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Bioactive Compounds in Cod (*Gadus morhua*) Products and Suitability of ^{1}H NMR Metabolite Profiling for Classification of the Products Using Multivariate Data Analyses

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This work investigates the suitability of ^{1}H NMR spectroscopy to identify the fate of some bioactive compounds in seafood submitted to several processing conditions and examines the possibility of using ^{1}H NMR spectroscopy profiling to classify such products. Perchloric acid extracts of cod white muscle from newly killed and (i) unprocessed, (ii) boiled, and (iii) fried fillets and from (iv) frozen fillets, (v) the frozen fillets after thawing, and (vi) their drip loss and from (vii) rehydrated cod klippfish ($n = 5$) were analyzed by 500 MHz ^{1}H NMR spectroscopy. It was possible to identify taurine, betaine, anserine, creatine, and trimethylamine oxide (TMAO) in all extracts examined, and frozen fish was recognizable by the presence of dimethylamine (DMA). None of the heating procedures seemed to induce the loss of bioactive compounds from the fillet, but freezing and thawing did: the compounds were lost in what is known as drip loss. About 80% of the samples were correctly classified using a probabilistic neural network procedure having as inputs the scores of the first 20 principal components of the principal component analysis of a selected region of the NMR spectra.

KEYWORDS: Bioactive compounds; taurine; betaine; anserine; TMAO; TMA; DMA; osmolytes; metabolite profiling; ^{1}H NMR; *Gadus morhua*; cod; fish; fish processing; multivariate data analysis; probabilistic neural networks

INTRODUCTION

Fish has a reputation as a healthy food based on the positive effects of some bioactive compounds including their lipid, osmolyte, and protein contents. Regarding the lipid content, the fatty acids 20:5n−3 (EPA) and 22:6n−3 (DHA) are considered of relevance in particular for the prevention and alleviation of cardiovascular diseases (*1*, *2*). The main function of osmolytes is to stabilize the structure of proteins (*3*, *4*), but they have additional relevant roles in preserving human health which make them true bioactive compounds. Thus, taurine is known to regulate heart rhythm, contractile function, blood pressure, platelet aggregation, neuronal excitability, eyesight, cell proliferation and viability, and bile acid synthesis (*5*); betaine is important for liver function, cellular replication, and detoxification reactions in addition to playing a role in the synthesis of carnitine and protecting the kidneys from damage (*6*); anserine and carnosine are known antioxidants (*7*, *8*); and trimethylamine oxide (TMAO) helps to stabilize the structure of proteins (*3*, *4*) and has recently been shown to prevent the misfolding of the prion protein (*9*). Osmolytes also help to protect cells against osmotic stresses (*10*) and prevent oxidative damage (*8*). Finally, fish dietary protein has been shown to improve insulin response in some insulin-resistant tissues (*11*, *12*).

The osmolyte content in a sample may be expected to vary depending on the tissue, physiological state, environmental conditions, and post-mortem processing. For example, freezing and thawing are accompanied by a loss of water containing salts and osmolytes (drip loss). Overrein et al. (*13*), have shown that the content of many osmolytes in *Calanus finmarchicus* was greatly decreased by freezing and that taurine was completely lost after 4 h of resting after thawing. Freezing and thawing are also known to increase protein denaturation and degradation, lipid oxidation, and drip loss, with the associated loss of the stabilizing osmolytes and, one should expect, also of bioactive peptides.

We are interested in developing analytical techniques to verify product composition and claims that may be of relevance for

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cusumers. Our previous works have dealt with the analyses of proteins and DNA for species and breeding stock identification (*14*, *15*) and authentication of fish and fish oils based on the analyses of lipids (*14*, *16*). Both lipids and osmolytes are of relevance to the claim of fish as "healthy" food, but although there are many works addressing the lipid content and their modifications, there are fewer examining the fate of the small bioactive molecules. For that, we selected common industrial processing and preservation methods such as freezing, drying, and salting and two usual cooking methods, that is, boiling and frying.

