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Differentiation of fresh and frozen-thawed fish samples using Raman spectroscopy coupled with chemometric analysis

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ABSTRACT

The potential of Raman spectroscopy was investigated in terms of its capability to discriminate the species of the fish samples and determine their freshness according to the number of freezing/thawing cycles they exposed. Species discrimination analysis was carried out on sixty-four fish samples from six different species, namely horse mackerel (*Trachurus trachurus*), European anchovy (*Engraulis encrasicolus*), red mullet (*Mullus surmuletus*), Bluefish (*Pomatamus saltatrix*), Atlantic salmon (*Salmo salar*) and flying gurnard (*Trigla lucerna*). Afterwards, fish samples were exposed to different numbers of freezing/thawing cycles and separated into three batches, namely (i) fresh, (ii) once frozen-thawed (OF) and (iii) twice frozen-thawed (TF) samples, in order to perform the freshness analysis. Raman data collected were used as inputs for chemometric analysis, which enabled us to develop two main PCA models to successfully terminate the studies for both species discrimination and freshness determination analysis.

1. Introduction

Fish is a life-sustaining source of food for humanity. It is reported that capture fisheries and aquaculture provide 148 million tonnes of fish and other marine species, with more than half of this used for human consumption. By containing low rates of saturated fats, carbohydrates and cholesterol, and high rates of essential micronutrients such as vitamins, minerals and polyunsaturated fatty acids (PUFA), fish offers an alternative source of high value protein (FAO., 2012).

Due to its high moisture, fat and protein content and weak muscle tissue, fish is classified as a highly perishable food product. Accordingly, from the time of capture to the time it is served for consumption, drastic changes occur in fish in terms of sensorial and nutritional quality (Hernández-Martínez et al., 2013). Decreasing fish quality mainly depends on fish species and storage conditions, where multiple deterioration mechanisms, such as enzymatic or microbial spoilage, mechanical damage and fat oxidation, are involved (Gram & Huss, 1996).

Even though numerous preservation methods have been used to extend the shelf life of fish, freezing still remains the most commonly used method. The freezing process aims to decrease bacterial growth and reaction rate of enzymes by converting the water in the fish body into the ice crystals, which also extends the rigor mortis period (Gram & Huss, 1996). Nevertheless, major textural and functional changes also occur along with improper practices of freezing, post-freezing and thawing processes (Matsumoto, 1979). Even though these major changes are taking place, it is still not possible for a consumer to differentiate between fresh and frozen-thawed fish due to their high similarity in sensorial characteristics (Karoui, Thomas, & Dufour, 2006).

Several methods based on different measurement parameters such as enzymatic changes (Alberio, Barbagallo, Todaro, Bono, & Spagna, 2014), dielectric properties (Kent et al., 2004) or chemical changes (De Abreu, Losada, Maroto, & Cruz, 2011) have been used to identify those changes which occur during the freezing and storage of fish and recognise the differences between fresh and frozen-thawed fish. In addition to existing methods, new technologies such as Fourier transform infrared (FTIR) spectroscopy (Chaijan, Benjakul, Visessanguan, & Faustman, 2006), front-face fluorescence spectroscopy (Karoui et al., 2006), impedance spectroscopy (Fernández-Segovia et al., 2012) and near-infrared spectroscopy (NIRS) (Ottavian, Fasolato, Facco, & Barolo, 2013) are gaining wide attention due to their advantages like rapidness, on-site usability and high accuracy. Raman spectroscopy is also one of the techniques that offer these advantages.

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Raman spectroscopy has been widely used as a promising technique for the determination of food adulterations and authenticity (Boyaci, Genis, Guven, Tamer, & Alper, 2012; Boyaci et al., 2014; Özbalci, Boyaci, Topcu, Kadılar, & Tamer, 2013; Uysal, Boyaci, 2013; Genis, & Tamer, 2013). By measuring fundamental vibrations of functional groups (rocking, wagging, scissoring and stretching) in the molecule, Raman spectroscopy provides detailed structural information without causing any alterations, and requires only a small amount of sample (Marquardt & Wold, 2004). Coupling Raman spectroscopy with chemometric analysis enables researchers to reduce the dimension of the data with minimum information loss (Nunes, 2013). Raman spectroscopy and chemometric methods have recently been utilised in a number of studies investigating fish quality (Herrero, Carmona, & Careche, 2004; Sarkardei & Howell, 2007).

In the present study, sixty-four samples of Horse mackerel (*Trachurus trachurus*), European anchovy (*Engraulis encrasicolus*), Red mullet (*Mullus surmuletus*), bluefish (*Pomatamus saltatrix*), Atlantic salmon (*Salmo salar*) and Flying gurnard (*Trigla lucerna*) fish species were analysed using Raman spectroscopy. Discrimination and quality assessment of these samples were successfully carried out based on the developed method, which uses Raman spectroscopy coupled with principal component analysis (PCA). Raman measurements were also compared with gas chromatography (GC) results to obtain detailed information about the fatty acid content of the analysed samples. In order to simulate the real life practice, the storage time was shortened to 24 h for freezing and 12 h for thawing. Although the shortened storage time also shortened the time available for changes to occur in the lipid structure, to the best of our knowledge, there have been no studies investigating the potential of Raman spectroscopy to detect changes in the lipid structures of fish species during very short storage times.

