci., 48: 91-99.

Nedreaas, K., Johansen, T. and Nævdal, G. (1994) Genetic studies of redfish (*Sebastes* spp.) from Iceland and Greenland waters. ICES J. Mar. Sci., 51: 461-467.

Nelson, D.L. and Cox, M.M. (2000) *Lehninger Principles of Biochemistry.* 3rd Edition. Worth Publishers, New York, USA.

Nichols, P.D., Mooney, B.D. and Elliot, N.G. (2002) Nutritional Value of Australian Seafood II. CSIRO Marine Research and FRDC, Hobart, Tasmania, Australia. 198 pp. ISBN 1 876 996 07 2.

Nordoy, A., Hatcher, L.F., Ullmann, D.L. and Connor, WE. (1993) Individual effects of dietary saturated fatty acids and fish oil on plasma lipids and lipoproteins in normal men. Am. J. Clin. Nutr., 57: 634-639.

Nordoy, A., Marchioli, R., Arnesen, H. and Videbaek, J. (2001) n-3 polyunsaturated fatty acids and cardiovascular diseases. Lipids, 36: S127-129.

Nott, K.P., Evans, S.D. and Hall, L.D. (1999a) Quantitative magnetic resonance imaging of fresh and frozen-thawed trout. Magn. Reson. Imag., 17: 445-455.

Nott, K.P., Evans, S.D. and Hall, L.D. (1999b) The effect of freeze-thawing on the magnetic resonance imaging parameters of cod and mackerel. Food Sci. Technol - LWT, 32: 261-268.

Official Journal of the European Community (1990), 33 L-272. 3 October, 1990.

O'Leary, M. (1981) Carbon isotope fraction in plants. Phytochemistry, 20: 553-567.

Olsson, B., Bradley, B.P., Gilek, M., Reimer, O., Shepard, J.L. and Tedengren, M. (2004) Physiological and proteomic responses in *Mytilus edulis* exposed to PCBs and PAHs extracted from Baltic Sea sediments. Hydrobiologia, 514: 15-27.

Oomen, C.M., Feskens, E.J., Rasanen, L., Fidanza, F., Nissinen, A.M., Menotti, A., Kok, F.J. and Kromhout, D.(2000) Fish consumption and coronary heart disease mortality in Finland, Italy, and The Netherlands. Am. J. Epidemiol., 151: 999-1006.

Paccioretty, L., Hickman, M.A., Morris, J.G. and Rogers, Q.R. (2001) Kinetics of taurine depletion and repletion in plasma, serum, whole blood and skeletal muscle in cats. Amino Acids, 21: 417-427.

Pastene, L.A., Goto, M. and Kanda, N. (2003) Further analysis of mtDNA RFLP data in the Antarctic minke whale from Areas III-VI. Document SC/55/IA no 8 to the International Whaling Commision.

Peddie, B.A. and Chambers, S.T. (1993) Effects of betaines and urine on the antibacterial activity of aminoglycosides. J. Antimicrob. Chemother., 31: 481–488.

Pedersen, H.T., Berg, H., Lundby F. and Engelsen, S.B. (2001) The multivariate advantage in fat determination in meat by bench-top NMR, Innov. Food Sci. and Emerging. Tech., 2: 87.

Perez-Martin, R.I., Franco, J.M., Aubourg, S. and Gallardo, J.M. (1988) Changes in free amino acids content in albacore (*Thunnus alalunga*) muscle during thermal processing. Z. Lebensm. Unter Forsch., 187: 432-435.

Phillipson, B.E., Rothrock, D.W., Connor, W.E., Harris, W.S. and Illingworth, D.R. (1985) Reduction of plasma lipids, lipoproteins, and apoproteins by dietary fish oils in patients with hypertriglyceridemia. New Engl. J. Med., 312: 1210-1216.

Pink, J., Naczk, M. and Pink, D. (1998) Evaluation of the quality of frozen minced red hake: Use of Fourier transform infrared spectroscopy. J. Agric. Food Chem., 46: 3667-3672.

Pink, J., Naczk, M. and Pink, D. (1999) Evaluation of the quality of frozen minced red hake: use of Fourier transform near-infrared spectroscopy. J. Agric. Food Chem., 47: 4280-4284.

Piñeiro, C., Barros-Velazquez, J., Perez-Martin, R.I., Martinez, I., Jakobsen, T., Rehbein, H., Kündiger, R., Mendes, R., Etienne, M., Jerome, M., Craig, A., Mackie, I.M. and Jessen, F. (1999) Development of a SDS-PAGE reference method for the analysis and identification of fish species in raw and heat-processed samples. A collaborative study. Electrophoresis, 20: 1425-1432.