The second aim of this work was to examine whether the variations in the metabolite profiles due to these processing conditions were important enough to permit the classification of the products. ^{1}H NMR spectroscopy was the selected technique to examine the metabolite profile due to its suitability to characterize complex biological mixtures (*17*−*22*). Some of the advantages it offers over more traditional techniques such as high-performance liquid chromatography are that (i) the detection is not limited by different properties of the molecules such as polarity or the presence of chromophores in the molecule, (ii) it renders information about the structure of the compounds from crude extracts with minimal sample preparation, (iii) unexpected and novel molecules will also be detected, (iv) the detection of the components is specific, and (v) all of the components are detected simultaneously. All of the previously mentioned bioactive compounds present in fish tissues—taurine, betaine, anserine, and TMAO—have been identified by one-dimensional ^{1}H NMR analyses, and their shift values have been published (*17*−*22*).

MATERIALS AND METHODS

Fish Samples. The authentic reference samples were five individuals of wild cod (*Gadus morhua*), of 3−4 kg of weight, that had been captured off the Trondheimsfjord in March. The fish were bled and gutted immediately after capture and delivered to our laboratory in ice within ∼3 h. Three cubes of approximately 4 × 4 × 2 cm were cut from the white skeletal muscle under the dorsal fin from each fish upon arrival: one cube was immediately frozen at −80 °C and then freeze-dried (control, fresh unprocessed samples), a second cube was submerged in boiling water for ∼5 min (boiled samples), and the third was fried in olive oil at ∼180 °C for ∼5 min (fried samples). After cooking, these samples were allowed to cool and freeze-dried.

Five frozen cod fillet products were purchased in a local supermarket. From each of the five samples, two cubes of approximately 4 × 4 × 2 cm were cut from the white skeletal dorsal muscle at the level of the dorsal fin: one cube was freeze-dried for perchloric acid extraction (frozen samples), and the second cube was allowed to thaw overnight at 4 °C and the liquid loss (drip loss) corresponding to each tube was collected. After thawing, both the fish flesh and the liquid loss were freeze-dried. These samples were called "frozen−thawed" and "drip loss" respectively.

Finally, five different products sold as dried salted cod (klippfish) were purchased in different local shops. They were soaked in a ratio of fish to cold water of 1:8 for 2 days with one change of water after 24 h. Cubes of approximately 4 × 4 × 2 cm were freeze-dried.

Perchloric Acid Extractions. Perchloric acid extraction was performed in ice according to the method of Glonek et al. (*23*). Approximately 15 mg of the freeze-dried powder was accurately weighed in an Eppendorf tube, and 1.5 mL of ice-cold 0.42 M $HClO_4$ was added. The mixture was kept for 5 min and then for an additional 15 min with regular mixing of the contents. The Eppendorf tubes were then centrifuged for 4 min at 1860*g* and 4 °C; 1.2 mL of each supernatant was taken out and precipitated with 675 μL of 0.36 M K_2CO_3. The tubes were centrifuged again for 4 min at 1860*g* and 4 °C. Then 1.2 mL of the supernatant was taken out into a new Eppendorf tube, frozen at −80 °C, and freeze-dried. The freeze-dried powder was stored at −80 °C until the NMR analysis was performed.

NMR Spectroscopy. The lyophilized samples were redissolved in 0.7 mL of 5 mM trimethylsilylpropinate-*2,2,3,3-d*$_4$ (TSP) in D_2O, and the pH was adjusted to 7.5 with 0.5 M NaOD. High-resolution ^{1}H NMR spectra were recorded at ambient temperature on a Bruker Avance DRX500 spectrometer operating at 500.13 MHz. The probe head used in the ^{1}H NMR was a 5 mm TXI probe (triple-resonance inverse probe 1H{13C.15N}) with shielded *z*-gradient (GRASP-II). The one-dimensional proton spectra were obtained using a water presaturation period of 5.5 s followed by a 90° excitation pulse. The sample was spun at 20 Hz to average field inhomogeneities in the *xy* plane, to improve resolution. A sweep width of 12 kHz was collected into 32K points, giving an acquisition time of 2.73 s. The number of scans was set to 512, and 8 dummy scans were applied. The raw data were multiplied with a 0.3 Hz exponential line-broadening factor before Fourier transformation into 64K data points. Chemical shift referencing was performed relative to the methyl groups of the standard TSP at 0.00 ppm.

