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ORIGINAL PAPER

Evaluation of NMR-based strategies to differentiate fresh from frozen-thawed fish supported by multivariate data analysis

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Abstract

The differentiation of fresh and frozen-thawed fish is a relevant authenticity aspect as in the European Union fish holds a high statistical risk of being adulterated. Here, nuclear magnetic resonance spectroscopy (NMR) in combination with principal components analysis followed by linear discriminant analysis (PCA-LDA) was used for a non-targeted based differentiation of fresh from frozen-thawed fish. To identify the most promising NMR approach(es), six different approaches were applied to 96 fish samples (mackerel, trout, cod). These approaches included different sample preparation procedures and different NMR methods to investigate both the lipid fraction and the polar fraction of the fish samples. After cross-validation embedded in a Monte Carlo resampling design, six independent classification models were obtained. Evaluation of the multivariate data analysis revealed that the most promising approaches were the ^{1}H NMR analysis of the lipid fraction (correct prediction of about 90.0%) and the ^{1}H NMR based screening of minor components of the lipid fraction with a correct prediction of about 91.9%. ^{1}H NMR analysis of the water extract of the fish samples showed a correct prediction of about 82.6%. Hence, a general differentiation of fresh from frozen-thawed fish via non-targeted NMR is feasible, even though the underlying sample batch contained different fish species. Additional fish samples need to be analyzed with the three most promising NMR approaches to further improve the developed classification models.

Keywords Food authenticity · Food fraud · Fresh frozen fish · Nuclear magnetic resonance spectroscopy (NMR) · Multivariate data analysis · Metabolomics

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Abbreviations

^{13}C NMR	13 Carbon nuclear magnetic resonance
^{1}H NMR	Proton nuclear magnetic resonance
^{31}P NMR	31 Phosphorus nuclear magnetic resonance
EDTA	Ethylene diamine tetra acetic acid
LDA	Linear discriminant analysis
NMR	Nuclear magnetic resonance
PCA	Principal components analysis
PCA-LDA	Principal components analysis in combination with linear discriminant analysis
TMS	Tetramethyl silane
TSP	3-(Trimethylsilyl)-propionic acid-2,2,3,3-d_4 sodium salt

Introduction

With the revelation of the horse meat scandal in 2013, the prosecution of food fraud became an increasingly important topic in the European Union and continues to be a challenging analytical topic. Adulterations may affect the whole

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spectrum of food categories, and a wide variety of adulterations have been detected in the past (e.g. adulterations regarding species and varieties, geographical origin, method of production, or valuable components) [1, 2]. Reliable analytical methods are needed for the prosecution of food fraud by food inspection authorities and to protect uninvolved food companies and retailers from financial loss as well as consumers from being misled [1, 2]. Fish and fishery products are assumed to be among the top 10 most adulterated food categories in the European Union [2, 3]. Here, one authenticity aspect is the differentiation of fresh from frozen-thawed fish. Due to the short shelf life of fishery products, freezing is a common practice for preservation [4, 5]. According to European law, frozen-thawed fish (e.g. gutted fish or fish fillets) needs to be labelled as such [6, 7]; a missing label would implicate that the fish product is fresh and unfrozen. As fresh fish is often higher-priced and an optical differentiation is often not possible, the labelling is important for the purchase decision of the consumer [4]. Additionally, frozen-thawed fish is more vulnerable for microbiological growth than fresh fish, whereby the extent depends on the freezing and thawing technology and further storage conditions [5].

Several analytical approaches for the differentiation of fresh from frozen-thawed fish were published in the past [4]. Histological examinations, enzymatic assays, and near-infrared spectroscopy based methods were often used. Changes in the microstructure of the fish flesh due to freezing and thawing, e.g. a shrinkage in muscle fibers and/or a larger extracellular space, were detected by histological analysis [4, 8–11]. As a consequence of cellular damage and osmotic effects in the fish flesh due to freezing and thawing, previously enclosed enzymes are released into intercellular and extracellular space. Thus, enzymatic assays were used to detect an increase in the activity of different enzymes in the fish juice [4, 12–17]. Near-infrared spectroscopy offers the advantage of minimal or no sample preparation, but generally requires extensive calibration and multivariate data analysis. In the past, this strategy was also applied to differentiate fresh from frozen-thawed fish [4, 18–24].

Besides the various analytical approaches that were published in the past, the outcome of the differentiation usually depends on the investigated fish species, the technology of freezing and thawing processes, and additional storage conditions (e.g. storage time and storage temperature). This complicates the implementation of a generally applicable and reliable analytical method, e.g. in the official food control.

Over the recent years, nuclear magnetic resonance spectroscopy (NMR) has been proven to be a powerful tool to detect potential food fraud [25–27]. NMR allows simultaneous detection and quantification of many analytes and has the advantage of providing additional structural information about the analyzed compounds. By using different extraction protocols, both hydrophilic and lipophilic metabolites can be investigated. In combination with multivariate data analysis, NMR data can also be used in non-targeted (metabolomics) approaches [25–27]. NMR based metabolomics has the potential to differentiate fresh from frozen-thawed fish as indicated by Shumilina et al. who studied the storage of salmon on ice [28].

In this work, the performance of NMR in combination with multivariate data analysis was further studied to differentiate fresh from frozen-thawed fish. To achieve a more general applicability of the methodology, different fish species with different fat contents, sea fish and freshwater fish from aquaculture as well as two different freezing methods were considered in the classification model.

Materials and methods

Fish samples

In total, 96 fish samples were collected in 2022 and 2023. Twenty-one samples were mackerel samples (*Scomber scombrus*, gutted fish (250–550 g), in one case a fillet (50 g)), 36 samples were trout samples (*Oncorhynchus mykiss*, *Oncorhynchus aguabonita* and *Salmo trutta fario*, gutted fish (300–1000 g), in one case a fillet (1250 g)), and 39 samples were cod samples (*Gadus morhua*, fillets (300–1500 g) and loins (200–600 g)). All samples were obtained raw and fresh, either from fish industry or from local merchants and supermarkets in south-western Germany. Overall, 58 fresh samples and 38 frozen-thawed samples were analyzed (see Supplementary Information, Table S1). Here, "fresh" indicated unprocessed fish samples, whether gutted or filleted, that have not undergone any treatment to ensure preservation other than chilling (in accordance with the definition of "fresh fishery products" in the European Law [29]). All fresh samples were stored on ice and directly analyzed the day after arrival at the laboratory. To obtain frozen-thawed samples, fresh samples were frozen either in a cold storage room (− 30 °C) or were quick-frozen with a blast freezer (4–6 m/s, − 30 °C, for at least 1 h 15 min). Frozen samples were stored in the cold storage room (− 30 °C) for at least seven and up to 30 days. Thawing was performed in a 2 °C-temperature controlled room for at least twelve and up to 24 h (depending on fish species and sample size). The frozen-thawed samples were stored on ice until analysis on the third day after thawing.