Pogson, G.H. and Fevolden, S.E. (2003) Natural selection and the genetic differentiation of coastal and Arctic populations of the Atlantic cod in northern Norway: a test involving nucleotide sequence variation at the pantophysin (*PanI*) locus Mol. Ecol., 12: 63-74.

Proctor, C.H., Thresher, R.E., Gunn, J.S., Mills, D.J., Harrowfield, I.R. and Sie, S.H. (1995) Stock structure of the southern bluefin tuna *Thunnus maccoyii*: An investigation based on probe microanalysis of otolith composition. Mar. Biol., 122: 511-526.

Quinteiro, J., Sotelo, C.G., Rehbein, H.,Pryde, S., Medina, I., Perez-Martin, R., Rey-Mendez, M. and Mackie, I. (1998) Use of mtDNA direct polymerase chain reaction (PCR) sequencing and PCR-restriction fragment length polymorphism methodologies in species identification of canned tuna. J. Agric. Food Chem., 46: 1662-1669.

Read, W.O. and Welty, J.D. (1963) Effect of taurine on epinephrine and digoxin-induced irregularities in the dog-heart. J. Pharmacol. Exp. Ther., 139: 283-289.

Rehbein, H., (1983) Differentiation of redfishes from the Northeast Atlantic (*Sebastes marinus* L., *S. mentella* Travin, *S. viviparus* Krøer and *Helicolenus dactylopterus* D. Delaroche 1809) by isoelectric focusing of sarcoplasmic proteins. ICES CM G: 40, p. 11.

Rehbein, H. and Cakli, S. (2000) The lysosomal enzyme activities of fresh, cooled, frozen and smoked salmon fish species (*Onchorhyncus keta* and *Salmo salar*). Turskish J. Vet. Animal Sci., 24: 103-108.

Rehbein, H., Kündiger, R., Malmheden Yman, Y., Ferm, M., Etienne, M., Jerome, M., Craig, A., Mackie, I., Jessen, F., Martinez, I., Mendes, R., Smelt, A., Luten, J., Piñeiro, C. and Perez-Martin, R. (1999). Species identification of cooked fish by urea isolectric focusing and sodium dodecylsulfate polyacrylamide gel electrophoresis: a collaborative study. Food Chem., 67: 333-339.

Rezzi, S., Guillou, C. and Reinero, F. (2003) The application of NMR in the implementation of European policies of consumer protection. In: *Magnetic Resonance in Food Science. Latest developments*. Belton, P.S., Gil, A.M., Webb, G.A. and Rutledge, D. (Eds.). The Royal Society of Chemistry-RSC. Cambridge, UK., pp: 62-69.

Robin, J.H., Regost, C., Arzel, J. and Kaushik, S.J. (2003) Fatty acid profile of fish following a change in dietary fatty acid source: model of fatty acid composition with a dilution hypothesis. Aquaculture, 225: 283-293.

Rodriguez-Ortega, M.J. (2003) Molecular biomarkers of pollution in bivalve molluscs from the Andalusian litoral (South of Spain). Ph D. Thesis Dept. Biochemistry and Molecular Biology, University of Cordoba, 3rd March, 2003.

Rodriguez-Ortega, M.J., Grosvik, B.E., Rodriguez-Ariza, A., Goksoyr, A. and Lopez-Barea, J. (2003) Changes in protein expression profiles in bivalve molluscs (*Chamaelea gallina*) exposed to four model environmental pollutants. Proteomics, 3: 1535-1543.

Roques, S., Pallotta, D., Sévigny, J.M. and Bernatchez, L. (1999a) Isolation and characterization of polymorphic microsatellite markers in the North Atlantic redfish (Teleostei: Scorpaenidae, genus *Sebastes*). Mol. Ecol., 8: 685-702.

Roques, S., Duchesne, P. and Bernatchez, L. (1999b) Potential of microsatellites for individual assignment: the North Atlantic redfish (genus *Sebastes*) species complex as a case study. Mol. Ecol., 8: 1703-1717.

Ruiz-Capillas, C. and Moral, A. (2001) Changes in free amino acids during chilled storage of hake (*Merluccius merluccius* L.) in controlled atmospheres and their use as a quality control index. Eur. Food Res. Technol., 212:302-302.

Sacchi, R., Medina, I., Aubourg, S.P., Addeo, F., Paolillo, L. (1993). Proton nuclear magnetic resonance rapid and structure-specific determination of n-3 polyunsaturated fatty acids in fish lipids. JAOCS, 70: 225-228.

Sambrook, J., Fritsch, E.F. and Maniatis, Y. (1989) *Molecular Cloning. A Laboratory Manual.* Cold Spring Harbor Laboratory Press.

dos Santos, J., Burkow, I.C. and Jobling, M. (1993) Patterns of growth and lipid deposition in cod (*Gadus morhua* L.) fed natural prey and fish-based feeds. Aquaculture, 110: 173-189.

