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Structural changes in sardine (*Sardina pilchardus*) muscle during iced storage: Investigation by DRIFT spectroscopy

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Abstract

Diffuse reflectance infrared Fourier transform (DRIFT) spectroscopy has been used for the first time to evaluate the postmortem changes in structure of components from sardine muscle in relation to quality loss. Sardines (*Sardina pilchardus)* were stored in ice for up to thirteen days. The spectroscopic study was focussed on the structural changes produced on the lipids and proteins.

The most significant results obtained from spectroscopic data are the following: (i) intensity decreasing of ν(=C–H) bands generated by olefinic bonds; (ii) protein structural changes involving mainly β-sheet formation; (iii) frequency increasing of the band near 1400 cm^{-1}, attributable to concentration decrease of salt bridges.

These results show that DRIFT spectroscopy is able to detect structural changes associated with deterioration in sardine muscle, even on the second day of storage. Some of those changes could be used in the development of applications for evaluation of the quality of sardines.

Keywords: Sardine; Fish; Protein; Lipid oxidation; Diffuse reflectance infrared Fourier transform spectroscopy; Quality; Sardines; Ice storage; Postmortem

1. Introduction

Freshness is regarded as one of the most important parameters of fish quality in markets for fresh and lightly preserved fish. The loss of freshness, and therefore of quality, depends on many factors, including the fish species, handling conditions and storage temperature. Fatty fish, such as sardine (*Sardina pilchardus*) are particularly sensitive to oxidative changes during storage, giving rise to rancidity due to the fat content (ranging from 3.0% to 25%, depending on season) and the presence of highly unsaturated fatty acids. The small fish size with its relatively high surface area/volume ratio, has been reported to be one of the causes of rapid deterioration (Shewan, 1971). The high metabolic rate of this species, with enzymes that remain active after death, causing protein hydrolysis, as well as other changes, is also, in part, responsible for the rapid quality deterioration (Sikorski, Kolakowska, & Burt, 1990).

The freshness and quality of the raw fish is usually investigated using a series of physical, chemical and sensory parameters (Olafsdottir et al., 2004). The measurement of some of these fish quality variables is time-consuming or expensive, especially regarding the analysis having to be performed by trained panels, and it is therefore desirable to limit the time required to obtain the information. One

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of the approaches is by using a set of instrumental devices or multisensor, which can be calibrated with a sensory panel, thus mimicking the human senses (Di Natale, 2003).

Since storage time is one of the most determinant factors involving loss of freshness and quality, another approach is to study changes occurring in fish muscle by some instrumental methods (e.g. spectral) that may give a 'fingerprint' of the material and then construct PLS models with time as the dependent variable. In order to get robust models, a knowledge of the structural changes occurring under different storage conditions is necessary prior to the modelling with these spectral data. In this connection, Raman spectroscopy has been used to get insights into protein and water structure in dependence on frozen storage conditions (Careche, Herrero, Rodriguez-Casado, Del Mazo, & Carmona, 1999; Herrero, Carmona, & Careche, 2004; Herrero, Carmona, Garcia, Solas, & Careche, 2005). As an analytical technique, being more sensitive than Raman spectroscopy, the use of Fourier transform infrared (FT-IR) spectroscopy offers an attractive possibility because it would provide a rapid, non-destructive on-line or at-line analysis in the industrial production chain. Nevertheless, transmission FTIR spectroscopy for study of fish muscle involves too much handling of samples, that may result in alterations of protein and muscle structure. An alternative to conventional transmission infrared spectroscopy is diffuse reflectance infrared Fourier transform (DRIFT) spectroscopy. This technique has several advantages: it is fast (5–20 min/sample), simple to use, sensitive, and inexpensive, requires only a few milligrammes of solid material, and allows the analysis of opaque samples, such as fish muscle particles. DRIFT spectroscopy has been used for the analysis of different compounds, including polymers, glass fibres, quartz, coals, and cellulose products (Graf, Koenig, & Ishida, 1984; Guimaraes, Stedile, & dos Santos, 2003; Sereti, Stamatis, Pappas, Polissiou, & Kolisis, 2001), and does not have the drawbacks encountered with the traditional infrared KBr pellet method, principally the incorporation of water. However, this technique has not been applied to fish food analysis.

The main objective here has been to investigate the changes in the DRIFT spectra during postmortem storage of sardines. Some physicochemical parameters have been analysed in parallel, to obtain a possible relationship with the above spectral measurements. Additionally, this research work offers an opportunity to develop a sampling method for DRIFT spectroscopy applied to infrared analysis of muscle from fatty species.

