Validation of near infrared spectroscopy analysis in authentication of fresh and frozen—thawed fish products

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Introduction

Providing label information about the preserving method used for fish conservation (eg. chilling or freezing) could have a strong influences on consumer acceptance. Thawed fish is more susceptible to microbial invasion than fresh fish, with a consequent reduction of its shelf life.[1] This should be strongly considered on management plans, such as the HACCP (Hazard Analysis and Critical Control Point) system, to reach higher standards of seafood quality and safety. The substitution of thawed products labeled as fresh is a common commercial fraud. There are numerous techniques available to distinguish fresh-chilled from frozen-thawed fish, having different performances. This study was the first part of a collaborative trial among different laboratories. The ring trial was carried out to evaluate the discriminating ability of three analytical techniques, a histological method, front-face fluorescence spectroscopy and infrared (IR) spectroscopy. This paper only reports the performances of IRS techniques, in terms of their specificity, sensitivity, accuracy, intra-laboratory reproducibility and agreement with the Standard test for authentication of fresh and thawed Gilthead Sea bream (*Sparus aurata*) fillets.

Materials and methods

80 fresh (F) and 80 frozen–thawed (T) fillets were used as a calibration set, while another set of samples was used to build up a validation set (F=71; T=71). The number (N) of samples for validation was chosen according to the formula $N = (1.96/e)^2\ \theta(1-\theta)$ when e is the accepted error of 0.07% and θ is a presumptive sensitivity (se) and specificity (sp) value of 0.9. T samples were

obtained by quickly cooling down to −80 °C and then stored at −20 °C for 6, 5 and 4 weeks. F and T white muscles were minced using a Retsch laboratory knife mill (10 s at 4000 rpm). Two aliquots per sample were scanned in small ring cups in reflectance mode with a FOSS NIRSystem 5000 (wavelengths, λ, 1100–2500 nm, at 2 nm intervals) and with a UNITY scientific SpectraStar 2500TW (680–2500 nm, at 1 nm intervals). Visible/NIR spectra were also collected in transmittance between 380–1080 nm at 2 nm intervals, using an in-house MMS1 (Zeiss) portable spectrophotometer, in Petri-dishes of 10 mm depth. Mathematical pre-processing techniques, such as the Standard Normal Variate and Detrend (SNVD) correction and first and second derivatives calculated with WinISI II (Infrasoft International, Port Matilda, USA), were performed on the averaged spectra. Discriminant analysis was performed by a multivariate analysis method through MPLS (Modified Partial Least Squares), with cross-validation. The robustness of calibration models was evaluated on the external validation data set. Each fillet was assigned a dummy dependent variable according to cold treatment (1 for F Fresh and 2 for T Frozen-thawed), using a cut-off of 1.5 to classify samples. During validation tests a threshold between 1.65 and 1.35 was also chosen to identify uncertain samples. Sensitivity, specificity and accuracy were calculated by using a free software WinEpiscope 2.0. The agreement between each instrument and the Standard test (Lab. 1 histological method) was evaluated at the 95% level of confidence by Cohen' s kappa. The histology of muscle tissue shows the physical damage due to the development of intra-extracellular ice crystals. The overall agreement among instruments (intra-laboratory reproducibility) was expressed with Fleiss' kappa.

Results and discussion

The discriminant models applied in this study considered all calibration samples. Removing outliers did not improve the classification ability of the models.

Classification abilities of all instruments are reported in table 1.

Table 1. Performances of calibration and validation.

Instruments/ modality	Groups	Number of fillets								Validation parameters				
		Cross-Validation				Validation								
		C	F	U	T	C	F	U	T	ac	se	sp	ag	
													K	Sign
FOSS/ reflectance	Fresh	71	9	41	80	35	36	13	71	0.74	0.96	0.51	0.45	NS
	Thawed	73	7	49	80	68	3	21	71					
UNITY/ reflectance	Fresh	79	0	40	80	57	14	7	71	0.89	0.97	0.80	0.75	**
	Thawed	80	1	35	80	69	2	10	71					
MMS1/ transmittance	Fresh	65	0	3	68	62	4	4	66	0.97	1.00	0.94	0.94	*
	Thawed	72	1	3	76	69	0	2	69					

C: correct; F: False; U: Uncertain; T: total samples analyzed; ac: accurateness; se: sensitivity; sp: specificity.

ag: agreement with standard test; K: Cohen's kappa. ** = $P < 0.001$; * = $P < 0.05$

Seven missing samples reduced the total number of MMS1 validation set. All instruments showed high sensitivity (true positives sample recognition: T fillets), ranging between 0.96 (FOSS) to 1.00 (MMS1). High specificity (sp: true negative samples: F fillets) was observed for instruments covering the short wavelength region (sp: FOSS 0.51, UNITY 0.80; MMS1 0.94). The highest global accuracy (ac) was reported for MMS1 validation, considering the missing values as error (ac: 0.93 data not shown) or in a restricted data set (ac 0.97).

Using the short wavelength region provided a reduction of uncertain samples according to the proposed thresholds (Percentage of uncertain samples FOSS 23.9%; UNITY 11.3%; MMS1 4.4%). Cohen's kappa describes the agreement between the histological technique (Standard test) and each instrument (Table 1). According to the scale proposed by Landis and Koch[2] the range of agreement was between moderate (FOSS K=0.45) and excellent agreement (MMS1 K=0.94). The significance of each contrast was influenced by the number of samples analyzed. Only the FOSS instrument did not show significant agreement with the reference test. Fleiss' kappa (k = 0.57) showed a positive agreement among instruments (moderate agreement; intra-laboratory reproducibility).