The peaks in the spectra corresponding to the bioactive compounds anserine, betaine, creatine, and taurine and to others known to be present in cod (*4*) such as TMAO, TMA, DMA, glutamate, and glycine were identified by spiking with pure compounds and by comparison with published shift values for these compounds (*16*−*23*).

Multivariate Data Analysis. For input to multivariate analysis, FIDs were Fourier transformed into 16K (16384) data points. Regions without resonances as well as the regions corresponding to the compounds TSP (reference internal standard at 0 ppm) and TMAO (the dominant compound common to all samples at 3.25 ppm) were not included in the calculations due to their lack of discriminant value.

Spectra were then peak aligned to a reference spectrum (one of the fresh samples) using the beam search modification (*24*) of the genetic algorithm of Forshed et al. (*24*). For dimension reduction, we performed principal component analysis (PCA) (*25*) and kept the first 20 scores. PCA is a projection and dimension reduction method for transforming the original measurement variables into new, uncorrelated variables called principal components. Each principal component is a linear combination of the original measurement variables where the first principal component (PC1) accounts for the maximum of the total variance, the second (PC2) is uncorrelated with the first one and accounts for the maximum of the residual variance, and so on, until the total variance is accounted for.

The scores of the PCA were then used as input for a probabilistic neural network (PNN) analysis (*26*, *27*) using the programs NeuroShell Classifier/Neuroshell2 /AI Trilogy (Ward Systems Group, Inc., Frederick, MD).

RESULTS AND DISCUSSION

^{1}H NMR Spectra and Bioactive Compounds. Relatively large sample to sample variation was noted, mainly in the relative intensity of the peaks, that can be attributed both to the considerable individual variability known to be found among fish samples (*28*) and to the extraction procedure. We chose to freeze-dry the muscle samples prior to perchloric acid extraction expecting that this would help in the extraction, because it is easier and more efficient to extract and dissolve a powder than a semifrozen muscle sample, with a minimal sample alteration, given that freeze-drying is considered to be a good preservation method. However, protein analyses of these extracts performed later (results not shown) indicated that we cannot disregard that some oxidation and/or proteolysis and/or protein aggregation may have occurred and affected the composition of the extracts. Variability in the acquisition of spectra by NMR is considered to be minimal because we used an automatic sample analyzer and all of the extracts were run under identical conditions. **Figure 1** shows the spectrum corresponding to the perchloric acid extract of a fresh unprocessed muscle indicating the peaks corresponding to the bioactive compounds anserine, betaine, creatine, and taurine, as well as additional peaks corresponding to TMAO, TMA, glutamate, and glycine.