2. Materials and methods

2.1. Fish sample preparation

Sardina pilchardus were purchased in November from a local supplier. The fish, with average size and weight of 15.9 ± 1.1 cm and 56.6 ± 10.8 g, respectively, were freighted in water and ice in expanded polystyrene boxes in an isothermal truck from the Port of Cádiz to the Madrid wholesale market, where they were collected approximately 20 h after preparation, and taken to the laboratory. On arrival, the boxes were re-iced and kept until analysis in a cold store at 1 °C and this procedure was performed when required. For each storage time, 5 boxes of approximately 7.0 kg of sardines were analysed. From these boxes, fifteen fishes were taken for analyses, and data averaged over boxes unless stated otherwise. Various storage times were analysed within 13 days.

2.2. Physicochemical analyses

Percent fat (Bligh & Dyer, 1959), protein, moisture and ash (Association of Official Analytical Chemists, 1984) were determined on portions taken from a homogenate of muscle belonging to at least 20 individuals.

For the measurement of thiobarbituric acid-reactive substances (TBARS) and pH, a homogenate of 20 individuals per box was prepared according to Careche, García, and Borderías (2002). Briefly, the fish were gutted, headed and the backbone, tail and dorsal fin removed. They were then lightly washed with water to remove any residue of blood and then ground for 1.0 min in a food processor machine.

The pH was measured as recommended by Vyncke (1981) from 10 g of homogenate. TBARS were determined on 20 g of the muscle homogenate, ground with 40 ml of 7.5% trichloroacetic acid, containing 0.1% propyl gallate and 0.1% EDTA, for 1 min (Osterizer 867-50E homogenizer, Schaumburg, Ill, TX, USA). The suspension was filtered through Whatman No. 1 paper (Whatman® 1 Qualitative, 110 mm Dia. Whatman International Ltd. Maidstone, England) and allowed to stand at room temperature for 30 min. The filtrate was divided into aliquots which were stored until analysis at −20 °C (three week maximum). The TBARS were measured according to Vyncke (1981) and expressed as μmol TBARS/kg homogenate.

2.3. Instrumentation and DRIFT spectroscopy analysis

For infrared analysis, each fish was filleted and a small portion of muscle from the dorsal part was taken through the DRIFT sample holder, as described below.

A Perkin-Elmer (PE) model 1725X spectrometer was used in this study. All measurements were made with the PE model L127 5001 diffuse reflectance accessory. Sample abrasive holders were prepared, putting, by vacuum, an aluminium coating on the optical surface of a piece of abrasive sandpaper, so that it reflected more light and, hence, to obtain spectra of higher signal-to-noise ratio than those collected using uncoated abrasive paper. The sandpaper had 700 mm mean particle size, the resulting aluminium coat being about 100 mm thick. This surface was cut into pieces 1.0 cm diameter and used as sample holders.

Before background and sample analysis, each fish was opened by cutting it along its back. The resulting muscle

surface was slightly scratched with the piece of abrasive paper by measuring the particles adhering to the paper for subsequent infrared analysis. The quality of the spectra collected from these abrasive sampling methods depends on the level of loading of the fish muscle sample on the pad: if there is too much sample on the pad, the bands in the spectrum will be too strongly absorbing. However, if the pad is loaded so that no loose fish muscle particles are visible, this sample loading has the same effect as diluting a sample with alkaline halide and provides spectra that are similar to transmission spectra. Since the distribution of lipid and proteins in sardine muscle is not homogeneous, repetitive sampling should be made. Moreover, difficulties in this regard can be overcome by considering spectral regions belonging only to lipids or proteins, as described later. To control the instrument energy conditions, a background measurement of an unloaded pad was performed before the spectral measurement of each fish sample, because the infrared energy detected by the spectrometer can fluctuate slightly, depending on the ambient humidity.

For the spectrum of each fish, 32 scans were collected between 4000 and 600 cm^{-1}, using a resolution of 2.0 cm^{-1} at 25 °C. The measurement time for each sample was around three minutes. To eliminate spectral contributions due to atmospheric water vapour, these were removed either by subtracting appropriate vapour spectra measured under identical conditions or by purging with dry nitrogen. The spectra shown here are the averaged spectra measured from the fish belonging to each storage time. For the study of lipid and protein structural features, spectral normalization was carried for the 2927 and 1550 cm^{-1} bands, respectively. The data processing was done by the PE Spectrum for Windows software and GRAMS/AI software from Thermo Galactic (MA, USA).