Other papers have tested the ability of IR spectroscopy to discriminate between fresh and frozen-thawed fish demonstrating interesting performances on products classification.[3–5] All trials have suggested that the differences between Fresh and Thawed spectra could be attributed to O-H functional groups and H_2O overtones. This is probably due to the lower water holding capacity (WHC) of thawed muscles than fresh ones, with a concomitant increase of drip loss. The study of the wavelength loadings factors on the first 3 PCs showed a large effect in the water regions (Figures 1, 2 and 3).

This comparative trial established that results based on measurements acquired in the short wavelength range, using transmittance as scanning mode with MMS1 portable instruments, provided the best ability to discriminate between Fresh and Thawed fillets. Furthermore, the

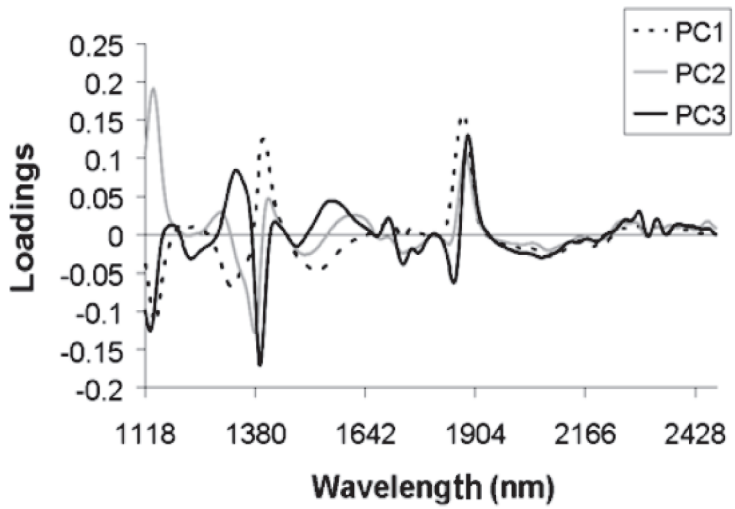

Figure 1. Loadings for the first 3 principal component axes for FOSS spectra.

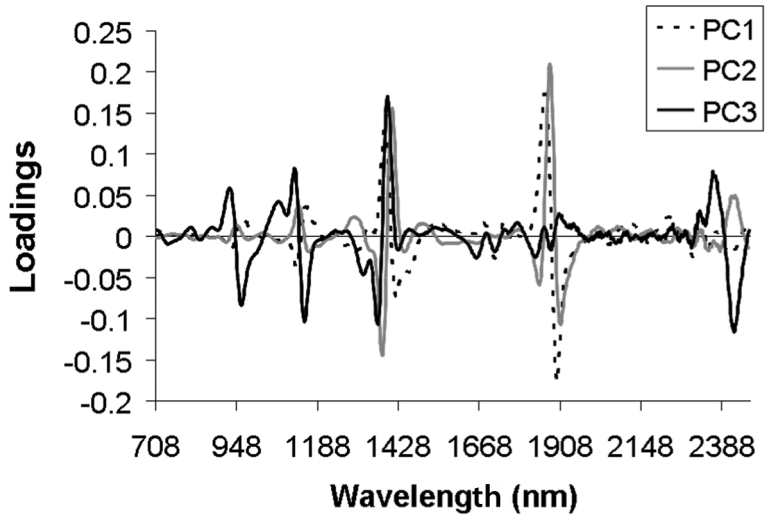

Figure 2. Loadings for the first 3 principal component axes for UNITY spectra.

method also seemed to be the best from a sensitivity point of view. The high accordance with standard test (histology) suggests that near infrared spectroscopy could be utilised as a screening method for official food control.

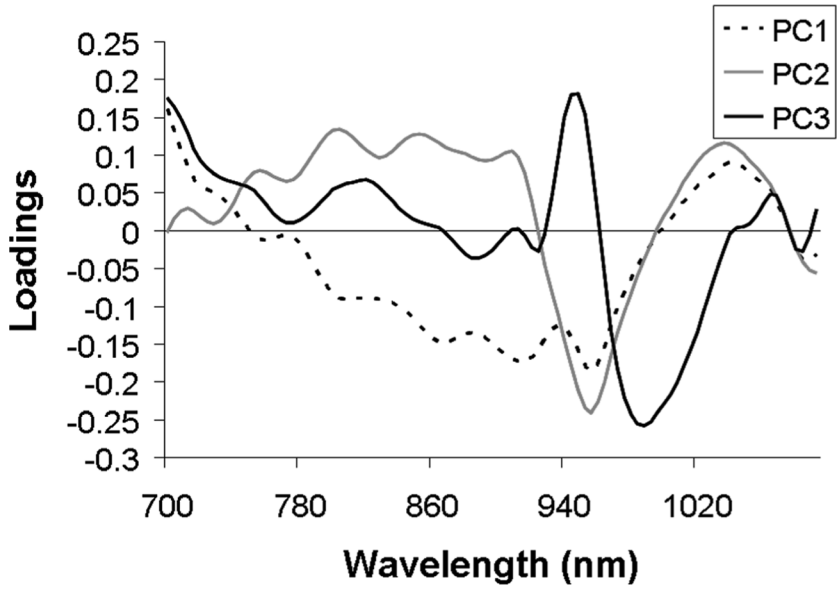

Figure 3. Loadings for the first 3 principal component axes for MMS1 spectra.

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