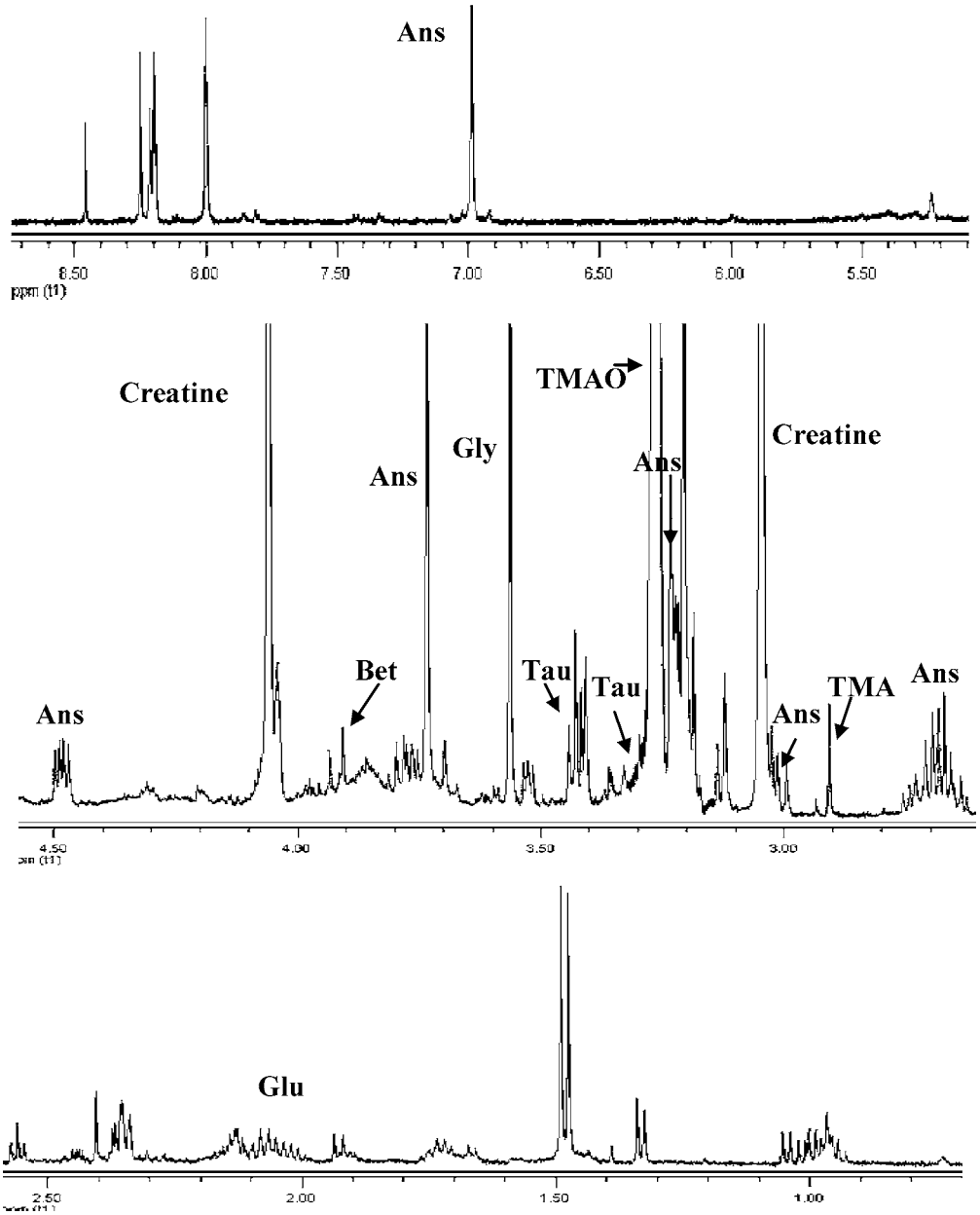

Figure 1. 500 MHz ^{1}H NMR spectrum of perchloric acid extract from white muscle of Atlantic cod (*Gadus morhua*) corresponding to one of the fresh unprocessed samples. Shift values (ppm) are from 0.7 to 2.6 (bottom), from 2.6 to 4.6 (middle), and from 5.1 to 8.7 (top). Abbreviations: Ans, anserine; Bet, betaine; Gly, glycine; Tau, taurine; Glu, glutamate.

Boiling and frying did not provoke major changes in the spectra (**Figure 2**), and the bioactive compounds did not seem to be reduced by these treatments: all of the identified compounds were present in relatively similar amounts, although both heat treatments seem to induce a loss of glycine from the fillet. Minor variations in the spectra are to be expected due to the treatment. Incorporation of substances from the oil cannot be totally discounted, but if it occurred, it did not seem to affect this analysis, which does not register changes in the lipid components.