Chemicals

All reagents and standard compounds were of analytical or high-performance liquid chromatography grade. Cyclohexane (anhydrous, 99.5%), isopropanol (ACS

reagent, ≥ 99.5%), methanol-d_4 (99.8 atom % D), sodium cholate hydrate (≥ 97.0%), ethylene diamine tetra acetic acid (EDTA) disodium salt dehydrate (Titriplex® III), sodium hydroxide, sodium dihydrogen phosphate (99.0%), the internal standards for normalization (dimethyl sulfone (98.0%), glyphosate (98.5%) and maleic acid (≥ 99.9%)), and the internal reference standard 3-(trimethylsilyl)-propionic acid-2,2,3,3-d_4 sodium salt (TSP, 98 atom % D) were obtained from Merck (Darmstadt, Germany). Sodium chloride (> 99.8%), chloroform-d_1 (≥ 99.8 atom % D), and the internal reference standard tetramethylsilane (TMS) were from Carl Roth (Karlsruhe, Germany). Deuterium oxide (99.9% atom % D) was obtained from Deutero (Kastellaun, Germany). Before usage of chloroform-d_1, reactive degradation products were removed by washing with concentrated disodium carbonate solution and subsequent dehydration with oven-dried disodium carbonate as described by Teipel et al. [30].

Sample preparation

In total six different approaches were examined. For this, the lipid fraction was extracted and analyzed by four different NMR approaches (see below). The polar fraction was extracted using the fish juice and the water extract and analyzed by ^{1}H NMR (two approaches) (see Results and Discussion, Experimental Design, and Fig. 2). All six approaches (sample preparations and NMR measurements) were carried out at the same day for each sample.

a) *Preparation of the lipid fraction (for NMR approaches 1–4)*

Fat extraction. Gutted fish samples were beheaded, filleted, and deboned. The fish fillet was homogenized in a regular blender. Due to the different fat contents of cod, trout, and mackerel, fat extraction was performed according to either a lean fish (cod) or medium-fat fish (trout) and fat fish (mackerel) protocol. For fat extraction, 75 mL (lean fish) or 20 mL (medium-fat and fat fish) of a mixture of cyclohexane and isopropanol (2/1, v/v) was added to the mixed fish sample (40.0 ± 1.0 g lean fish, 8.0 ± 0.1 g medium-fat and fat fish). The sample was shaken for 30 min (lean fish: KL 2-Shaker, Edmund Bühler GmbH, Bodelshausen, Germany; medium-fat and fat fish: Multi Reax, Heidolph, Schwabach, Germany). A 5% (w/v) sodium chloride solution was added (20 mL (lean fish) or 5 mL (medium-fat and fat fish)), and the sample was shaken for another 30 min. The suspension was passed through a filter (MN 615 ¼ ff, 150 mm, Macherey Nagel, Düren, Germany) into a separating funnel, followed by rinsing with another 5 mL of a mixture of cyclohexane/isopropanol/5% sodium chloride (10/5/4, v/v/v). After 20 min, a complete phase separation was achieved, and the upper organic phase was collected. The organic phase was vaporized at 40 °C under a gentle stream of nitrogen to obtain the lipid fraction. The sample vessel was weighed before transferring the organic phase into the vessel and again after vaporization to document the weight of the extracted lipid fraction of the sample.

Preparation of the sample solution for ^{1}H NMR (first and second approaches) and ^{13}C NMR measurements (third approach). Different sample weighting protocols were adapted depending on the amount and nature (liquid or waxy/solid) of the gained lipid fraction. For lean fish, all of the extracted fat (about 100 mg) was used. If applicable for medium and fat fish, 40.0 ± 5.0 mg were weighed in; otherwise, all of the extracted fat (about 100 mg) was used. Each was dissolved in a mixture of chloroform-d_1 (containing 0.5% (v/v) TMS) and methanol-d_4 (containing 1.0 mg/mL dimethyl sulfone as an internal standard for normalization) (mixture ratio 1/1, v/v, 2.0 mL (if all of the extracted fat was used), 1.0 mL (if 40.0 ± 5.0 mg fat was used)). The fat sample weight per mL of sample solution was then calculated. A 600 µL-aliquot of the sample solution was transferred into an NMR tube (5-mm Boro 300–5-8, Deutero, Bad Kreuznach, Germany).

Preparation of the sample solution for ^{31}P NMR based phospholipid screening (fourth approach). To the lipid fraction (lean fish: all extracted fat (about 100 mg); medium-fat and fat fish: 100.0 ± 10.0 mg), 1.0 mL of an aqueous detergent solution [containing 10% (w/v) sodium cholate, 1% (w/v) EDTA, 20% (v/v) deuterium oxide, and 0.15 mg/mL glyphosate as an internal standard for referencing and normalization, with the pH adjusted to 7.4 with 6 M sodium hydroxide] was added. After short vortexing, the sample was treated for 10 min in an ultrasonic bath (Bandelin, Berlin, Germany) and centrifuged at 10,000 × *g* for 15 min at room temperature (Centrifuge 5415D, Eppendorf AG, Hamburg, Germany). A 600 µL-aliquot of the bottom transparent layer of the sample solution was transferred into an NMR tube (5-mm Boro 300–5-8, Deutero, Bad Kreuznach, Germany).

b) *Preparation of the polar fraction (for NMR approaches 5 and 6)*

Extraction of the fish juice (fifth approach). Gutted fish samples were beheaded, filleted, and deboned. Four pieces of fish muscle (see Fig. 1, only piece No. 1 in case of cod loins) were cut out of the fillet and each piece was centrifuged at 39,412 × *g* for 30 min at 15 °C (Sorvall RC 6, Thermo Fischer Scientific Inc., Karlsruhe, Germany). The obtained fish juice was collected, and the juices from all four fish pieces were combined. To remove lipids, the sample was centrifuged at 4,000 × *g*

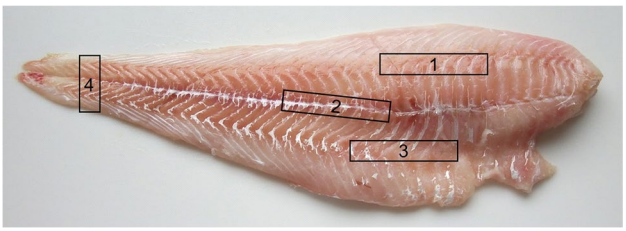

Fig. 1 Selected pieces of a fish fillet (here: cod) for the extraction of the fish juice. Each piece was centrifuged and the obtained fish juice was combined. In case of cod loins, fish juice was only extracted from piece No. 1

for 10 min at 21 °C. The lower aqueous layer was collected.

