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ORIGINAL PAPER

# Non-targeted analysis of lipophilic and hydrophilic metabolites to distinguish between fresh and frozen-thawed fish of certain fish species using comprehensive $^{1}$H NMR spectroscopy and multivariate data analysis

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**Abstract**

Food fraud along the production chain is a well-known issue that requires an effective authenticity control. For the differentiation of fresh and frozen-thawed fish, $^{1}$H nuclear magnetic resonance (NMR) spectroscopy based methods in combination with multivariate data analysis have proven to be suitable in principle. Here, from a total of 317 samples (cod, rainbow trout, mackerel; fresh and frozen-thawed), the lipid and polar fractions of the fish flesh were analyzed, and classification models based on a principal components analysis with linear discriminant analysis (PCA-LDA) including cross-validation were generated. Additionally, data fusions were carried out. The obtained average accuracies of > 90% (94.0% based on the lipid fraction, 92.8% based on the polar fraction) and > 95% (95.6% based on a low-level data fusion, 95.5% based on a mid-level data fusion) demonstrated a promising differentiation. Further examinations confirmed that the non-targeted analysis appears to be mandatory as no marker substances were indicated in the loadings plots of the models. To evaluate whether the generated classification models are suitable to be used in a broader manner, they were applied to 13 fresh and 13 frozen-thawed samples from twelve other common edible fish species in a preliminary study. The classification model based on the low-level data fusion gave the best results (84.6% of all 26 samples correctly predicted). Thus, although these models are very suitable for analyzing cod, rainbow trout, or mackerel for a classification as fresh or frozen-thawed, they cannot generally be applied to samples of other fish species.

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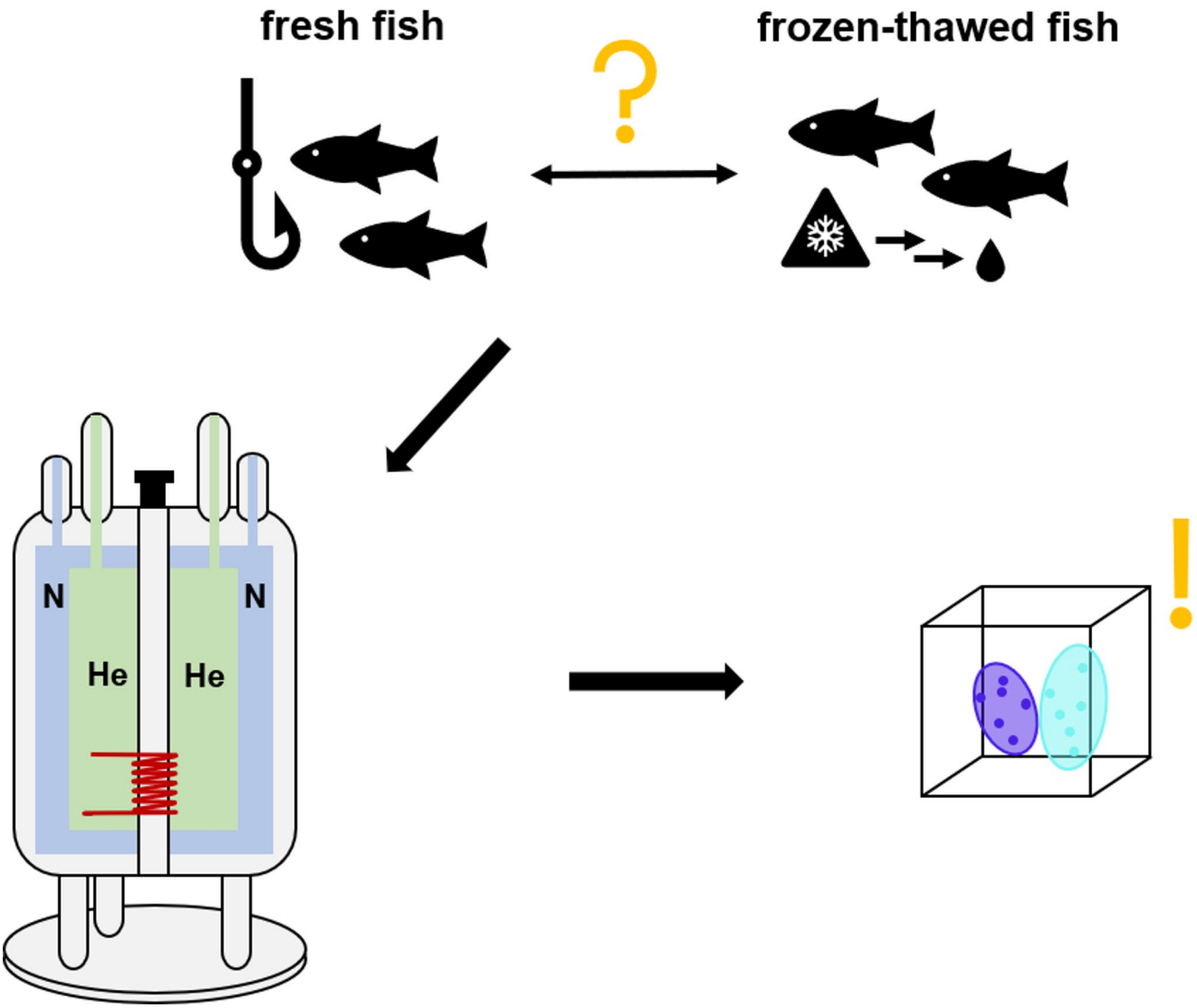
**Graphical Abstract**




**Keywords** Food authenticity · Food fraud · Fresh fish · Frozen-thawed fish · NMR spectroscopy · Multivariate data analysis

**Abbreviations**

| | |
|---|---|
| $^1$H NMR | Proton nuclear magnetic resonance |
| LDA | Linear discriminant analysis |
| NIR | Near-infrared |
| NMR | Nuclear magnetic resonance |
| PCA | Principal components analysis |
| PCA-LDA | Principal components analysis in combination with linear discriminant analysis |
| TMS | Tetramethylsilane |
| TSP | 3-(Trimethylsilyl)-propionic acid-2,2,3,3-$d_4$ sodium salt |

## Introduction

Concealing actual properties of a food product and/or advertising incorrect properties with the aim of gaining a higher financial profit means food fraud. Estimations of the actual financial damage account to, e.g., globally up to 50 billion US dollars per year [1]. This practice represents a challenge for quality control and food monitoring because the non-conformity of a food product with its labeling can only be proven by analytical examination, especially when accompanying documents are not accessible or are also suspected to be counterfeited. As new fraudulent practices arise

analytical methods have to be adapted, too [2, 3]. Fish and fishery products are among the top ten most adulterated food categories in the European Union [4, 5]. Besides fish species substitution [2, 6], mislabeling of geographic origin [7] or method of production (farmed vs. wild) [7–10], unlabeled reddening processes [6, 11] or addition of external water [2, 12, 13], the unlabeled sale of frozen-thawed fish as fresh fish is one further possibility that is recently discussed [14–18]. Due to a lack of studies and control programs there are currently no statistics about this adulteration [14].