Freezing, on the other hand, is known to induce the appearance of DMA in gadoids: upon death and during ice storage, the TMAO is metabolized to TMA due to bacterial activity (*29*). However, bacterial activity is arrested upon freezing and the TMAO is metabolized during the frozen storage period in gadoids to DMA and formaldehyde by the endogenous enzyme TMAOase (*30*). The formaldehyde is responsible for the toughening of these species during frozen storage (*31, 32*). Thus, unless the fish is badly spoiled prior to freezing, the DMA may be used as a marker for freezing in these species (*33*). Fresh fish is more highly priced than frozen fish, and it is not unusual to find that frozen fish is thawed and sold as fresh. DMA is therefore important as a marker for freezing in the life history of the product and to uncover this type of fraud. In the present work, DMA was detectable in all of the samples that we knew had been frozen, regardless of whether the extracts were performed on the frozen or on the frozen and thawed muscle and in the drip loss from these muscles (**Figure 3**). Interestingly, all of the klippfish samples contained DMA, indicating that they had been manufactured from frozen fish (**Figure 3)**. This information was not given in the label of the products.

As performed, the analysis did not permit the absolute quantification of the compounds, but it is possible to carry out relative estimations within each spectrum. Thus, although the cooking procedures used did not seem to affect the relative content of the identified bioactive compounds, freezing and

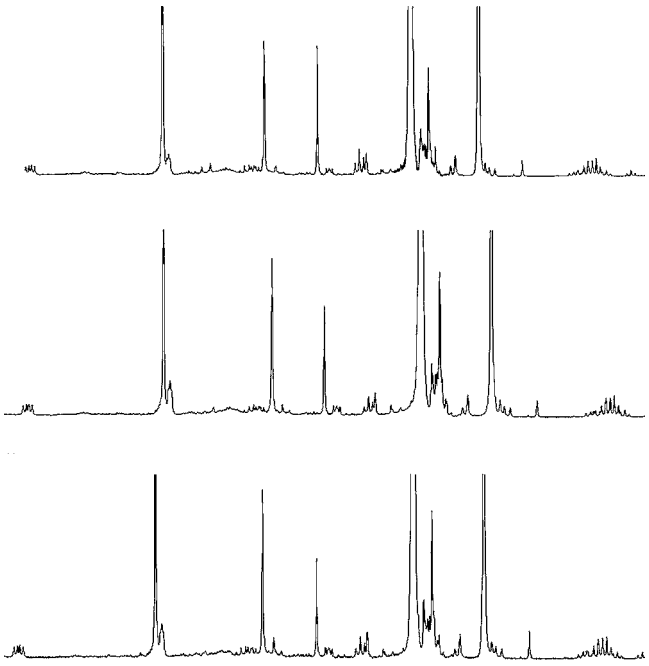

Figure 2. 500 MHz ^{1}H NMR spectrum of perchloric acid extract from white muscle of Atlantic cod (*Gadus morhua*) in the region of 2.5–4.5 ppm corresponding to the same fish: fresh and unprocessed (top), boiled (middle), and fried (bottom).

thawing did. There seemed to be an increase in the amount of detectable peaks in the thawed samples and especially in their corresponding drip water. This is not unexpected due to the degradation of bigger molecules or to their "liberation" from forms or compartments where they may have been bound in the live tissue, thus giving rise to more mobile small molecules than in the intact live muscle. In addition, one should expect an increase in the number and type of small compounds in the frozen and in the frozen and thawed samples than in the newly killed fish, because the post-mortem deterioration may have proceeded to a greater extent in these samples than in the fresh ones, taken about 3 h post-mortem. The spectra of the rehydrated klippfish samples were the poorest with regard to the number and amount of perchloric acid-soluble compounds. Again, this is to be expected because the drying and salting processes to which these products are submitted are known to induce protein aggregation, denaturation, and oxidation and some of the water soluble compounds that may have remained in the product must have been washed away during the rehydrating procedure. In addition, the fact that these samples had been frozen—shown by the presence of DMA—probably prior to salting may have added to the loss of these compounds from the fillet.