Sargent J.R. and Tacon, A.G.J. (1999) Development of farmed fish: a nutritionally necessary alternative to meat. Proc. Nutr. Soc. 58: 377–383.

Schaffer, S.W., Lombardini, J.B. and Azuma, J. (2000a) Interaction between the actions of taurine and angiotensin II. Amino Acids, 18: 305-318.

Schaffer, S., Takahashi, K. and Azuma, J. (2000b) Role of osmoregulation in the actions of taurine. Amino Acids, 19: 527-546.

Schmidt, E.B., Skou, H.A., Christensen, J.H. and Dyerberg, J. (2000) N-3 fatty acids from fish and coronary artery disease: implications for public health. Public Health Nutr., 3: 91-98.

Schönherr, J. (2002) Analysis of products of animal origin in feeds by determination of carnosine and related dipeptides by high-performance liquid chromatography. J. Agric Food Chem., 50: 1945-1950.

Schubring, R. (2004) Differential scanning calorimetric (DSC) measurements on the roe of rainbow trout *(Oncorhynchus mykiss*): influence of maturation and technological treatment. Thermochim. Acta , 415: 89-98.

Secor, D.H. (1992) Application of otolith microchemistry analysis to investigate anadromy in Chesapeake Bay striped bass. Fish. Bull. 90: 798-806.

Secor, D.H. and Piccoli, P.M. (1996) Age- and sex-dependent migrations of the Hudson River striped bass population determined from otolith microanalysis. Estuaries, 19:778-793

Secor, D.H. and Zdanowicz, V.S. (1998) Otolith microconstituent analysis of juvenile bluefin tuna (*Thunnus thynnus*) from the Mediterranean Sea and Pacific Ocean. Fish. Res., 36: 251-256.

Secor, D.H., Campana, S.E., Zdanowicz, V.S., Lam, J.W.H., Yang, L. and Rooker, J.R. (2002) Inter-laboratory comparison of Atlantic and Mediterranean bluefin tuna otolith microconstituents. ICES J. Mar. Sci., 59: 1294-1304.

Selhub, J. (1999) Homocysteine metabolism. Annu. Rev. Nutr., 19: 217–246.

Shepard, J.L. and Bradley, B.P. (2000) Protein expression signatures and lysosomal stability in *Mytilus edulis* exposed to graded copper concentrations. Mar Environ. Res., 50: 337-340.

Shepard, J.L., Olsson, B., Tedengren, M. and Bradley, B.P. (2000) Protein expression signatures identified in *Mytilus edulis* exposed to PCBs, copper and salinity stress. Mar Environ. Res., 50: 337-340.

Shirai, T., Fuke, S., Yamaguchi, K. and Konosu, S. (1983) Studies on extractive components of salmonids—II. Comparison of amino acids and related compounds in the muscle extracts of four species of salmo. Comp Biochem. Physiol., 74B: 685-689.

Simopoulos, A.P., Kifer, R.R., Martin, R.E., Barlow, S.M. (1991) Health effects of 3 polyunsaturated fatty acids in seafoods. World Rev. Nutr. Diet., 66: 581-592.

Simopoulos, A.P., Leaf, A. and Salem, N. Jr. (1999) Essentiality of and recommended dietary intakes for n-6 and n-3 fatty acids. Ann. Nutr. Metab., 43: 127-130.

Soderling, E., Le Bell, A., Kirstila, V. and Tenovuo J. (1998) Betaine-containing toothpaste relieves subjective symptoms of dry mouth. Acta Odontol. Scand., 56: 65-69.

Sørland G.H., Larsen P.M., Lundby F., Rudi A.P. and Guiheneuf T. (2004) Determination of total fat and moisture content in meat using low field NMR. Meat Sci., 66: 543-550.

Sotelo, C.G., Franco, J.M., Aubourg, S.P. and Gallardo, J.M. (1995) Changes in free amino acids of hake (*Merluccius merluccius* L.) muscle during frozen storage. Food Sci. Technol. Int., 1: 19-24.

Sundt, R.C. and Johansen, T. (1998) Low levels of interspecific DNA sequence variation of the mitochondrial 16S rRNA in North Atlantic redfish *Sebastes* (Pisces, Scorpaenidae). Sarsia, 83: 449-452.

Thorarinsdottir, K.A., Arason, S., Geirsdottir, M., Bogason, S.G. and Kristbergsson, K. (2002) Changes in myofibrillar proteins during processing of salted cod (*Gadus morhua*) as determined by electrophoresis and differential scanning calorimetry. Food Chem., 77: 377-385.

Thresher, R.E., Proctor, C.H., Gunn, J.S. and Horrowfield, I.R. (1994) An evaluation of electron-probe microanalysis of otoliths for stock delineation and identification of nursery areas in a southern temperate groundfish, *Nemadactylus macropterus* (Cheilodactylidae). Fish. Bull. 92: 817-840.