Methods that have been proposed in the literature are mainly based on enzymatic assays [14, 19–24], histological examinations [14, 25–28], near-infrared (NIR) spectroscopy [14, 17, 18, 29, 30] and mass spectrometry [31, 32]. To the best of our knowledge, standardization of the methods is, however, missing. Another possibility to differentiate fresh from frozen-thawed fish is the use of nuclear magnetic resonance (NMR) spectroscopy. Although extractions as sample preparation are needed [16], the NMR spectroscopy captivates with allowing a broad metabolite analysis in a specific and quantitative manner [33–35]. It is non-destructive, enabling different measurements on the same sample solution [33, 35]. Compared to other analytical techniques, NMR spectroscopy is highly reproducible [33, 34], allowing data exchange and the creation of multivariate data analysis models among different laboratories [33]. To differentiate fresh from frozen-thawed fish, NMR based analysis of either the lipid fraction or the polar fraction of the fish flesh in order to generate classification models using multivariate data analysis is feasible [16]. In the cited study, 96 fish samples of fresh and frozen-thawed cod, trout, and mackerel samples were analyzed, and classification models were created using a principal components analysis with linear discriminant analysis (PCA-LDA). After internal validation, two classification models derived from the lipid fraction resulted in higher average accuracies than a classification model derived from the polar fraction. However, only a few samples were used for the creation of the classification models. Moreover, for a broader evaluation, it is still unknown which spectral areas are responsible for the differentiation using these models (e.g., identification of marker substances, if applicable) and whether the models can be used independent of the fish species, as only three fish species were considered. Other studies cited above also focused only on one or a few fish species, prohibiting a general implementation of the methodologies.

Here, the aim was to develop an effective method for a broader application, so the classification models based on $^{1}$H NMR and PCA-LDA were expanded and further investigated. Data fusions (combinations of $^{1}$H NMR data of the lipid and polar fraction) were implemented, and the spectral areas that are important for the classification were examined. Additionally, a preliminary application of these models to predict fish samples from other species than cod, rainbow trout, and mackerel as “fresh” or “frozen-thawed” was performed.

## Materials and methods

An overview of the experimental design can be found in Fig. 1.

### Fish samples underlying the classification models

In total, 317 fish samples (152 fresh, 165 frozen-thawed) were analyzed from 2022 to 2024 for the creation of the classification models (Supplementary Information, Table S1). One hundred thirty-one samples were mackerel samples (*Scomber scombrus*, whole fish (350–450 g), gutted fish (250–550 g), fillets (50–100 g)), 95 samples were rainbow trout samples (*Oncorhynchus mykiss*, gutted fish (300–1,000 g), fillets (150–1,250 g)), and 91 samples were cod samples (*Gadus morhua*, fillets (250–1,500 g) and loins (200–600 g)). All samples were obtained raw and fresh, either from fish industry or from supermarkets and local merchants in south-western Germany. All fresh samples were stored on ice (in accordance with the definition of “fresh fishery products” in the European Law [36]) and analyzed the day after arrival at the laboratory. To obtain frozen-thawed samples, the same procedure as described before [16] was implemented. In short, 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 chamber (-30 °C) for at least seven and up to 78 days. Thawing was performed in a 2 °C-temperature controlled room for up to 24 h and the frozen-thawed samples were stored on ice until analysis on the third day after thawing.

### Fish samples from other fish species used as an external data set

Twenty-six fish samples (13 fresh, 13 frozen-thawed) from twelve other common edible fish species (Table 1) were analyzed. All samples were obtained raw and fresh, in accordance with the fish samples that were used to generate the classification models. One fresh and one frozen-thawed sample was analyzed for each fish species, frozen-thawed samples were obtained by freezing in a cold storage room (-30 °C) and storing for twelve days at -30 °C before thawing in accordance to the described procedure in the previous section. Deviating from this, two fresh river trout samples and two frozen-thawed river trout samples were analyzed

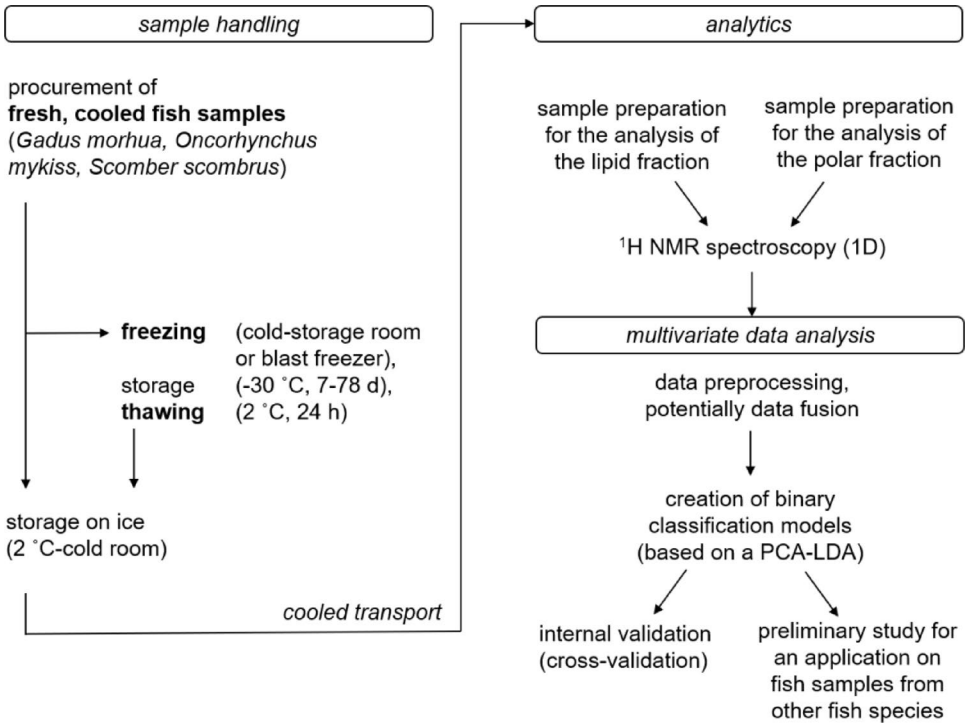



**Fig. 1** Experimental design.

(one frozen in the cold storage room, one frozen in the blast freezer).

## 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 dihydrogen phosphate (99.0%), disodium carbonate (99.9%), the internal standards for normalization (dimethyl sulfone (98.0%) 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, 99.9 atom % D) were from Carl Roth (Karlsruhe, Germany). Deuterium oxide (99.9 atom % D) was obtained from Deutero (Kastellaun, Germany). Before usage of chloroform-$d_1$, the chemical was washed with concentrated disodium carbonate solution and subsequently dehydrated with oven-dried disodium carbonate to remove reactive degradation products as described by Teipel et al. [37].

## Sample preparation

The lipid fractions and the polar fractions of the fillet of all fish samples were analyzed based on previously developed and reported $^1$H NMR methods [16]. All analyses were carried out on the same day for each sample. In brief, the lipid fraction was obtained by fat extraction of the fish fillet using cyclohexane/isopropanol (2/1, v/v) and 5% sodium chloride. The sample solution for $^1$H NMR was prepared using 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). The polar fraction was obtained as the water extract after protein removal (ultrafiltration, 2 kDa) of the fish fillet. Here, the obtained filtrate was mixed with a sodium dihydrogen phosphate buffer (1 M, containing 0.20 mg/mL maleic acid as an internal standard for normalization, pH adjusted to 6.65) and TSP (0.06 M in deuterium oxide) for the measurement.

## NMR analysis

One-dimensional $^1$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. Bruker Biospin software Topspin (version 3.5) was used for data acquisition and processing.

Previously published NMR measurement and processing procedures [16] were applied.