Klippfish were the products with the lower content in bioactive compounds. However, peptides produced from fish protein hydrolysates are also known to have clear positive effects (*11*, *12*). It would be very interesting to examine differences in the peptides produced from different fish products from the same species and tissues and whether they display a different effect on some selected human cells. It seems also clear from this work that drip loss is rich in antioxidants and amino acids, and it would be desirable to reduce their loss from the fillet or to somehow use it in the preparation of the final dish or add it back to the product. Due to that loss, products that are frozen and thawed are indeed poorer in positive compounds relevant for human health, which should be taken into account when correct product information and labeling are required. On the other hand, heating the cod fillets by either of the two selected methods did not seem to induce obvious changes in the metabolite profile or content in bioactive compounds.

Multivariate Data Analysis and Classification of Products. In principle, there is no need to identify all of the peaks or compounds in the spectra to classify the samples correctly. In some cases it can be decisive to be able to identify compounds that, by themselves, can be used as clear markers for some processing conditions. In these cases, the presence or absence of the diagnostic compound is sufficient to identify processing conditions that produce the marker compound. An example, as mentioned above, is DMA, a diagnostic compound for freezing. In the absence of diagnostic compounds valid for each processing condition analyzed (boiled, fried, salted, and dried) classification of samples based on the complex data obtained from ^{1}H NMR spectroscopy is best achieved by a suitable statistical treatment, usually based on multivariate data analyses. For the present work, we selected PCA of the spectra to reduce the number of dimensions, followed by a PNN analysis of the data (*27*). This approach, however, presents one problem, namely, the redundancy in the NMR data: in the NMR spectra each peak is the signal given by a proton type, so that sometimes one compound gives only one "peak" signal (as TMAO, TMA, or DMA) but, more often than not, biochemical compounds contain more than one type of proton and therefore originate several peaks that may be scattered along the whole spectrum, anserine being an example (**Figure 1**). This redundancy together with the fact that one should expect a significant part of the information contained in the spectra to be common to all samples, because all of them are cod white muscle, makes more difficult the correct classification of samples in reduced datasets such as the present one.

The results of the PCA for all 35 samples are shown in **Figure 4**. For reasons unknown to us, one of the drip loss samples behaved as an outlier in the model. Not unexpectedly, due to the more drastic processing conditions of salting and drying that induce oxidation (*34*), protein aggregation (*35*, *36*), and proteolysis (our unpublished results), the five samples of klippfish were shown to be very different from all of the other ones.

The agreement matrix resulting from the PNN analysis of 20 principal component scores from the 35 samples and 5194 data point matrix is shown in **Table 1**. The outlier drip loss sample could not be allocated to any class by the model. The group of frozen and thawed samples was correctly classified. In the rest of the groups the number of misclassifications varied between one and three samples (20−60%). Some of the misclassified samples were allocated to related classes; thus, one frozen sample was classified as frozen and thawed, whereas one sample in each group of cooked samples was incorrectly classified with regard to cooking procedure, the other four being correctly classified.

More serious were the misclassifications in the groups of fresh unprocessed fillet and klippfish. In the former, two samples were misclassified as frozen and one was misclassified as boiled; in the latter, one sample was misclassified as boiled cod and another as drip loss. This situation may have its origin in a well-known potential problem with neural network models with a relatively small number data, as is the present case: PNNs do not extrapolate, which implies that one must have training samples that are representative of the extremes of the distributions of input parameters in the original spectra and the subsequently derived scores. This may also be the reason for the higher number of misclassified samples in the group of fresh unprocessed samples: the individual variations noted among