2.4. Statistical analysis

Analysis of variance (ANOVA) was carried out with iced storage time as the factor. Five replicates, which consisted of the average spectra of individuals belonging to the same box, were used per storage time. The Levene test has been used to check the homogeneity of variances. On the assumption of the equality of variances and the difference between means, the Bonferroni test was used to determine the means differing from each other. The software used was SPSS 11.5 (SPSS Inc. Chicago, IL).

3. Results and discussion

FT-IR spectroscopy monitors the vibrational modes of functional groups within biomolecules and allows a correlation between chemical and structural information. Shifts in peak positions and changes in intensities of the infrared bands are used to obtain structural and functional information about the system of interest.

Fig. 1 shows the average DRIFT spectrum in the 4000–500 cm^{-1} region, corresponding to sardine samples obtained from the first storage time. The band assignments are presented in Table 1. The infrared sardine spectrum is an absorption system consisting of several bands arising from functional groups belonging to proteins, lipids and water, which are the major components of sardine muscle, as is shown from the proximate composition: 16.8 ± 0.4% protein, 17.0 ± 2.0% fat, 64.7 ± 0.2% moisture, and 1.4 ± 0.1% ash. The 4000–3100 cm^{-1} region is the spectral region that contains strong absorptions arising from N–H and O–H stretching modes of proteins and water molecular species involved in intermolecular hydrogen bonding. Water was not removed during sample preparation and, since the absorptivity of the band close to 2100 cm^{-1} changes in going from pure water to water interacting with protein–lipid matrix, the spectrum of water cannot be removed by subtraction. Therefore, the water spectral regions could not be used for structural study.

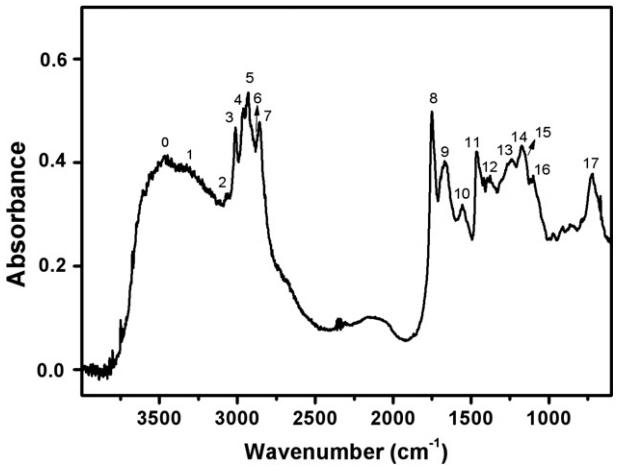

Fig. 1. The averaged DRIFT spectrum obtained from of sardine muscle sampled on the first day of iced storage. The band assignment is presented in Table 1. The three major absorption regions used for spectral comparison in this study are indicated. (i) 3050–2800 cm^{-1}, dominated by absorption bands of the asymmetric and symmetric C–H stretching vibrations of CH_2 and CH_3 groups contained in fatty acids in fish muscle. (ii) 1800–1500 cm^{-1}. This spectral region is shaped mainly by the C=O stretching absorption band of ester carbonyl (1738 cm^{-1}), the various conformation-sensitive amide I band (1700–1600 cm^{-1}) and amide II band components (1600–1500 cm^{-1}) of proteins, and (iii) 1300–1000 cm^{-1}, the "fingerprints region" originate from P=O, C–C and C–O stretching vibrational modes.

Consequently, three major absorption regions are used for spectral comparison in this study, namely: (i) 3100–2800 cm^{-1} which is dominated by absorption bands of the asymmetric and symmetric C–H stretching vibrations of CH_2 and CH_3 groups, mainly arising from fatty acids of fish muscle; (ii) the 1800–1500 cm^{-1} spectral region, which includes mainly the C=O stretching absorption band of ester carbonyl (1738 cm^{-1}), the various conformation-sensitive amide I band (1700–1600 cm^{-1}) and amide II band (1600–1500 cm^{-1}) components of proteins; (iii) the 1300–1000 cm^{-1} range, the "fingerprints region" stemming from P=O stretching vibrational modes of phospholipids and from protein amide III band and C–O or C–C stretching

Table 1
General band assignments of FT-IR peaks of sardine muscle based on the literature (Socrates, 2001)