Tocher, D.R., Bell, J.G., Dick, J.R., Henderson, R.J., McGhee, F., Michell, D. and Morris, P.C. (2000) Polyunsaturated fatty acid metabolism in Atlantic salmon (*Salmo salar*) undergoing parr-smolt transformation and the effects of dietary linseed and rapeseed oils. Fish Physiol. Biochem., 23: 59-73.

Toften, H. and Jobling, M. (1996) Development of spinal deformities in Atlantic salmon and Arctic charr fed diets supplemented with oxytetracycline. J. Fish Biol., 49: 668-677.

Townsend, W.E., J.E. Thomson and J.R. Hutchins (1984) Coagulation test for cooked meat temperature: effect of variations in filtration. J. Food Sci. 49: 853.

Uddin, M. (2001) Determination of the maximum internal temperature of previously heated saury (*Cololabis saira*) meat. Asian J. Chem., 13: 99-106.

Uddin M. and Okazaki E (2004) Classification of fresh and frozen-thawed fish by near-infrared spectroscopy. J Food Sci., 69: C665-C668.

Undeland, I., Hultin, H.O. and Richards, M.P. (2002) An aqueos fraction of cod muscle inhibits haemoglobin-mediated oxidation of cod muscle membrane lipids. 32nd Annual WEFTA Meeting, May 13-15th, Galway, Ireland.

Urbon Artero, A., Aldana Gomez, J., Reig del Moral, C., Nieto Conde, C. and Merinero Cortes, B. (2002) Neonatal onset methylmalonic aciduria and homocystinuria: biochemical and clinical improvement with betaine therapy. An. Esp. Pediatr., 56: 337-341.

Veliyulin, E., van der Zwaag, C., Burk, W. and Erikson, U (2005) In-vivo determination of fat content in Atlantic salmon (*Salmo salar*) with a mobile NMR spectrometer, J. Sci. Food Agric., (in press)

Vielma, J., Makinen, T. Ekhol, P. and Koskela, J. (2000) Influence of dietary soy and phytase levels on performance and body composition of large rainbow trout (*Oncorhynchus mykiss*) and algal availability of phosphorus load. Aquaculture, 183: 349-362.

Vielma, J., Ruohonen, K. and Peisker, M. (2002) Dephytinization of two soy proteins increases phosphorus protein utilization by rainbow trout, *Oncorhynchus mykiss*. Aquaculture, 204: 145-156.

Villareal, B.W., Rosenblum, P.M. and Fries, L.T. (1994) Fatty-acid profiles in red drum muscle. Comparison between wild and cultured fish. Trans. Am. Fish. Soc., 123: 194-203.

Waples, R. (1998) Separating the wheat from the chaff: patterns of genetic differentiation in high gene flow species. J. Heredity, 89: 438-450.

Ward, R.D., Woodwark, M. and Skibinski, D.O.F. (1994) A comparison of genetic diversity levels in marine, freshwater, and anadromous fishes. J. Fish Biol., 44: 213-232.

Watabe, S., Hirayama, Y., Nakaya, M., Kakinuma, M., Kikuchi, K., Guo, X.F., Kanoh, S., Chaen, S. and Ooi, T. (1997) Carp expresses fast skeletal myosin isoforms with altered motor functions and structural stabilities to compensate for changes in environmental temperature. J. therm. Biol., 22: 375-390.

Watanabe, T. (1982) Lipid nutrition in fish. Comp. Biochem. Physiol., 73B: 3-15.

Wendel, U. and Bremer, H.J. (1984) Betaine in the treatment of homocystinuria due to 5,10-methylenetetrahydrofolate reductase deficiency. Eur. J. Pediatr., 142: 147-150.

Wilson, R.P and Cowey, C.B. (1985) Amino acid composition of whole-body tissue of rainbow trout and Atlantic salmon. Aquaculture, 48: 373-376.

Wolff, S.D., Yancey, P.H., Stanton, T.S. and Balaban, R.S. (1989) A simple HPLC method for quantitating major organic solutes of renal medulla. Am. J. Physiol. -Renal Physiol.- 256: F954-F956.

Wu, H.C. and Shiau, C.Y. (2002) Proximate composition, free aminoacids and peptide contents in comemrcial chicken and other meat essences. J. Food Drug Anal., 10: 170-177.

Yamori, Y., Nara, Y., Iritani, N., Workman, R.J. and Inagami, T. (1985) Comparison of serum phospholipid fatty acids among fishing and farming Japanese populations and American islanders. J. Nutr. Sci. Vitaminol. (Tokyo), 31: 417-422.

Ziegler, H., Osmond, C.B., Stichler, W. and Trimborn, P. (1976) Hydrogen isotope discrimination in higher plants: correlations with photosynthetic pathway and environment. Planta, 128: 85-92.