*a) Lipid fraction*. Two measurements were performed, spectra were referenced to the TMS signal at 0.00 ppm. *i)*

**Table 1** Characteristics of fish samples of other common edible fish species that were used for an external validation

| Analyzed samples[†] of the fish species | Binomial name | Habitat | Method of production | Category (fat content) [‡] |
|---|---|---|---|---|
| Atlantic salmon | *Salmo salar* | saltwater | aquaculture | fat fish |
| Brook trout | *Salvelinus fontinalis* | freshwater | aquaculture | medium-fat fish |
| River trout | *Salmo trutta fario* | freshwater | aquaculture | medium-fat fish |
| Gilt-head bream | *Sparus aurata* | saltwater | aquaculture | medium-fat fish |
| European seabass | *Dicentrarchus labrax* | saltwater | aquaculture | medium-fat fish |
| Bigeye tuna | *Thunnus obesus* | saltwater | wild catch | medium-fat fish |
| Red fish | *Sebastes norvegicus* | saltwater | wild catch | medium-fat fish |
| European plaice | *Pleuronectes platessa* | saltwater | wild catch | medium-fat fish |
| Swordfish | *Xiphias gladius* | saltwater | wild catch | medium-fat fish |
| Saithe | *Pollachius virens* | saltwater | wild catch | lean fish |
| Angler | *Lophius piscatorius* | saltwater | wild catch | lean fish |
| Eurasian carp | *Cyprinus carpio* | freshwater | aquaculture | medium-fat fish |

[†]For each fish species, one fresh sample and one frozen-thawed sample (frozen by storing in the -30 °C cold room, stored for twelve days at -30 °C) were examined. In case of the river trout, two fresh samples and two frozen-thawed samples (one frozen by storing in the -30 °C cold room, one frozen in the blast freezer, each stored for twelve days at -30 °C) were examined.

[‡]Division of fish according to their fat content: Lean fish (< 1.0 g fat per 100 g fish fillet), medium-fat fish (≥ 1.0 < 10.0 g fat per 100 g fish fillet), fat fish (≥ 10.0 g fat per 100 g fish fillet), modified after [11, 36, 51].

*$^1$H NMR.* A standard Bruker pulse program zg (300 K, P1 calibration, D1 of 10 s, acquisition time of 4 s, 64 scans, receiver gain of 20.2) was used. *ii) $^1$H NMR based minor component screening.* Two $^1$H NMR experiments were performed for each sample in an automated procedure. Experiment 1 (preparation): A standard Bruker pulse program zg (300 K, P1 calibration, D1 of 4 s, acquisition time of 4 s, 16 scans, receiver gain of 4) was used, then 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 (300 K, D1 of 4 s, acquisition time of 2 s, presaturation pulse of 25 Hz, 64 scans, receiver gain of 16) was used, characterized by the suppression of the 20 frequencies of the major signals in the spectrum gained from experiment 1.

*b) Polar fraction, $^1$H NMR.* A standard Bruker pulse program noesygppr1d (300 K, P1 calibration, D1 of 15 s, acquisition time of 8 s, presaturation pulse of 25 Hz (1880.7 Hz), 64 scans, receiver gain of 128) with a relaxation delay (D1) of 15 s and an acquisition time of 8 s was used. The spectra were referenced to the TSP signal at 0.00 ppm.

## 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. i. Lipid fraction ($^1$H NMR and $^1$H NMR based minor component screening, respectively).* 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 0.30–9.50 ppm was divided into 1,000 segments, equal in width (buckets, bucket size in ppm), and integrated. 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), and cyclohexane (1.40–1.47 ppm) were excluded. *ii. Polar fraction.* Spectra were normalized to the signal of maleic acid (6.02–6.06 ppm). The spectral region 0.50–11.0 ppm was divided into 1,000 buckets and integrated. 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 step (lipid and polar fraction, respectively).* A pseudo-scaling effect was achieved by a log type transformation [38] after bucketing, to improve an equal treatment of higher and lower values and to reduce the influence of size-related noise. In short, integrals > 1 were transformed to one plus the logarithm of the integral, whereas integrals with values ≤ 1 remained untreated.

## Multivariate data analysis

MATLAB version 2019b (The Math Works, Natick, MA, USA) was used for the analysis.

*Creation of classification models.* For the differentiation of fresh and frozen-thawed fish, binary classification models based on the 317 samples (Table S1) were created independently for the data sets based on the lipid fraction ($^1$H NMR and $^1$H NMR based minor component screening, separately) and the data set based on the polar fraction, respectively. Here, principal components analysis (PCA) was used for dimension reduction and linear discriminant analysis (LDA) for class separation. Classification was based on the distances between the test object and the class means of the training set in the LDA-scores: an object was assigned according to the nearest class mean. Multiple dimensions can potentially be used in the multivariate data analysis, so

that the number of principle components that offered a satisfying highpredictivity was identified first (10-times repeated tenfold cross-validation to avoid overfitting). Finally, the classification performance was evaluated by a tenfold cross-validation. To avoid any segmentation bias, a Monte Carlo resampling design with a random segmentation for every cycle was used (10-times repeated 90% to 10% training and test sample splitting).

*Data fusion for additional classification models.* The data obtained from the analysis of the lipid fraction ($^1$H NMR, without suppressions) and the polar fraction were combined in a low-level data fusion and in a mid-level data fusion. *a) Low-level data fusion*. Preprocessed data, i.e., buckets, were combined. *b) Mid-level data fusion*. Respectively for the lipid and the polar fraction, preprocessed data (i.e., buckets) underwent a PCA to calculate the PCA scores. For the data fusion, three different combinations using 37 PCA scores each, 65 PCA scores each, and 37 PCA scores of the lipid fraction and 65 PCA scores of the polar fraction were tried. *a)+b)* The fused data were used for the creation of a classification model as described in the previous section, respectively.

*Identification of relevant spectral areas for the classification.* During the creation of the classification models using a PCA-LDA, additional loadings plots were generated. Here, variables with the greatest impact on the separation of the two groups were highlighted. Loadings plots of the classification models based either on the lipid fraction or the polar fraction were generated and analyzed.

*Classification of external samples (samples from other fish species).* Each sample was predicted as "fresh" or "frozen-thawed" by applying classification models based on a) the lipid fraction ($^1$H NMR, without suppressions), b) the polar fraction, c) the low-level data fusion, and d) the mid-level data fusion. In detail, the $^1$H NMR data of each sample were preprocessed in accordance with the samples of the classification models, and the LDA scores were calculated. A confidence value (conf-value) was obtained for each group, describing the distance of the sample to the group mean in the LDA discriminant space (the group mean equals a conf-value of 0). A conf-value < 1.00 demonstrated that the sample was within the prognosis ellipsoid of the group, whereas a conf-value > 1.00 described that the sample was outside of the prognosis ellipsoid of the group. Each sample was assigned to the group with the lower conf-value (also in cases where the conf-values of a sample was < 1.00 for both classes).