2. Material and methods

2.1. Samples

All fish samples except Atlantic salmon were caught (January, 2013) off the Tekirdag city coast of Marmara Sea, Turkey (the area around latitude 40°50′ N, longitude 27°40′ E). Fresh Atlantic salmon was bought from a local fish store where fish that had been imported alive were put up for daily sale, after being headed and gutted. The fish species of interest in the present study were selected according to their seasonally market share in Tekirdag fresh fish market in January, 2013.

2.2. Sample preparation

All samples were transported to the laboratory under cold conditions (4 °C) in thermo-boxes, 10 h after mortem. Fish samples were divided into three batches for further analysis: (i) fresh, (ii) once frozen-thawed (OF), and (iii) twice frozen-thawed (TF). Fresh fish samples were washed and filleted immediately. OF and TF fish samples were put into the deep-freezer (−18 °C) and stored for 24 h. Thawing was performed in refrigerated conditions for 12 h and the OF fish samples were washed and filleted; the TF fish samples were frozen again at −18 °C for 24 h and thawed using the same conditions as described for fresh and OF fish samples.

2.3. Fat extraction

Fat extraction was performed with some modifications according to the procedure described previously (Boyaci et al., 2014). Fish fillets were homogenised using a laboratory type blender. In order to avoid errors originating from fat contamination, the blender was carefully washed with boiling water and detergent, rewashed with distilled water and completely dried prior to the next blending. Twenty grams of each homogenised sample was placed in a mortar and 20 ml of analytical grade *n*-hexane (Sigma–Aldrich, Munich, Germany) was added. After pounding in a mortar, the mixture of sample and hexane was transferred to the sampling bag and blended (BagMixer® 400 P, Interscience, St Nom, France) for 120 s to ensure the effectiveness of fat extraction. The fat-hexane mixtures were taken from the upper side of the stomacher bag and pipetted into the eppendorf tube. The tubes were centrifuged (Universal 32 R, Hettich, Tuttlingen, Germany) at 4 °C for 10 min at 10,000 rpm (9391 g) to remove impurities originating from the fish sample. The supernatant was passed through filter paper and placed in a water bath at 70 °C and purged with N_2 gas to facilitate the evaporation of hexane from the filtrate. The pure fat samples were stored in glass vials under refrigerated conditions until analysis.

2.4. Instruments and data collection

Raman spectra of the fat samples were obtained using a DeltaNu Examiner Raman Microscopy system (DeltaNu Inc., Laramie, WY, USA) with a 785 nm laser source and a cooled charge-coupled device (CCD at 0 °C) detector. Instrument parameters were set as follows: integration time of 15 s and 100 mW laser power. Prior to analysis, the sample was placed in a water bath (Memmert, W350, Schwabach, Germany) for 1 h and heated to 50 °C, then immediately entered into the system. Spectra were obtained in the range of 200–2000 cm^{-1} with a resolution of 2 cm^{-1}. Measurements were conducted in duplicate for each sample.

The fatty acid profiles of the fat samples were determined as fatty acid methyl esters by gas chromatography. The fatty acid methyl esters (FAMEs) were prepared according to the AOCS method (AOCS Official Method., 1997). Gas chromatographic analysis of the fat samples was performed using a Trace GC (ThermoScientific, Thermo Fisher Scientific, Inc., Waltham, MA, USA) equipped with a flame ionisation detector. A capillary column (TR-WaxMS, Thermo Fisher Scientific, Inc., Waltham, MA, USA) with 60 m × 0.25 mm internal diameter and 0.25 μm film thickness was used for the separation. The injection volume was 1 μL. Samples were injected in the split mode with a split ratio of 1:50. The temperature of the GC oven was programmed to 120 °C for 1 min then to increase by 6 °C/min to a final temperature of 240 °C, at which point the samples were held for 10 min at this temperature. The injector and detector temperatures were 250 °C. The helium carrier gas flow rate was 2 ml/min. Fatty acid methyl esters (FAMEs) were identified by comparison of retention times with authentic standards (Supelco 37 comp. FAME mix). Results were expressed as a percentage (% wt/wt) of all fatty acids detected.

2.5. Data processing

Chemometric analysis was used to evaluate the Raman data while statistical analysis was used for GC results. All data were given as mean ± error (SE) and subjected to analysis of variance (ANOVA) using the statistical software SPSS 15.0 for Windows. Means were compared by one-way procedure tests at $\alpha = 0.05$ ($n = 2$). Stand-alone Chemometrics Software (Version Solo, 6.5 for Windows 7, Eigenvector Research Inc., Wenatchee, WA, USA) was used for the PCA analysis.

3. Results and discussion

Fresh and frozen-thawed fish samples from six different species were collected and their pure fat samples were obtained according