Water extraction (sixth approach). Gutted fish samples were beheaded, filleted, and deboned. The fish fillet was homogenized in a regular blender. Water (10 mL) was added to the mixed fillet (2.5 ± 0.1 g), and the sample was briefly vortexed. After 10 min of shaking (Multi Reax, Heidolph, Schwabach, Germany), the suspension was centrifuged at $4{,}000 \times g$ for 15 min at 21 °C. The aqueous layer (water extract) was collected.

Protein removal and sample solution for ^{1}H NMR measurements (fifth and sixth approach). An ultrafiltration filter (2 kDa, Hydrosart membrane, Vivaspin, Sartorius, Göttingen, Germany) was rinsed four times with 2 mL of water each to remove glycerol (centrifugation at $4{,}000 \times g$ and 21 °C for 20 min for the first three cycles, for 30 min for the fourth cycle (Heraeus Magefuge 16R, Thermo Fischer Scientific Inc., Karlsruhe, Germany)). After glycerol removal, 1.5 mL of the fish juice resp. the water extract was transferred to the ultrafiltration filter, followed by centrifugation at $4{,}000 \times g$ for 1.5 h at 21 °C for protein removal. An aliquot of the filtrate (500 µL) was mixed with 250 µL of a sodium dihydrogen phosphate buffer (1 M, containing 0.20 mg/mL maleic acid as an internal standard for normalization, with the pH

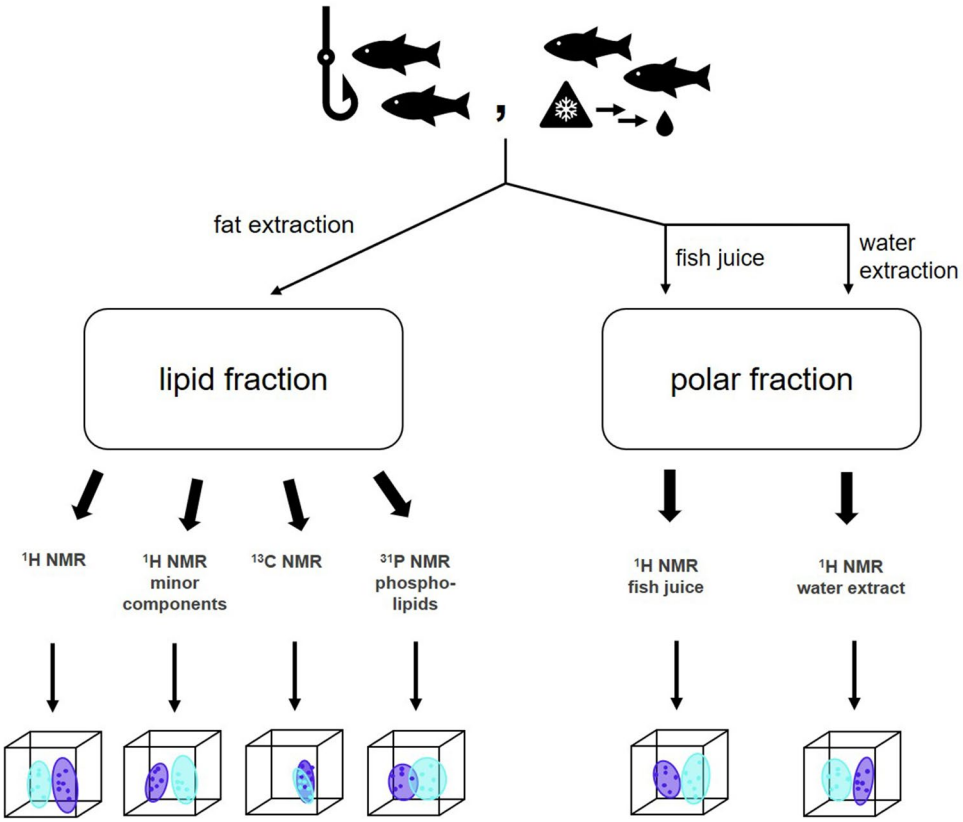

Fig. 2 Overview of the applied experimental design to identify a useful NMR method to differentiate fresh from frozen-thawed fish in terms of authenticity aspects. The lipid fraction (lipophilic metabolites; left side) was analyzed after a fat extraction of the fish samples. The fat extract of each sample was measured with ^{1}H NMR, a ^{1}H NMR based minor component screening, ^{13}C NMR and—after another sample preparation step—with ^{31}P NMR (phospholipid screening). The polar fraction (hydrophilic metabolites; right side) was analyzed in the fish juice of the samples as well as in the water extract of the mixed fillets. Both sample preparations were measured with ^{1}H NMR after protein removal. Multivariate data analysis (principal components analysis with linear discriminant analysis (PCA-LDA)) was applied to all datasets, resulting in four independent classification models for the lipid fraction and two independent classification models for the polar fraction

adjusted to 6.65) and 75 µL of TSP (0.06 M in deuterium oxide). Finally, 600 µL of the sample solution was transferred to an NMR tube (5-mm Boro 300–5-8, Deutero, Bad Kreuznach, Germany).

NMR analysis

NMR instrumentation. All ^{1}H NMR spectra were acquired on a Bruker 400 MHz AVANCE III HD spectrometer (Bruker Biospin GmbH, Rheinstetten, Germany) equipped with a 5-mm BBI (broadband inverse) probe and a Bruker automatic sample changer Sample Xpress. Deviating from this, a 5-mm BBO (broadband observe) probe was used to acquire ^{13}C NMR and ^{31}P NMR spectra.

NMR measurement and processing. The one-dimensional spectra of the samples for each approach were recorded under the same conditions. Bruker Biospin software Topspin (version 3.5) was used for data acquisition and processing. Prior to every measurement, automated tuning and matching as well as locking and shimming were performed. Spectra were acquired at 300 K, the time for temperature equilibration was 5 min for each sample. The detailed acquisition parameters for each approach are listed in the following sections. For processing, in each approach exponential line broadening was set to 0.3 Hz, followed by a Fourier transformation. Automatic spectral phasing and baseline correction were performed. Spectra were referenced to internal standards as listed in the following sections, too.

Lipid fraction, ^{1}H NMR (first approach). The P1 pulse length was calibrated for each sample. A standard Bruker pulse program zg with a relaxation delay (D1) of 10 s and an acquisition time of 4 s was used. Spectra were acquired with 64 k time domain data points, 64 scans, 2 dummy scans, a spectral width of 20.5617 ppm, and a receiver gain of 20.2. Spectra were referenced to the TMS signal at 0.00 ppm.

Lipid fraction, ^{1}H NMR based minor component screening (second approach). The P1 pulse length was calibrated for each sample and the presaturation pulse was adjusted to 25 Hz. Two ^{1}H NMR experiments were performed for each sample in automation procedure.