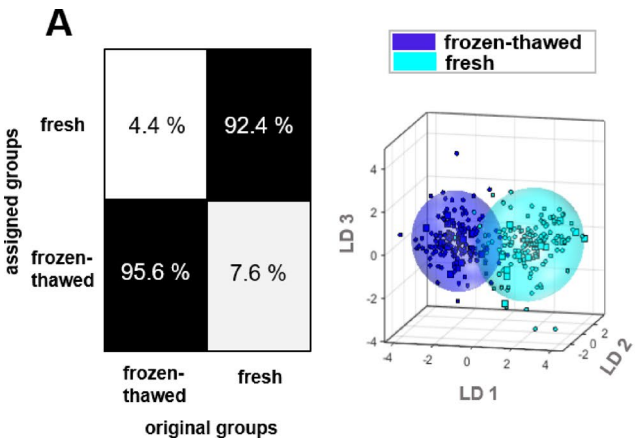
## Results and discussion

### Differentiation of fresh and frozen-thawed fish based on the lipid fraction

Lipophilic metabolites in the fish flesh are mainly triglycerides, diglycerides, monoglycerides, cholesterol, cholesterol esters, as well as amphiphilic phospholipids and free fatty acids. The bound and free fatty acids contain both saturated and mono- or polyunsaturated structures according to the specific fatty acid distribution [11, 39–41]. For the analysis of the lipid fraction of the 317 fish flesh samples (152 fresh, 165 frozen-thawed), a fat extraction followed by two $^1$H NMR measurements were performed: One $^1$H NMR experiment characterized by a 90° pulse for a general acquisition of the metabolites (in the following: $^1$H NMR), and one $^1$H NMR method with suppression of the most intense signals to improve the detection of minor components in complex samples like fish fat extracts (in the following: $^1$H NMR based minor component screening, further information in [16]). For each of the two data sets, a classification model based on a PCA-LDA was constructed to differentiate fresh from frozen-thawed fish using multivariate data analysis.

The model based on the $^1$H NMR spectra of the fat extracts resulted in an average accuracy for correct classification of 94.0% (Fig. 2A, the first 37 principal components of the 924 variables used explained 99.7% of the variance), whereas the model based on the $^1$H NMR based minor component screening spectra showed an average accuracy for correct classification of 93.0% (Fig. 2B, the first 35 principal components of the 924 variables used explained 99.7% of the variance). In the authenticity control, "fresh" fish samples are to be examined in order to verify this quality parameter. In this case, false-positive results (actual fresh samples but predicted as "frozen-thawed") are more relevant than false-negative results (actual frozen-thawed samples but predicted as "fresh") because false-positive results generate unwarranted accusations. In the internal validation of the two classification models, a false-positive rate of 7.6% and a false-negative rate of 4.4% ($^1$H NMR) or 6.4% ($^1$H NMR based minor component screening) respectively, were achieved.

According to internal validation, both classification models indicate a sufficient and comparable prediction. However, internal validation is only partially suitable for a final performance evaluation of a created classification model; external validation is recommended and required to evaluate the statistical relevance of the differentiation based on independent data. It should be noted that the $^1$H NMR based minor component screening led to a different weighting of the signal intensities across the entire spectrum without a notable gain in signal intensity for the minor signals



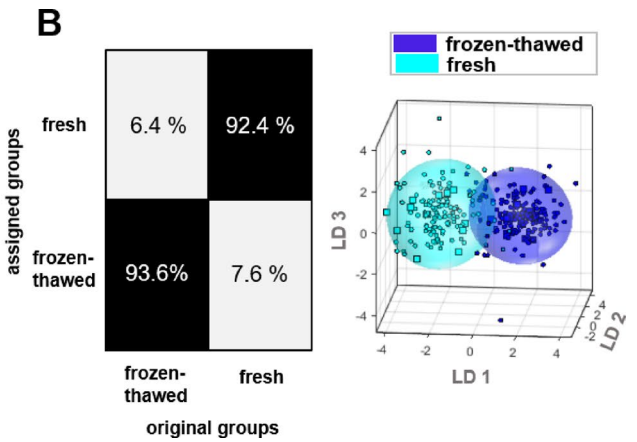




**Fig. 2** Classification models based on the lipid fraction. Results of the embedded Monte Carlo cross-validation (MCCV) to evaluate the performance of the obtained PCA-LDA based classification models for the prediction of fresh fish (turquoise, 152 samples) and frozen-thawed fish (blue, 165 samples). The underlying data were obtained from **A)** $^{1}$H NMR spectra of the fat extract of the fish flesh **B)** $^{1}$H NMR spectra of the screening regarding minor components of the fat extract of the fish flesh. The left figures in **A**, **B** show the confusion matrices of the MCCV. The confusion matrix demonstrates the accuracies about the frequency of the prediction result in percent. The figures on the right side in **A**, **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. Number of principal components used: **A** 37 **B** 35. Explained variance: **A** 99.7% **B** 99.6%.

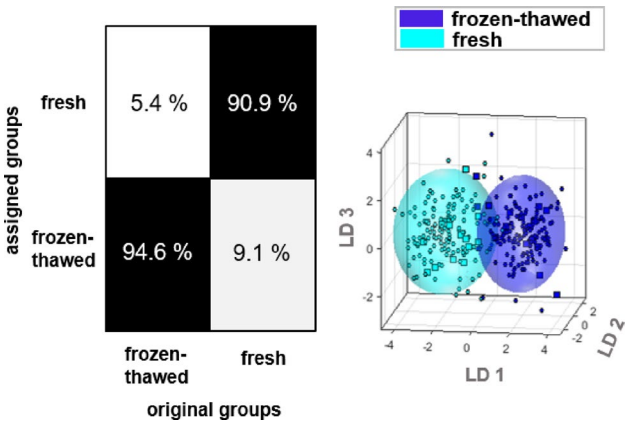



**Fig. 3** Classification model based on the polar fraction. Results of the embedded Monte Carlo cross-validation (MCCV) to evaluate the performance of the obtained PCA-LDA based classification models for the prediction of fresh fish (turquoise, 152 samples) and frozen-thawed fish (blue, 165 samples). The underlying data were obtained from $^{1}$H NMR spectra of the water extract after protein precipitation of the fish flesh. The left figure shows the confusion matrices of the MCCV. The confusion matrix demonstrates the accuracies about the frequency of the prediction result in percent. The figure on the right side shows 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. Number of principal components used: 65. Explained variance: 97.8%.

(Supplementary Information, Figure S1). Due to the irradiation of suppression frequencies, the inherent quantification possibilities of NMR can no longer be used in the spectra generated in this way. For a later application of the developed NMR method for examining the lipid fraction of the fish flesh samples, with a possible different purpose, the $^{1}$H NMR measurement without suppressions is therefore recommended and was further pursued in this study.

In comparison to the classification models based on 96 fish samples constructed by using the same procedure [16], the expansion to 317 fish samples resulted in a higher average accuracy ($^{1}$H NMR: 94.0% compared to 90.0%, $^{1}$H NMR based minor component screening: 93.0% compared to 91.9%).

## Differentiation of fresh and frozen-thawed fish based on the polar fraction

Non-protein nitrogen compounds such as creatine, trimethylamine oxide, adenosine nucleotides, free amino acids, and dipeptides [11], taurine, betaine, purine derivatives such as inosine and hypoxanthine, and organic acids such as lactic acid and acetic acid represent relevant hydrophilic metabolites in the fish flesh [11, 42–44]. The water extracts of the 317 fish flesh samples were measured by $^{1}$H NMR after protein removal. The obtained spectra served as a data set to create another classification model to distinguish between fresh and frozen-thawed fish.

After internal validation, the classification model resulted in an average accuracy of 92.8%, a false-positive rate of 9.1%, and a false-negative rate of 5.4% (Fig. 3, the first 65 principal components of the 956 variables used explained 97.8% of the variance). With an accuracy of > 90% analogous to the classification models based on the lipid fraction, the prediction was considered sufficient. Different from the classification models based on the lipid fraction more principal components had to be used to separate the two

classes (Figs. 2 and Fig. 3, 65 instead of 37 or 35 principal components in the models based on the lipid fraction). Furthermore, 97.8% of the variance of the data was explained in the present model, while 99.7% was explained in both models based on the lipid fraction. Thus, the data set of $^{1}$H NMR spectra of the water extracts showed a larger variance than the two data sets of $^{1}$H NMR spectra of the fat extracts. However, this larger variance did not contribute to a better discrimination between fresh and frozen-thawed fish.