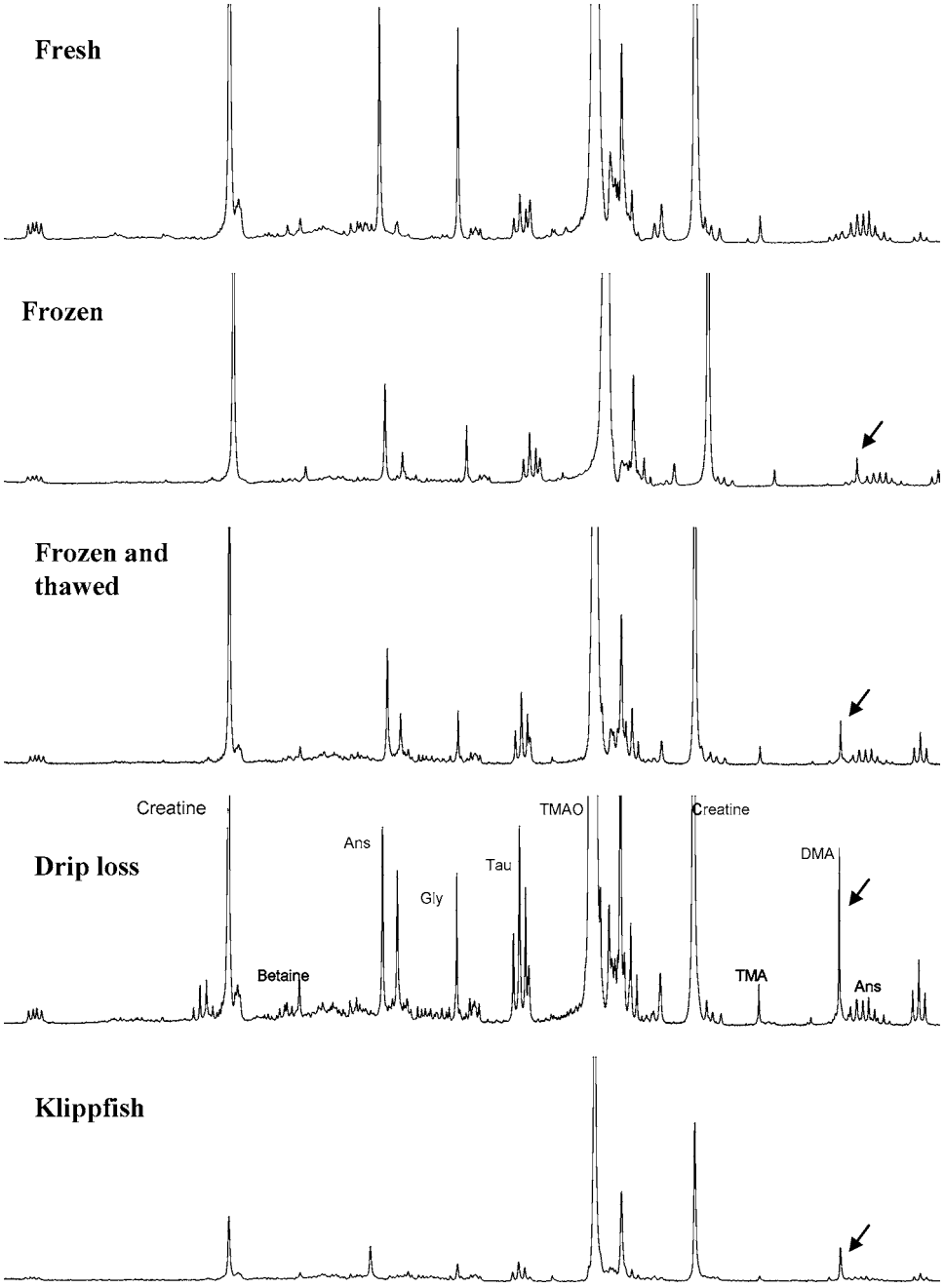

Figure 3. 500 MHz ^{1}H NMR spectrum of perchloric acid extract from white muscle of Atlantic cod (*Gadus morhua*) in the region of 2.5–4.5 ppm, showing DMA. Comparison of, from top to bottom, fresh unprocessed, frozen, frozen and thawed, drip loss, and klippfish samples. Relevant compounds are indicated in the drip loss spectrum; the abbreviations used are the same as in **Figure 1**. The arrow indicates the presence of DMA in the extracts of frozen and klippfish samples.

these samples should not apparently be a cause for misclassifications in this group only, because these samples were used to prepare the fried and boiled groups as well; therefore, one should expect a related degree of variation in these three groups. Moreover, relatively large individual variations are the rule rather than the exception in measurements of physiological data from fish (*28*).