Experiment 1 (only used as preparation for the main measurement): A standard Bruker pulse program zg was used, applying a relaxation delay (D1) of 4 s, an acquisition time of 4 s, 64 k time domain data points, 16 scans, 4 dummy scans, a spectral width of 20.5617 ppm, and a receiver gain of 4. Spectra were referenced to the TMS signal at 0.00 ppm. Twenty frequencies of signals with the highest intensity in decreasing order were automatically identified in the spectrum.

Experiment 2 (main measurement): A standard Bruker pulse program noesygpps1d.comp2 was used, characterized by the suppression of the 20 frequencies of the major signals in the spectrum gained from experiment 1. The NMR experiment was acquired with a relaxation delay (D1) of 4 s, an acquisition time of 2 s, with 32 k time domain data points, 64 scans, 4 dummy scans, a spectral width of 20.5617 ppm, and a receiver gain of 16. A shaped pulse for presaturation was applied during the relaxation delay with a frequency spectrum of 20 highly selective bands to achieve highly selective suppression of the selected signals, leaving the rest of the spectrum undistorted. Two additional spoil gradients were applied to improve the signal suppression quality. Spectra were referenced to the TMS signal at 0.00 ppm.

Lipid fraction, ^{13}C NMR (third approach). A standard Bruker pulse program zgig with a relaxation delay (D1) of 5 s and an acquisition time of 2.5 s was used. The NMR experiment was acquired with 128 k time domain data points, 1024 scans, 4 dummy scans, a spectral width of 250.9622 ppm, and a receiver gain of 203. Spectra were referenced to the dimethyl sulfone signal at 43.90 ppm.

Lipid fraction, ^{31}P NMR based phospholipid screening (fourth approach). A standard Bruker pulse program zgig was used with a relaxation delay (D1) of 5 s and an acquisition time of 2.5 s, 16 k time domain data points, 1024 scans, 4 dummy scans, a spectral width of 19.9928 ppm, and a receiver gain of 203. Spectra were referenced to the glyphosate signal at 7.20 ppm.

Polar fraction: Fish juice, ^{1}H NMR (fifth approach). The P1 pulse length was calibrated for each sample and the presaturation pulse was adjusted to 25 Hz. A standard Bruker pulse program noesygppr1d (water suppression at 1880.7 Hz) with a relaxation delay (D1) of 15 s and an acquisition time of 8 s was used. The NMR experiment was acquired with 128 k time domain data points, 64 scans, 4 dummy scans, a spectral width of 20.5617 ppm, and a receiver gain of 128. Spectra were referenced to the TSP signal at 0.00 ppm.

Polar fraction: Water extract, ^{1}H NMR (sixth approach). Same NMR measurement and processing as for the fish juice samples.

NMR data preprocessing

Before conducting the multivariate data analysis of the spectra, NMR data were preprocessed using MATLAB version 2019b (The Math Works, Natick, MA, USA).

a) *Individual preprocessing steps for the approaches*

Lipid fraction, ^{1}H NMR (first approach). Spectra were normalized to the signal of dimethyl sulfone (2.95–3.08 ppm) and to a fat sample of 40.0 mg per 1.0 mL. The spectral region -0.50—9.50 ppm was divided into segments equal in width (buckets) and integrated. Three different numbers of buckets were tried (500, 1000 and 1500) and the regions around the signals of chloroform (7.54–7.66 ppm), residual water (4.50–4.94 ppm), meth-

anol (3.30–3.37 ppm), cyclohexane (1.40–1.47 ppm), and TMS (-0.20–0.20 ppm) were excluded.

Lipid fraction, ^{1}H NMR based minor component screening (second approach). Spectra were normalized to the signal of dimethyl sulfone (2.95–3.08 ppm) and to a fat sample of 40.0 mg per 1.0 mL. The spectral region -0.50 to 9.50 ppm was divided into buckets and integrated, three different numbers of buckets were tried (500, 1000 and 1500). The regions around the signals of chloroform (7.54–7.66 ppm), residual water (4.50–4.94 ppm), methanol (3.30–3.37 ppm), cyclohexane (1.40–1.47 ppm), and TMS (-0.20 to 0.20 ppm) were excluded.

Lipid fraction, ^{13}C NMR (third approach). Spectra were normalized to the signal of dimethyl sulfone (43.8–44.1 ppm) and to a fat sample of 40.0 mg per 1.0 mL. The spectral region -5.0 to 180.0 ppm was divided into buckets and integrated, three different numbers of buckets were tried (500, 1000 and 1500). The regions around the signals of chloroform (76.50–79.00 ppm), methanol (47.00–49.50 ppm), and dimethyl sulfone (43.80–44.10 ppm) signals were excluded.

Lipid fraction, ^{31}P NMR based phospholipid screening (fourth approach). Spectra were normalized to the signal of glyphosate (7.0–7.6 ppm) and to a fat sample of 100.0 mg per 1.0 mL. The spectral region -1.50 to 0.50 ppm was divided into buckets and integrated, three different numbers of buckets were tried (100, 250 and 500). No exclusions were needed.

Polar fraction: Fish juice, ^{1}H NMR (fifth approach). Spectra were normalized to the signal of maleic acid (6.02–6.06 ppm). The spectral region 0.50–9.20 ppm was divided into buckets and integrated, three different numbers of buckets were tried (500, 1000 and 1500). The regions around the signals of maleic acid (6.02–6.06 ppm) and residual water (4.72–5.06 ppm) were excluded.

Polar fraction: Water extract, ^{1}H NMR (sixth approach): Spectra were normalized to the signal of maleic acid (6.02–6.06 ppm) and to a fillet sample weight of 2.5 g. The spectral region 0.50–9.20 ppm was divided into buckets and integrated, three different numbers of buckets were tried (500, 1000 and 1500). The regions around the signals of maleic acid (6.02–6.06 ppm) and residual water (4.72–5.06 ppm) were excluded.

b) *Additional preprocessing steps performed in each approach*

After bucketing, a pseudo-scaling effect was achieved by a log type transformation [31]. Here, integrals > 1 were transformed to one plus the logarithm of the integral, whereas integrals with values ≤ 1 remained untreated.