Here, the average accuracy increased notably with the expansion of the data set from 96 to 317 samples (82.6% [16] to 92.8%).

## Data fusion

Due to the different solubility of the metabolites, the lipid fraction and the polar fraction of the fish flesh have to be analyzed separately. However, by subsequent data fusion, it was possible to evaluate both data sets together. Both a low-level and a mid-level data fusion were conducted using the $^{1}$H NMR data of the fat extracts (without suppressions in the measurement) and the $^{1}$H NMR data of the water extracts of the 317 fish samples (152 fresh and 165 frozen-thawed), resulting in two additional classification models.

In the low-level data fusion approach, the $^{1}$H NMR spectra of the lipid fraction and the $^{1}$H NMR spectra of the polar fraction were initially pretreated in a manner analogous to the creation of the individual models. Following log transformation, the two data sets (924 resp. 965 variables of the $^{1}$H NMR spectra of the lipid resp. polar fraction) were combined and then subjected together to the PCA-LDA for the creation of the classification model. As a result of the low-level data fusion, an average accuracy of 95.6%, a false-positive rate of 4.3%, and a false-negative rate of 4.5% were obtained (Fig. 4A, the first 27 principal components of the 1,889 variables used explained 98.5% of the variance).

For the mid-level data fusion, a PCA was carried out after data preprocessing of the individual data sets, and the first 37 principal components were used to calculate the PCA scores of the individual samples in both data sets. These were then combined in the sense of a mid-level data fusion (resulting in a total of 74 variables). Afterwards, a PCA was carried out again with the generated data set as part of the subsequent PCA-LDA procedure for building the classification model. In addition to using 37 PCA scores each, other combinations were also tried out. For example, 37 PCA scores from the data set based on the lipid fraction were fused with 65 PCA scores from the data set based on the polar fraction in order to consider the optimal number of principal components used in the individual models. Due to the unequal number of PCA scores in the two data sets the polar fraction was weighted slightly more heavily. In order to take both data sets into account more equally, the first 65 PCA scores were calculated for both data sets and combined afterwards. Since all three possible combinations provided a similar average accuracy according to the internal validation of the classification models (Table S2), data fusion based on the combination of the first 37 PCA scores was selected and pursued. This mid-level data fusion showed an average accuracy of 95.5%, a false-positive rate of 4.4%, and a false-negative rate of 4.7% (Fig. 4B, the first 27 principal components of the 74 variables used explained 99.6% of the variance).

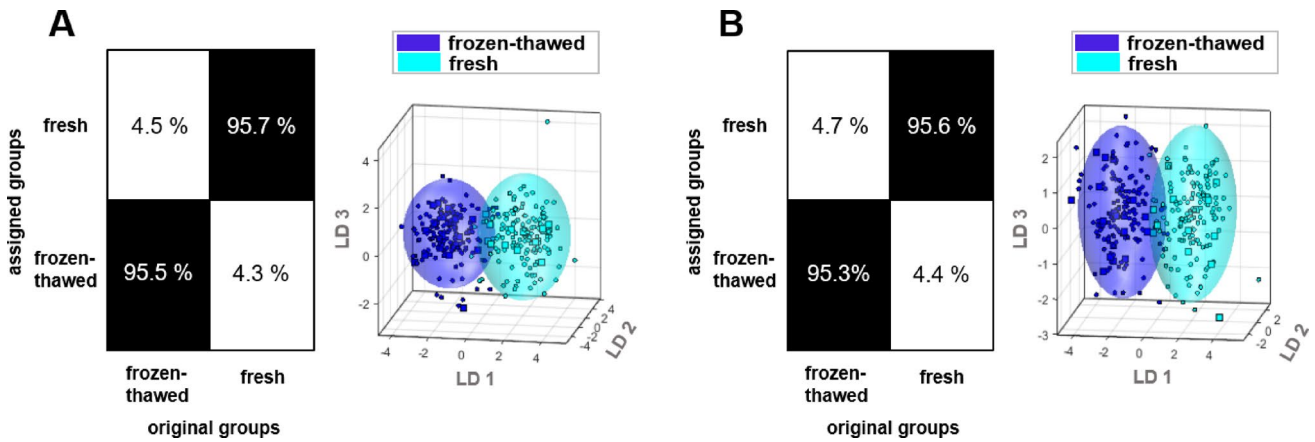



**Fig. 4** Classification models based on data fusion (lipid and polar fraction). Results of the embedded Monte Carlo cross-validation (MCCV) to evaluate the performance of the obtained PCA-LDA based classification models for the prediction of fresh fish (turquoise, 152 samples) and frozen-thawed fish (blue, 165 samples). Two datasets were evaluated, **A** after low-level data fusion of the preprocessed buckets and **B** after mid-level data fusion of the first PCA scores of the NMR data. The left figures in **A**, **B** show the confusion matrices of the MCCV. The confusion matrix demonstrates the accuracies about the frequency of the prediction result in percent. The figures on the right side in **A**, **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. Number of principal components used: **A** 27 **B** 27. Explained variance: **A** 98.5% **B** 99.6%.

Both the low-level and mid-level data fusion resulted in accuracies of > 95%, being slightly higher than the accuracies of the individual models (94.0% and 92.8% in the classification models based on the lipid and polar fraction, respectively). Thus, fusion of the data of the lipophilic and hydrophilic metabolites should be considered for the differentiation of fresh and frozen-thawed fish. In a direct comparison of the two classification models of data fusion, neither model was preferable to the other due to the very similar prediction accuracy (95.6% by the classification model based on the low-level data fusion, 95.5% by the classification model based on the mid-level data fusion). However, a conclusive evaluation and comparison of the performance of the individual models could be only conducted after external validation.

Methods to distinguish between fresh and frozen-thawed fish have been published in the past as summarized in a review by Hassoun et al. in 2020 [14]. However, NMR based classification models were not yet mentioned. Published classification models are based on other analytical techniques with accuracies of > 90% (NIR, e.g., [29, 30]) or 100% (mass spectrometry, [31, 32]) being stated. The achieved accuracies of the classification models in this study are therefore comparable with the cited literature, whereas the experimental design varies within the studies (e.g., fish species, origin, freezing and thawing techniques, storage time).

## Investigation of spectral areas that are relevant for the differentiation

In order to further evaluate the classification models, the related loadings plots were analyzed. In a loadings plot, the original variables (i.e., the preprocessed data or buckets) are displayed according to their impact on the classification model, enabling the identification of spectral ranges that are relevant for the separation.