When all of the unfrozen samples (the groups of unprocessed, boiled, and fried) were pooled as one, the 15 sample were correctly classified (**Table 2**). This regrouping also changed the classification of klippfish, the two misclassified samples of this group being now allocated to the drip loss group. The classification of the frozen and drip loss groups did not improve, with one misclassified sample in each group. Analysis of only the fresh samples, unprocessed, and cooked permitted a total correct classification of these samples (**Table 3**).

Evaluation of the suitability of metabolite fingerprinting for product classification is therefore complicated. Some of the main problems, as already mentioned, reside in the very nature of the data, the existence of redundancy, and the fact that unless the samples are very different, smaller differences may be obscured by the main bulk of common compounds found in closely related samples. It is therefore perhaps not surprising that frozen samples were not fully discriminated from unfrozen ones: the peak corresponding to DMA, although diagnostic of freezing, is reduced in significance by the presence of the multitude of remaining resonances, which are in essence irrelevant as diagnostic markers for this type of processing.

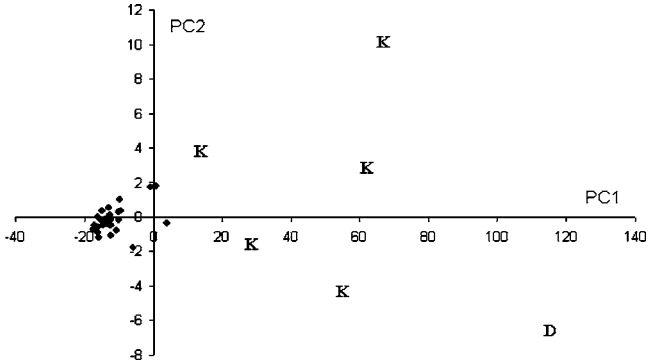

Figure 4. Principal component analysis scores plot of the 35 samples on PC1 (OX) and PC2 (OY). K represents the five klippfish samples, and D represents the outlier drip loss sample. The rest of the samples are represented by dots.

Table 1. Results of the PNN Using Seven Classes as Input[a]

	actual class of sample							
classified as	fresh unprocessed	boiled	fried	frozen	frozen thawed	drip loss	klippfish	total
fresh unprocessed	2							2
boiled	1	4	1				1	7
fried		1	4					5
frozen	2			4				6
frozen thawed				1	5			6
drip loss						3	1	4
klippfish						1	3	4
total	5	5	5	5	5	4	5	34

[a] The sample not classified here was the same "D" sample behaving as an outlier in the PCA model shown in **Figure 4**.

Table 2. Results of the PNN Using Five Classes as Input

	actual class of sample					
classified as	not frozen	frozen	frozen thawed	drip loss	klippfish	total
not frozen	15					15
frozen		3				3
frozen thawed		1	5			6
drip loss				4	2	6
klippfish				1	3	4
total	15	4	5	5	5	34

Table 3. Results of the PNN Using Three Classes as Input

	actual class of sample			
classified as	unprocessed	boiled	fried	total
fresh unprocessed	5			5
boiled		5		5
fried			5	5
total	5	5	5	15

Therefore, when clear, unambiguous, diagnostic resonances/compounds exist for certain processing conditions, they should be used for this purpose, and the remaining shift intensity information should be excluded. No multivariate analysis is required for such situations. This approach, easily applied to freezing, is not so straightforward when applied to temperature treatments, because changes in the sample composition start to occur as the temperature increases, and these changes do not need to be linear: there may be critical temperatures after which changes occur rapidly. In any case, use of metabolic fingerprinting for product classification demands the construction of databases containing as many samples as possible and with samples representing all extreme cases regarding natural variations in sample composition.

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