Multivariate data analysis

The potential to differentiate fresh from frozen-thawed fish based on NMR data was evaluated using a combination of established multivariate data analysis tools including principal components analysis (PCA) with linear discriminant analysis (LDA) and a cross-validation embedded in a Monte Carlo resampling design. MATLAB version 2019b (The Math Works, Natick, MA, USA) was used for the analysis. Binary prediction models were created independently for each of the six data sets, respectively. A PCA was performed to reduce the dimensions, followed by an LDA to separate the two classes [26, 32]. The classification rule is based on the distances between the test object and the class means of the training set in the LDA-scores. That is, an object is assigned according to the nearest class mean. The number of the used principal components in the LDA was determined by a 5-times repeated fourfold cross-validation, and was selected based on the highest predictivity. To evaluate the classification performance a tenfold cross validation was applied. The cross-validation consisted a 10-times repeated 90% to 10% training and test sample splitting, whereas the splitting was performed after the same algorithm with the identical seed for each approach. The mean over the ten repetitions of the rates of correct and false class predictions were calculated for each class as percentages and presented in a confusion matrix. Additionally, the discrimination space is shown with a 95% prognosis ellipsoid of each group for one cross-validation step.

Results and discussion

Experimental design

Cod, trout, and mackerel were chosen as fish samples in the experimental design as they represent different fish species that cover the range of commercially relevant fish. With cod as a lean fish, trout as a medium-fat fish and mackerel as a fat fish, fish species with different fat contents were considered. Also, cod and mackerel are sea fish species, whereas trout is a typical freshwater fish from aquaculture. Only fresh fish samples were sourced. Freezing and thawing were performed in the pilot plant under realistic conditions to obtain frozen-thawed samples: Besides freezing in a cold storage room, blast freezing as a rapid freezing method was implemented in the experimental design. Fish flesh freezes in the critical core temperature range of 0 °C to − 5 °C. A rapid freezing technology is characterized by passing this range in less than 2 h [5]. In this pilot plant, blast freezing for 1 h and 15 min was sufficient to safely pass the critical core temperature range in gutted fish samples as well as fillet or loin samples. Therefore, all samples were blast-frozen for at

least 1 h and 15 min before being regularly stored in the cold storage room at − 30 °C. To avoid storage of the sourced samples for a longer period of time, six different analytical approaches were applied in parallel (see Fig. 2).

The applied sample preparation procedures (extraction of lipophilic metabolites, extraction of hydrophilic metabolites) as well as the NMR measurement and processing conditions were the results of extensive preliminary experiments (details in Tables S2, S3 and S4). The selection process took into account that subsequent multivariate data analysis requires both an efficient and (even more important) a highly reproducible metabolite extraction [27]. Thus, lipid extraction was optimized regarding the nature and ratio of organic solvents, potential acid catalysis (which was not applied), improved phase separation, and potential two-step extraction. Also, mixing was studied (shaker vs. ultra-turrax). Due to the wide range of fat contents in fish species, it was necessary to establish adapted protocols for the fat extraction of lean fish (in this case cod) and for the fat extraction of medium-fat and fat fish (in this case trout and mackerel). For the ^{1}H NMR measurements of the fat extracts, the solvent (namely the ratio of chloroform-d_1 and methanol-d_4) was optimized. Additional sample preparation steps to investigate the phospholipids in the fat extract via ^{31}P NMR were performed in accordance with the described optimized method of Ahmmed et al. [33], except the usage of a non-heated ultrasonic bath. For the analysis of the hydrophilic metabolites, the following parameters have been optimized: protein removal method, (after choosing ultrafiltration as protein removal method) type of ultrafiltration filters, filter washing-protocol for glycerol removal, ultrafiltration time, and buffer of the sample solution (buffer capacity and pH value, no additional pH titration needed). The NMR measurements of the six approaches were each optimized regarding D1-delay, receiver gain, number of scans, acquisition time, and measurement temperature. In case of the ^{1}H NMR measurements of the fish juice and the water extract, water suppression was also optimized.

As a result of the optimization procedure, fat extraction was performed with cyclohexane/isopropanol (2/1, v/v), followed by four different NMR measurements of the fat extracts. In the first approach, a common ^{1}H NMR measurement of the fat extract was performed to analyze all extracted lipophilic metabolites. As shown in the literature, ^{1}H NMR based analysis of lipids in combination with multivariate data analysis was proven to be a promising tool for different authenticity aspects in different kinds of food [34–37]. Precise suppression of some major signals during the NMR measurement improves the detection of minor components in complex samples. Therefore, in the second approach, the frequencies of the major signals were suppressed in the ^{1}H NMR based analysis (minor component screening). In the third approach, ^{13}C NMR measurements of the fat extracts were performed. ^{13}C NMR measurements have the disadvantage of a relative sensitivity of only 1.74 * 10^{-4} in comparison to ^{1}H NMR due to both the lower natural abundance and the lower gyromagnetic ratio [38]. However, ^{13}C NMR is known to be better suited to study the different fatty acids as compared to ^{1}H NMR because fatty acid signals overlap more heavily in ^{1}H NMR spectra. Thus, a better resolution is achieved in the ^{13}C NMR spectra [39]. Amphiphilic phospholipids are increasingly captured in the lipid fraction if the fat extraction is performed with a mixture of solvents including more polar solvents such as isopropanol. Ahmmed et al. [33] developed a ^{31}P NMR based method to precisely analyze phospholipids and their degradation products (e.g. glycerophosphocholine as a hydrolysis product). An ^{31}P NMR based analysis of the fat extract (after transferring the phospholipids in a H_2O/D_2O-phase in accordance with the method of Ahmmed et al.) was incorporated into the experimental design, too (fourth approach).

Also based on the optimization procedure, two different sample preparations were pursued to study hydrophilic metabolites. Accordingly, fish juice, which is also used in enzymatic assays to differentiate fresh from frozen-thawed fish, was analyzed (fifth approach). Fish juice represents the polar metabolome of a fish sample without greater impact (e.g. blending of the fish fillet). Additionally, fish samples were homogenized and then extracted with water (sixth approach). Sample preparation in the latter case followed the method of Decker et al. [40] who provided a reliable differentiation of meat species based on the water extract of meat samples. Both approaches for the polar fractions were analyzed using ^{1}H NMR. Protein removal was necessary to avoid signal overlapping with the broad protein signals in the spectral regions 0.5–5.0 and 6.5–9.0 ppm.