In the loadings plot of the classification model based on the lipid fraction of the fish flesh, the variables scattered narrowly over a small range around the zero point (Fig. 5A). The only variables that were shifted slightly to the left (significance for the class "frozen-thawed") were the buckets at 3.680 ppm, 2.289 ppm, and 2.271 ppm. On the right side (significance for the class "fresh"), the buckets at 3.928 ppm and 3.385 ppm stood out slightly. By comparing them with an underlying $^{1}$H NMR spectrum (Fig. 5B), these spectral regions were examined in more detail (Fig. 5C): The five buckets contained information of signal shoulders or signals of greatly reduced intensity. Consequently, there were no isolated signals that would have been characteristic of either class. In addition to the buckets mentioned, the numerous buckets that scattered around the zero point were also responsible for the separation. It had to be considered that 37 dimensions of the PCA were used to build the model, while a two-dimensional loadings plot was evaluated. Although the first dimension of the loadings plot (horizontal

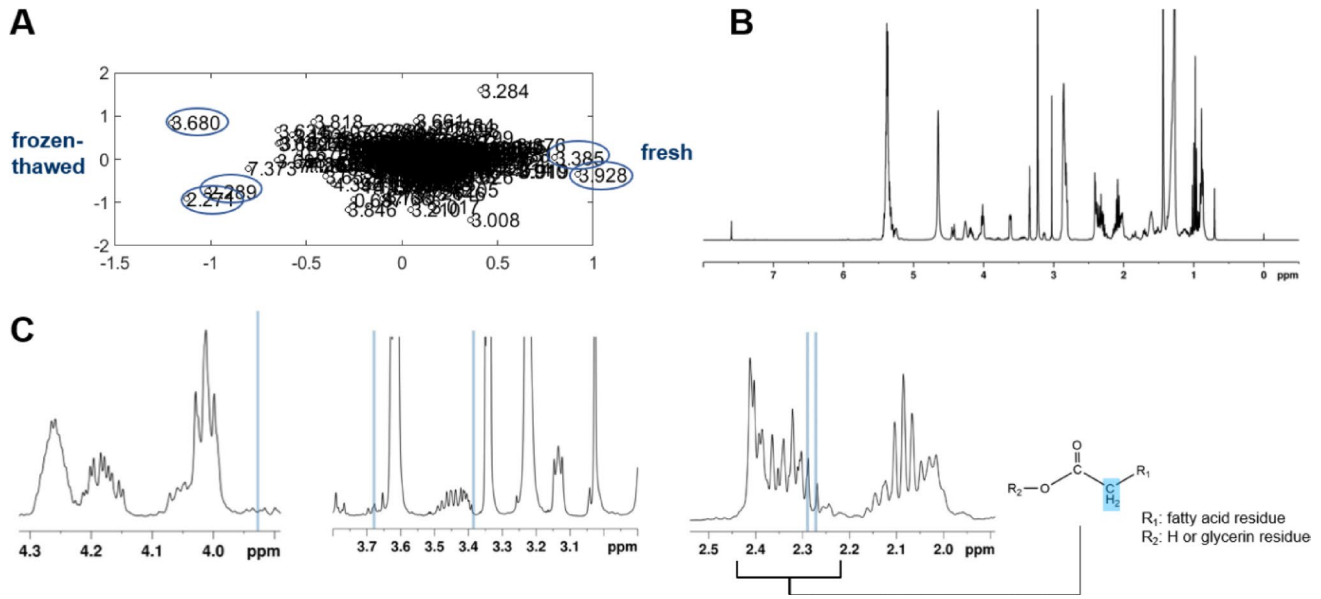



**Fig. 5** Further analysis of the classification model based on the lipid fraction (without suppressions) to identify spectral ranges that are relevant for the differentiation of fresh and frozen-thawed fish. **A** Two-dimensional loadings plot which shows the original variables (preprocessed data i.e. buckets) in relation to the classification by the model (x-axis is primarily substantial). **B** Representative $^{1}$H NMR spectrum (400 MHz, 300 K, in chloroform-$d_1$/methanol-$d_4$ (1/1, v/v), referenced to $\delta_{tetramethylsilane} = 0.00$ ppm) of the lipid fraction of one frozen-thawed cod fillet which served as sample in the model. **C** Details from **B**, to examine the in blue highlighted relevant spectral ranges of the loadings plot (**A**, circled in blue). The overlaying signals in the range 2.20–2.45 ppm could be assigned to protons neighboring the carboxyl group in fatty acid structures (see [41, 49]).

axis) is the most important for the separation of the model, the number of 37 dimensions shows that this was a more complex separation problem. Thus, it was not possible to identify an individual marker compound that is responsible for the separation of fresh and frozen-thawed fish. Therefore, the separation is based on the interaction of many lipophilic metabolites in the fat extract.

Changes in the lipid fraction of fish flesh during the freezing and thawing process are described in the literature, with lipid oxidation and lipolysis often being mentioned [14, 45–47]. Studies that evaluated quality aspects among the storage time show specific changes that can be directly attributed to metabolites (e.g., free fatty acids in mackerel and salmon samples [48] or in rainbow trout samples [47]) or characteristic values (e.g., peroxide number in salmon samples [48] or rainbow trout samples [47]). Also, eicosapentaenoic acid and docosahexaenoic acid were suggested as relevant lipophilic metabolites for the separation of fresh and frozen-thawed European sea bass samples [31, 32]. Contrary to expectations from the literature, the loadings plot of the classification model based on the lipid fraction did not reveal any individual marker metabolites for the separation of the fresh and frozen-thawed fish. With the buckets at 2.289 ppm and 2.271 ppm examined above, relevant spectral ranges of fatty acids (according to [41, 49]) were identified in the loadings plot, but they contributed to the class separation alongside many other buckets. The characteristic $^{1}$H NMR signal ranges of the monounsaturated and polyunsaturated fatty acids (5.5–5.3 ppm, 2.9–2.7 ppm, 2.1–1.9 ppm, according to [41, 49]) did not indicate any particular significance for the class separation in the loadings plot. Thus, they did not demonstrate any major changes in the course of lipid oxidation. In the cited studies, the relevant metabolites were identified by direct comparison of samples from storage experiments. Despite the multivariate data analysis, this also applied to the studies by Massaro et al. (2021) and Stella et al. (2022), as both studies had used samples from only one source to build the model [31, 32]. In the present work, the classification model considered a more comprehensive separation problem, as there were larger variations in the data set (e.g., three fish species, two freezing methods, different producers/wild catches, different storage times, seasonal fluctuations). It could be attributed to the larger variations in the data set that there was more comprehensive information needed than individual signals to successfully separate the two classes.

The analysis of the loadings plot for the classification model based on the polar fraction of the fish flesh also gave a similar picture (Fig. 6A). The variables scattered within a small range around the zero point, with only the buckets 2.802 ppm and 2.413 ppm being shifted slightly to the left (significance for the class "frozen-thawed") and the buckets 1.204 ppm, 1.929 ppm, and 1.194 ppm being slightly shifted to the right (significance for the class "fresh"). The evaluation of a sample spectrum (Fig. 6B) assigned these buckets to spectral areas with signal shoulders (including acetic acid) or signals of greatly reduced intensity (Fig. 6C). Analogous to the evaluation of the loadings plot of the

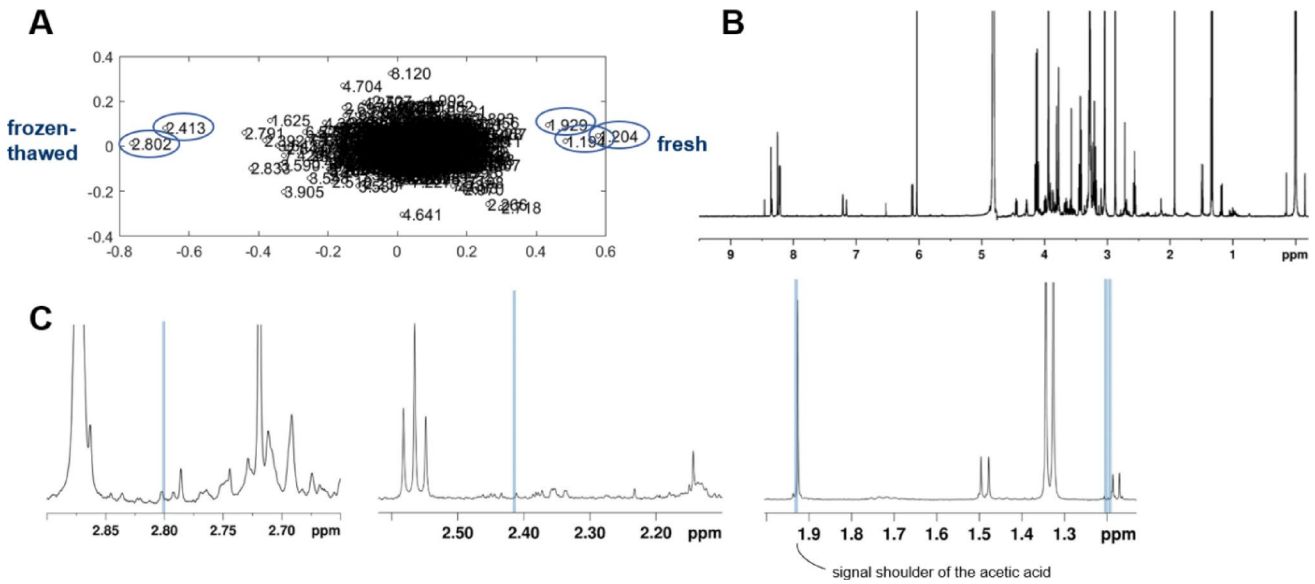



**Fig. 6** Further analysis of the classification model based on the polar fraction to identify spectral ranges that are relevant for the differentiation of fresh and frozen-thawed fish. **A** Two-dimensional loadings plot which shows the original variables (preprocessed data i.e. buckets) in relation to the classification by the model (x-axis is primarily substantial). **B** Representative $^{1}$H NMR spectrum (400 MHz, 300 K, in $H_2O$/$D_2O$, pH 6.7, referenced to $\delta_{3\text{-(trimethylsilyl)-propionic acid-2,2,3,3-d4}} = 0.00$ ppm) of the polar fraction of one frozen-thawed cod fillet which served as sample in the model. **C** Details from **B**, to examine the in blue highlighted relevant spectral ranges of the loadings plot (**A**, circled in blue). The singlet at 1.927 ppm could be assigned to the acetic acid.

classification model based on the lipid fraction, the separation can therefore be interpreted as an interaction of many metabolites from the water extract.

Differently, Shumilina et al. [50] suggested aspartic acid as a marker substance for frozen-thawed Atlantic salmon, whereas Chiesa et al. [51] suggested arginine and its derivatives as marker substances for frozen-thawed Atlantic salmon, and arginine as well as phosphorylated choline and ethanolamine derivatives as marker substances for thawed bullet tuna. Moreover, two studies demonstrated that both the storage time of fresh and frozen-thawed fish [50] and the number of freezing and thawing cycles [52] affect the composition of the hydrophilic metabolites of the fish flesh as determined by $^{1}$H NMR. This could merit further studies for the evaluation of the performance of a classification model based on hydrophilic metabolites to differentiate fresh from frozen-thawed fish.

In the literature, other studies demonstrated a differentiation of fresh and frozen-thawed fish using enzymatic essays: In frozen-thawed fish samples, a higher activity of mitochondrial and/or lysosomal enzymes was found in the fish juice [19–24]. Enzyme-catalyzed reactions potentially affect lipophilic and hydrophilic metabolites, too (e.g., higher amounts of free fatty acid or free amino acids in terms of lipolysis (lipase) or proteolysis (protease), respectively). In the published studies, specific enzyme activities were proven to be increased, although the addition of (partially artificial) substrates was necessary for the determination (e.g., addition of *p*-nitrophenyl-α-glucopyranoside to determine the activity of α-glucosidase [24]). Therefore, these publications do not necessarily contradict the results of the present study.

## Investigation of the impact of the fish species on the classification

In the literature, individual fish species or a small selection of fish species were investigated. However, genetics affect the metabolome [11, 39, 42, 53, 54] and the fish species also determines the fat class (lean, medium-fat, or fatty) and the habitat (saltwater or freshwater) [11, 39, 55]. In order to assess to which extent these criteria affect the classification models, further investigations were carried out. To interpret the influence of the fish species on the classification models, separate classification models were additionally generated for each fish species (cod, rainbow trout, mackerel) with regard to the differentiation of "fresh" from "frozen-thawed".

First, classification models for each fish species were built based on the lipid fraction of the fish flesh. While the classification model based only on the rainbow trout samples delivered an average accuracy of 99.6%, a false-positive rate of 0.2%, and a false-negative rate of 0.7% for the prediction of the groups "fresh" and "frozen-thawed" (95 samples, Table 2, Figure S2B), the model based on the cod samples showed an average accuracy of only 88.6% with a false-positive rate of 13.5% and a false-negative rate of 9.3% (91 samples, Table 2, Figure S2A). The third classification model that included the mackerel samples (131 samples, Table 2, Figure S2C) was characterized by an average accuracy of 96.9%, a false-positive rate of 2.4%, and a false-negative rate of 3.9%. Only the models for rainbow trout and mackerel thus promised a very good prediction. In addition to the average accuracy, differences were also found in the number of principal components required from the PCA for further model building. For the model based on rainbow trout, five dimensions of the 924 variables, which explained

**Table 2** Overview of classification models where the former analyzed fish samples were divided according to the three fish species [cod (*Gadus morhua*), rainbow trout (*Oncorhynchus mykiss*) and mackerel (*Scomber scombrus*)]

| | Percentage of the prediction of | | | |
|---|---|---|---|---|
| | originally "fresh" as "fresh" | originally "fresh" as "frozen-thawed" | originally "frozen-thawed" as "frozen-thawed" | originally "frozen-thawed" as "fresh" |
| Classification model based on the lipid fraction | | | | |
| Cod samples (*Gadus morhua*) | 86.5% | 13.5% | 90.7% | 9.3% |
| Rainbow trout samples (*Oncorhynchus mykiss*) | 99.8% | 0.2% | 99.3% | 0.7% |
| Mackerel samples (*Scomber scombrus*) | 97.6% | 2.4% | 96.1% | 3.9% |
| Classification model based on the polar fraction | | | | |
| Cod samples (*Gadus morhua*) | 93.9% | 6.1% | 93.3% | 6.7% |
| Rainbow trout samples (*Oncorhynchus mykiss*) | 96.5% | 3.5% | 100% | 0% |
| Mackerel samples (*Scomber scombrus*) | 91.4% | 8.6% | 97.1% | 2.9% |

Classification models for the differentiation of fresh and frozen-thawed fish were created with fish samples from only one fish species, respectively. The percentage of the prediction is calculated after the internal validation (cross-validation) as the frequency of the prediction result in percent. The graphical confusion matrix and the discrimination space of one cross-validation step could be found in Figure S2 and Figure S3.