NMR analysis of fish samples

In Fig. 3, representative NMR spectra of the lipid extracts using the four different approaches are shown. The lipid fraction of fish is characterized by triglycerides, free fatty acids, phospholipids, and esters of cholesterol. Fish is known for being rich in unsaturated fatty acids [39, 41], resulting in characteristic signals in the ^{1}H and ^{13}C NMR spectra [39] (Fig. 3A-C). The advantage of the ^{31}P NMR based phospholipid analysis is demonstrated by comparing the ^{31}P NMR spectrum (Fig. 3D) to the ^{1}H NMR spectrum (Fig. 3A). In the ^{31}P NMR spectrum, phospholipids and their degradation products result in multiple signals in the range of -1.5 to 0.5 ppm as they differ structurally in the organic base molecule and, more importantly, in the number and kind of fatty acids [33]. In the ^{1}H NMR spectrum, phospholipids can only be assigned by signals of their organic base molecule (e.g., phosphatidyl choline, signal at around 3.2 ppm [39]). The signals of the protons of the phospholipid fatty acids overlap

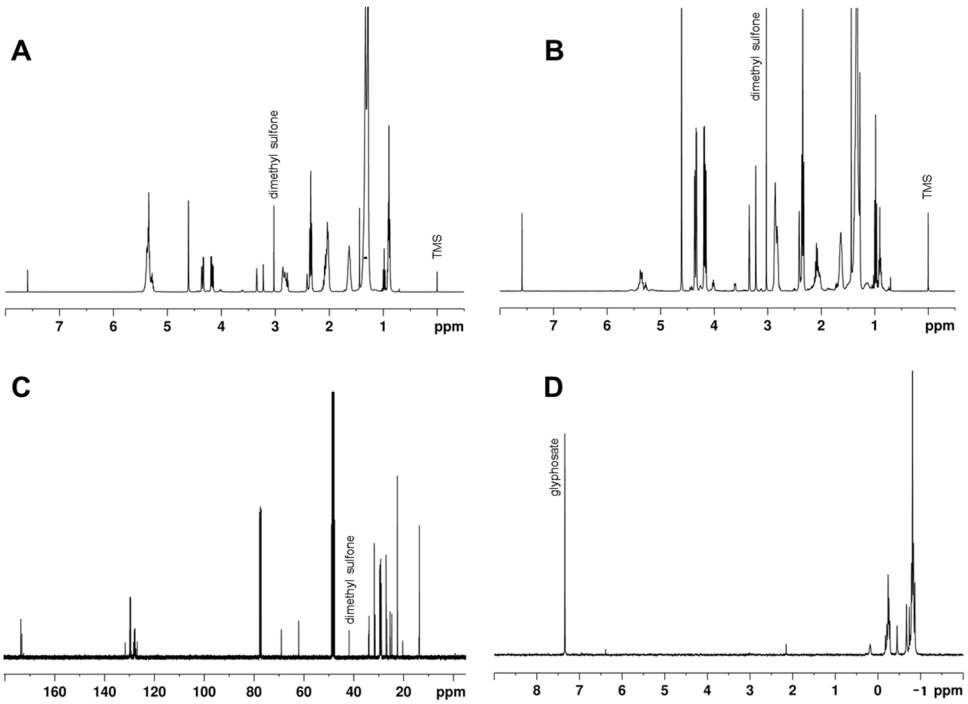

Fig. 3 Representative NMR spectra of the lipid fraction of one fresh trout sample (*Oncorhynchus aguabonita*). **A** ^{1}H NMR spectrum in $CDCl_3$/$MeOD_4$ (1/1, v/v), 400 MHz, 300 K, referenced to δ_{TMS} = 0.00 ppm **B** ^{1}H NMR spectrum of the minor component screening in $CDCl_3$/$MeOD_4$ (1/1, v/v), 400 MHz, 300 referenced to δ_{TMS} = 0.00 ppm **C** ^{13}C NMR spectrum in $CDCl_3$/$MeOD_4$ (1/1, v/v), 400 MHz, 300 K, referenced to $\delta_{dimethyl\ sulfone}$ = 43.9 ppm. **D** ^{31}P NMR spectrum in H_2O/D_2O pH 7.40, 400 MHz, 300 K, referenced to $\delta_{glyphosate}$ = 7.20 ppm

with the signals of free fatty acids or triglyceride-bound fatty acids. Thus, ^{31}P based signals of the different metabolites showed a better resolution than ^{1}H NMR based signals.

Representative NMR spectra of the two approaches to study the polar fraction of the fish samples are shown in Fig. 4. Free amino acids and free dipeptides, nucleotides and related compounds, organic acids, creatine, quaternary ammonium compounds (e.g. trimethyl aminoxide), and breakdown products from amino acids (e.g. taurine) are typical metabolites that could be found in ^{1}H NMR spectra of the polar fraction of fish, as they have been identified in the literature before [41, 42]. In this pilot study, no concluding attempts were made to assign the signals unambiguously to the compounds mentioned, as the focus has been on a general non-targeted differentiation of fresh and frozen-thawed fish. The ^{1}H NMR spectra of the fish juice (Fig. 4A, fifth approach) and of the water extract (Fig. 4B, sixth approach) consistent mostly of signals with the same chemical shift and multiplicity, differing only in signal intensity. For the multivariate data analysis, NMR measurements and preprocessing steps were carried out equally for each of the fish samples.

Multivariate data analysis of the lipid fraction

In total, 96 fish samples (58 fresh, 38 frozen-thawed) were analyzed by each of the four approaches to study the lipid fraction. To evaluate each approach regarding the ability

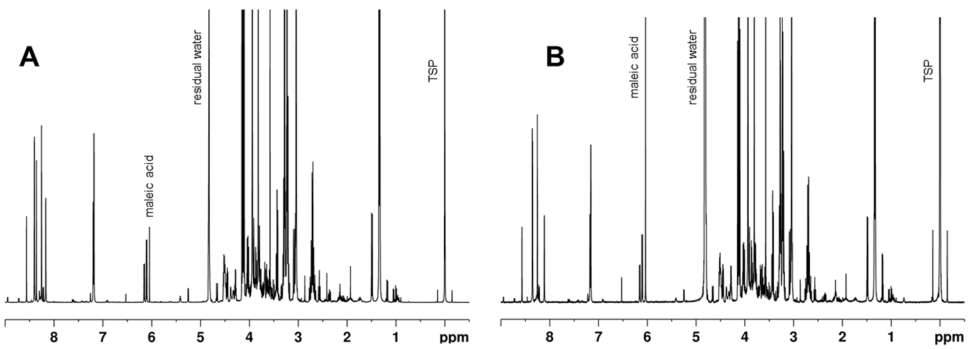

Fig. 4 Representative NMR spectra of the polar fraction of one fresh trout sample (*Oncorhynchus aguabonita*). **A** ^{1}H NMR spectrum of the fish juice after protein removal in H_2O/D_2O pH 6.65, 400 MHz, 300 K, referenced to δ_{TSP} = 0.00 ppm **B** ^{1}H NMR spectrum of the water extract after protein removal in H_2O/D_2O pH 6.65, 400 MHz, 300 K, referenced to δ_{TSP} = 0.00 ppm

to differentiate the group "fresh" from the group "frozen-thawed", a PCA-LDA for multivariate data analysis was applied in each case. Preprocessing included normalization to an internal standard and normalization to sample weight to avoid variations due to pipetting, weighing, or dilution. Bucketing was performed for data reduction and to provide the input variables for the following multivariate data analysis. After trying three different numbers of buckets, the number of buckets was chosen based on the highest predictivity after PCA-LDA in each approach. Following bucketing and integration, the data were subjected to a log-transformation that provided a pseudo-scaling effect of higher and lower values. To distinguish between fresh and frozen-thawed fish, PCA was used for dimension reduction and LDA for class separation and prediction. As multiple dimensions can potentially be used in the multivariate data analysis, the number of the principle components that offered the highest predictivity were identified first. Finally, the classification performance was evaluated by a cross-validation. Here, a Monte Carlo resampling design with a random segmentation for every cycle was used to avoid any segmentation bias.