88.4% of the variance, were sufficient for the very good prediction result of the internal validation. For the model based on the mackerel samples, 21 dimensions were used, which explained 99.5% of the variance. In comparison, the model for cod required 29 dimensions, which, despite explaining 99.5% of the variance of the data set, only resulted in an average accuracy of < 90% (Figure S2). Analysis of the cross-validation results of the classification models based on the polar fraction of the fish flesh revealed different accuracies for each fish species (Table 2, Figure S3). While the model based on the rainbow trout samples achieved a very good average accuracy (98.2%, false-positive rate 3.5%, false-negative rate 0%) for distinguishing between the groups "fresh" and "frozen-thawed", the models based on cod and mackerel showed a good average accuracy (93.6%, false positive rate of 6.1%, false negative rate of 6.7% and 94.3%, false-positive rate of 8.6%, false-negative rate of 2.9%, respectively). There were minor differences between the models for the individual fish species with regard to the number of dimensions required and the variance explained. To summarize, the prediction of fresh and frozen-thawed fish in classification models for each fish species produced different results, especially in the models based on the lipid fraction. It can be concluded that when applying the presented classification models (from the previous sections, containing all 317 fish samples) to an unknown sample, the fish species likely affects the prediction result as "fresh" or "frozen-thawed".

To investigate this further, a preliminary study for an application of the models built (based on the 317 samples) to samples of other fish species was carried out. A total of 26 fish samples (13 fresh, 13 frozen-thawed) from twelve other common edible fish species were obtained analogously to the fish samples that were used to generate the classification models. Then, they were analyzed according to the sample preparation protocols for lean fish or medium-fat and fatty fish to obtain the fat extracts or water extracts for $^{1}$H NMR measurement. The obtained spectra were used as an external data set and, after analogous data pretreatment, were predicted as "fresh" or "frozen-thawed" by the classification models [a) based on the lipid fraction ($^{1}$H NMR without suppressions), b) based on the polar fraction, c) based on the low-level data fusion, and d) based on the mid-level data fusion].

Saithe (*Pollachius virens*) was examined as a fish species that is more closely related to the cod (family Gadidae). The Atlantic salmon (*Salmo salar*), the river trout (*Salmo trutta fario*), and the brook trout (*Salvelinus fontinalis*) were chosen as fish species that are more closely related to the rainbow trout (family Salmonidae), and the bigeye tuna (*Thunnus obesus*) was analyzed as a fish species that is more closely related to the mackerel (family Scombridae). In addition, carp (*Cyprinus carpio*), monkfish (*Lophius piscatorius*), gilthead sea bream (*Sparus aurata*), European sea bass (*Dicentrarchus labrax*), redfish (*Sebastes norvegicus*), European plaice (*Pleuronectes platessa*), and swordfish (*Xiphias gladius*) samples were examined. Nine of the twelve fish samples examined were saltwater fish, and three were freshwater fish. The samples of six fish species came from aquaculture, whereas the other samples were wild catches. Two fish species were lean and one was fatty, whereas the other nine fish species were medium-fat fish (Table 1).

**Table 3** Results of the classification ("fresh" or "frozen-thawed") of 26 test samples (13 fresh, 13 frozen-thawed, see Table 1) from twelve other fish species than those within the classification models. Details regarding specific classification results of the samples can be found in Table S3.

| | Classification model for the differentiation of fresh and frozen-thawed fish based on | | | |
|---|---|---|---|---|
| | The lipid fraction | The polar fraction | A low-level data fusion | A mid-level data fusion |
| Percentage of correct predicted samples | 69.2% | 73.1% | 84.6% | 80.8% |
| Percentage of false-positive results[†] | 15.4% | 11.5% | 7.69% | 11.5% |
| Percentage of false-negative results[†] | 15.4% | 15.4% | 7.69% | 7.69% |
| Samples outside of the prognosis ellipsoids | 3.85% | 0% | 0% | 0% |

[†]Here, positive means a classification as "frozen-thawed", and negative means a classification as "fresh" (in terms of the methodology serving as a verification of fish samples against unlabeled freezing and thawing).

Application of the models revealed that only one of the test samples (frozen-thawed Atlantic salmon sample in the classification model that is based on the lipid fraction, conf-values > 1.00, Table 3, Table S3) was outside the prediction ellipsoids of both classes ("fresh", "frozen-thawed") whereas all other test samples were within the prediction ellipsoids in each classification model, respectively. Consequently, the LDA scores calculated from the test samples when predicting them as "fresh" or "frozen-thawed" did not deviate greatly from those of the model data. As a visual illustration, Figure S4 shows the discrimination space of the model based on low-level data fusion, where the prediction ellipsoids are pictured and the test samples are shown as numbers.

Following the classification rule, a test sample was assigned to the class with the smaller conf-value. Across all results, only 6.73% of the test samples had conf-values of both classes of ≤ 1.00, meaning that the samples were within the prognosis ellipsoid of both groups. Here, the samples

were assigned according to the classification rule although the prediction result was ambiguous.

The model based on the lipid fraction predicted 69.2% of all test samples correctly, whereas the model based on the polar fraction achieved a correct assignment of 73.1%. Consequently, the prediction results of the test data set as a whole were assessed as insufficient (Table 3). It was noticeable that some samples were differently predicted in both models; for example, the fresh European plaice sample was correctly predicted as "fresh" by the model based on the lipid fraction, but incorrectly predicted as "frozen-thawed" by the model based on the polar fraction (Table S3). The prediction of the test samples by the models based on data fusion yielded better results, especially the model based on low-level data fusion with a correct classification of 84.6% of the test samples (Table 3). It can be assumed that for some of the samples that yielded different prediction results in the individual models, data fusion resulted in an information gain, thereby improving the prediction. However, the percentage of the correct prediction results of the test samples was overall lower than the average accuracy of the internal validation of the classification models. No connection was observed between systematics or genetics, habitat, production method, or fat class and the prediction results (Table S4).

Apparently, the species of a fish sample affects the prediction result as "fresh" or "frozen-thawed". Although it seems possible in principle to apply the created classification models to fish species other than cod, rainbow trout, and mackerel, sufficient certainty of prediction cannot be expected for every fish species. The model based on low-level data fusion showed the most promising result. The results of the fish samples in the test data set (Table S3) thus could enable a first assessment of whether a certain fish species can be analyzed using a certain model. For a more precise assessment of the prediction accuracy for a specific fish species, further investigations or validations with a higher number of samples are required.

Publication in the field often only consider one fish species or a small selection of fish species [14, 29–32, 50, 51]. However, it is known that an activity increase of lysosomal or mitochondrial enzymes in the pressed juice of frozen-thawed fish depends on the fish species [15], limiting studies based on enzymatic assays. Thus, a non-targeted analysis of the metabolites from the fish flesh is likely more robust than a targeted enzymatic examination. However, the transfer of a non-targeted method to differentiate fresh and frozen-thawed fish to fish species other than those used to build the model needs to be confirmed by appropriate method validation.

## Conclusion

In principal, non-targeted $^{1}$H NMR based methods are very suitable to differentiate fresh from frozen-thawed fish. Good to very good prediction results (i.e., accuracy averaged across the classes "fresh fish" and "frozen-thawed fish" of > 90% (based on the lipid fraction or of the polar fraction) or > 95% (based on the lipid and the polar fraction in a data fusion)) were achieved for fresh and frozen-thawed cod, rainbow trout, and mackerel. However, fish species affect the applicability of the generated models and, thus, the prediction results. Consequently, further investigations or validations are required if the developed classification models will be used to predict fresh or frozen-thawed samples of fish species other than cod, rainbow trout, and mackerel. On the other hand, models that are built on single fish species only, e.g. rainbow trout, differentiate very well between fresh and frozen-thawed fish if this fish species is analyzed.

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**Data availability** A dataset (including 5 files with zipped NMR data, metadata and readme file) will be published in Zenodo under DOI 10.5281/zenodo.14289795.

## Declarations

**Conflict of interest** The authors declare no competing interests.

**Human and animal rights** This article does not contain any studies with human or living animal subjects.

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