As shown in Fig. 5, ^{1}H NMR analysis (Fig. 5A) and ^{1}H NMR based minor component screening (Fig. 5B) of the lipid fractions showed the best results. In the first approach, the integrals of 1500 buckets (after exclusions: 1358 buckets) led to the use of the first 33 principle components for the LDA-model. These 33 dimensions described 99.8% of the total variance of the data. For the ^{1}H NMR based minor component screening, 905 buckets (originally 1000 buckets, after exclusions) and the first 31 principle components were used, which described 99.8% of the total variance. The resulting classification models of both approaches successfully separated the two groups "fresh" and "frozen-thawed" with average accuracies for correct classification of 90.0% (^{1}H NMR, Fig. 5A) and 91.9% (^{1}H NMR based minor component screening, Fig. 5B). These promising results were achieved even though different fish species with different fat contents were used to build the models. Data analysis of the ^{13}C NMR spectra led to the use of 1000 buckets, whereof

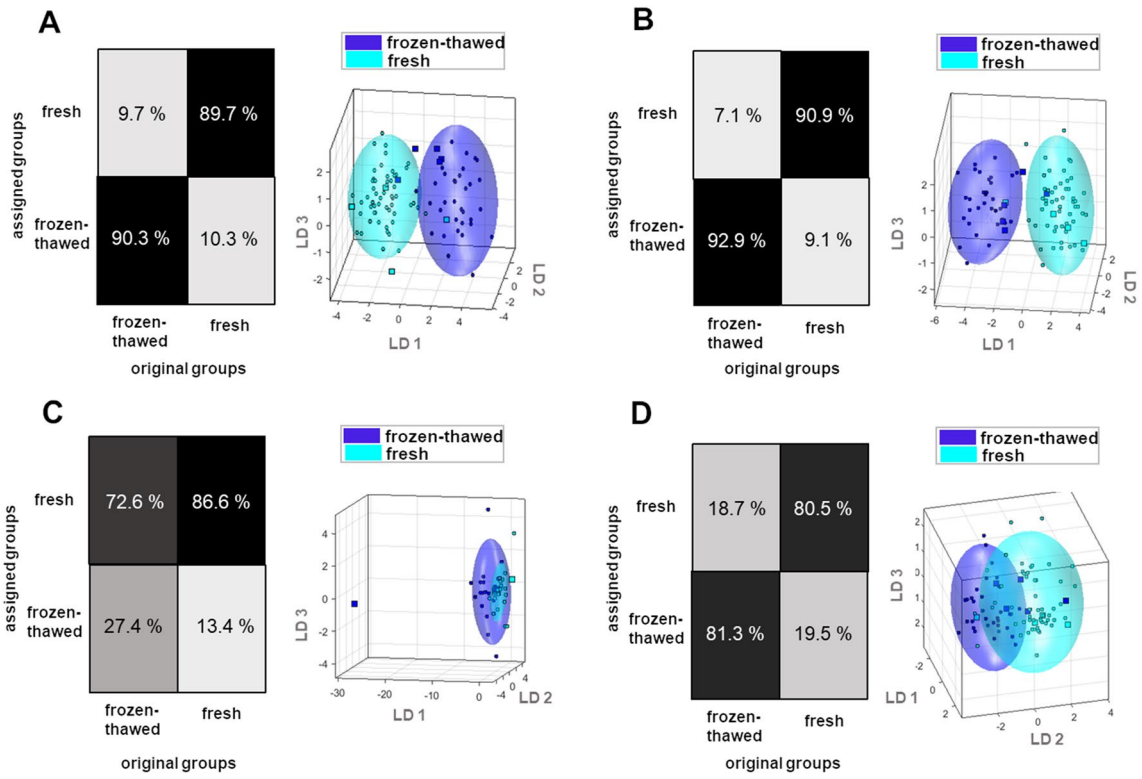

Fig. 5 Results of the embedded Monte Carlo cross-validation (MCCV) to evaluate the performance of the obtained PCA-LDA based classification models for the prediction of fresh fish (turquoise, 58 samples) and frozen-thawed fish (blue, 38 samples) based on the lipid fraction. The underlying data were obtained from **A** ^{1}H NMR spectra of the lipid fraction **B** ^{1}H NMR spectra of the minor component screening of the lipid fraction **C** ^{13}C NMR spectra of the lipid fraction, and **D** ^{31}P NMR spectra of the lipid fraction (including phospholipids). The left figures in **A** to **D** show the confusion matrices of the MCCV. The original groups are horizontally depicted, whereas the assigned groups are vertically depicted. The confusion matrix demonstrates the accuracies about the frequency of the prediction result in percent. The figures on the right side in **A** to **D** show the discrimination space of one cross-validation step, characterized by the 95 % prognosis ellipsoid of each group. The test set samples are marked as rectangles, whereas the samples of model building are marked as dots

971 buckets remained after the exclusions. The first 23 principle components of the PCA that described 99.9% of the total variance of the data were used in the LDA. However, PCA-LDA based group separation was not satisfactory (see Fig. 5C). With PCA being an unsupervised method, class affiliations are not considered while maximizing the total variance of the data. Despite the high percentage of explained variance, the ^{13}C NMR data may not include sufficient useful information for the differentiation of fresh from frozen-thawed fish. This might be due to the lower sensitivity of the ^{13}C NMR measurements. The good prediction results of the ^{1}H NMR based minor component screening suggests that the classification is based—at least in part—on minor components of the lipid fraction. Because ^{1}H NMR based approaches were successful and ^{1}H NMR has the advantage of shorter measurement times compared to ^{13}C NMR, additional data analysis of the ^{13}C spectra (such as applying a supervised method for classification or data reduction) was not further investigated. Multivariate data analysis of the ^{31}P NMR spectra showed a tendency for separation and classification. The scores of the first 15 principle components described 96.7% of the total variance of the 250 buckets. The average accuracy for correct classification using PCA-LDA was about 80.9% (Fig. 5D). However, due to the better prediction rates of the ^{1}H NMR based models, ^{1}H NMR analysis or ^{1}H NMR based minor component screening should be preferred for the analysis of the lipid fraction to differentiate fresh from frozen-thawed fish.

Fish oils were already widely studied with both ^{1}H and ^{13}C NMR [39]. In addition, ^{13}C NMR based classifications of fish samples regarding different gadoid species [43] or the wild, farmed, and geographical origins of salmon [44] were possible. To the best of our knowledge, however, differentiation of fresh from frozen-thawed fish based on ^{1}H or ^{13}C NMR analysis of the lipid fraction in combination with multivariate data analysis has not been attempted before. Also, although ^{31}P NMR based authentication approaches were described in the literature (with olive oils, milk and milk powder being the targets [45–48]), fish has not been extensively studied with ^{31}P liquid-state NMR.

Multivariate data analysis of the polar fraction

Two approaches to differentiate fresh from frozen-thawed fish were based on the analysis of the polar fraction of the fish samples. Multivariate data analysis was performed as described for the lipid fraction. Application of PCA-LDA to the data of the 96 samples resulted in a tendency for separation and classification for both approaches (Fig. 6). 500 buckets (479 buckets after exclusions) were defined for the analysis of the ^{1}H NMR spectra of fish juice after protein removal. The first 37 principle components, which described 98.7% of the total variance of the data, were used for LDA. The average accuracy for correct classification was 84.3% (Fig. 6A). The average accuracy for correct classification based on the analysis of the ^{1}H NMR spectra of water extracts after protein removal was slightly lower (82.6%, Fig. 6B). Here, the first 37 principle components, which described 97.2% of the total variance of the 1436 buckets (originally 1500 buckets, after exclusions), were used for LDA.

However, in addition to the average accuracy for correct classification a few more (practical) aspects need to be considered. Extraction of the fish juice can be challenging.

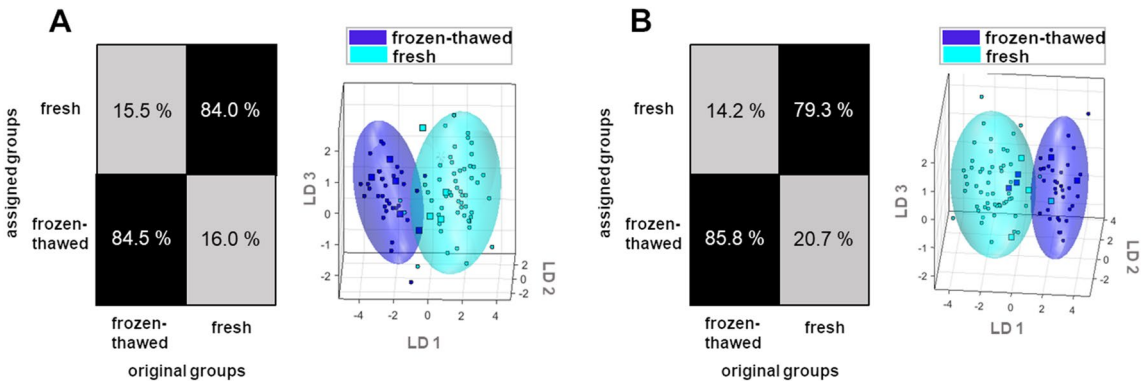

Fig. 6 Results of the embedded Monte Carlo cross-validation (MCCV) to evaluate the performance of the obtained PCA-LDA based classification models for the prediction of fresh fish (turquoise, 58 samples) and frozen-thawed fish (blue, 38 samples) based on the polar fraction. The underlying data were obtained from **A** ^{1}H NMR spectra of the fish juice after protein removal **B** ^{1}H NMR spectra of the water extract after protein removal. The left figures in **A** and **B** show the confusion matrices of the MCCV. The original groups are horizontally depicted, whereas the assigned groups are vertically depicted. The confusion matrix demonstrates the accuracies about the frequency of the prediction result in percent. The figures on the right side in **A** and **B** show the discrimination space of one cross-validation step, characterized by the 95% prognosis ellipsoid of each group. The test set samples are marked as rectangles, whereas the samples of model building are marked as dots

Especially for fish species with higher fat content, e.g. mackerel, higher centrifugation performance (namely 39,412 × *g*) was needed; mechanical squeezing of the fish samples was not successful. During ultrafiltration-based protein removal of the fish juice samples, filters tended to clog early, especially when trout or mackerel samples were prepared. This suggests different protein contents or different protein structures in the juice of the different fish species. As the goal of methods for fish authenticity analysis is also their application in laboratories of the official food control, an easy and reliable method is preferred. The approach that is based on the water extract does not require special equipment (high-performance centrifuge) and protein removal is unproblematic. Therefore, although a little weaker in its classification accuracy, the water extract based approach appears to be more suitable and should be further investigated.

A previous study that focused on salmon demonstrated the differentiation of fresh from frozen-thawed fish by ^{1}H NMR based analysis of hydrophilic metabolites [28]. Shumilina et al. stored fresh and frozen-thawed salmon over several days on ice and analyzed the samples on different days, quantifying several metabolites. Additionally, they observed a separation of the groups "fresh salmon" and "frozen-thawed salmon" in the discrimination space after applying a PCA to the data. Based on their quantification study over several storage days, they proposed aspartate as a possible marker substance for frozen-thawed salmon [28]. Besides the focus on salmon only, the cited study differs in experimental details, too. The salmon samples were extracted with 7.5% (w/v) aqueous trichloroacetic acid [28]. Although this procedure has the advantage of performing extraction and protein removal in one step, acid-induced modifications of the hydrophilic metabolites cannot be excluded. Also, a more efficient and reproducible extraction of the metabolites was found when the native fish juice or the extract with water was analyzed after protein removal via ultrafiltration.

Conclusion

Although several NMR-based non-targeted metabolomics approaches to differentiate fresh from frozen-thawed fish are theoretically possible, to the best of our knowledge, many have not been attempted before. In this work, it is shown that the approaches differ in their classification accuracy as well as in their feasibility. Two ^{1}H NMR based methods that were applied to the lipophilic metabolites (measuring all metabolites or with a focus on the minor components) and one ^{1}H NMR based method applied to the hydrophilic metabolites are the most promising approaches. Focusing on the lipophilic metabolites results in the best classification accuracies (≥ 90%). Consequently, these approaches merit further studies that increase the number of fish samples to refine the classification models. Additionally, the spectral areas (and ideally some maker compounds after signal assignment) that are relevant for the differentiation need to be identified. Whether these classification models based on mackerel, trout, and cod are also suitable for the differentiation of fresh and frozen-thawed fish samples from other fish species, should be further examined, too.

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Data availability The authors confirm that the data supporting the findings of this study are available within the article and its supplementary materials. The raw datasets obtained in this study will be deposited with the Zenodo repository.

Declarations

Conflict of interest The authors declare that there are no conflicts of interest.

Financial interests The authors declare they have no financial interests.

Compliance with ethics requirements This article does not contain any studies with human or living animal subjects.

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