Band	Wavenumber (cm^{-1})	Assignment
0	3480 s	O–H stretching of water
1	3308 s	Mainly N–H stretching (amide A) of proteins with little contribution from O–H stretching of water
2	3055 vw	Olefinic =C–H stretching vibration: lipids, cholesterol esters
3	3015 m	Olefinic =C–H stretching vibration: lipids, cholesterol esters
4	2959 sh	CH_3 asymmetric stretching: mainly lipids, with little contribution from proteins
5	2927 vs	CH_2 asymmetric stretching: mainly lipids, with little contribution from proteins
6	2896 sh	CH_3 symmetric stretching: mainly proteins, with little contribution from lipids
7	2857 s	CH_2 symmetric stretching: mainly lipids, with little contribution from proteins
8	1739–1744 vs	Ester C=O stretching: triglycerides, cholesterol esters
9	1655 s	Amide I (protein C=O stretching) of α-helix, with contribution from water
10	1550 m	Amide II (protein N–H bend, C–N stretch): α-helix
	1525 m	Amide II (protein N–H bend, C–N stretch): β-sheets
11	1461 m	CH_2 bending: mainly lipids, with little contribution from proteins
12	1395 w	COO^- symmetric stretching: Glu and Asp amino acids residues
13	1238 m	PO_2^- asymmetric stretching of phospholipids, protein amide III band, and C–O–C asymmetric stretching of lipid esters
14	1170 s	C–O–C asymmetric stretching of lipid esters
15	1118 sh	C–O and C–C stretching of lipids and proteins
16	1098 m	PO_2^- symmetric stretching, and C–C and C–O of lipids and proteins
17	725 m	Rocking CH_2 of lipids

motions of ester groups or hydrocarbon chains, respectively. The spectral region below 700 cm^{-1} consists mainly of a great absorption background caused by vibration and translation modes of water molecules which mask the low frequency bands of lipids and proteins, and therefore we have omitted this spectral range. In order to analyse, in more detail, the compositional and structural changes during storage, we have examined the spectral profiles of the above three regions, independently of each other.

Fig. 2 shows the averaged DRIFT spectra in the 3100–2800 cm^{-1} (C–H stretching) for the 1st, 2nd and 13th day. The infrared absorption in this region is mainly due

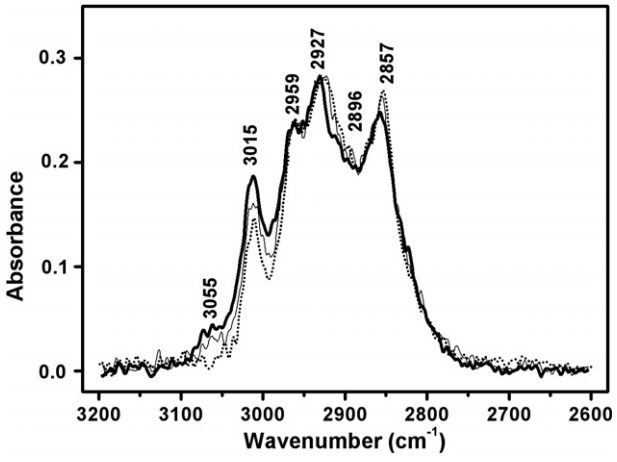

Fig. 2. The averaged DRIFT spectra in the 3100–2800 cm^{-1} region (C–H stretching region) of sardine muscle samples on ice for the first day (bold line), the second (thin line) and the thirteenth (dotted line). The spectra were normalized with respect to the 2927 cm^{-1} CH_2 asymmetric stretching band. Baseline correction has been made through the spectral points at 3150 and 2700 cm^{-1}.

to lipid hydrocarbon chains as a consequence of the relatively high content of lipids in sardines (Chaijan, Benjakul, Visessanguan, & Faustman, 2006), which reached values as high as 17.0%, in accordance with the expected values in autumn for this species. The most significant changes occur for the =C–H stretching bands located at 3055 and 3015 cm^{-1}, whose intensities decrease throughout the storage due to oxidation and concomitant lowering of unsaturated C=C bonds. This change constitutes the common behaviour of lipid oxidation by which saturated lipid chains are considerably more stable to lipid oxidation than are unsaturated lipid fragments. In support of this interpretation, it is the frequency downshifting of the 2927 and 2857 cm^{-1} CH_2 bands due to surrounding chemical changes as a consequence of the oxidation process. In fact, the frequency decrease of these bands may be ascribed to increase of intermolecular order of the lipid acyl chains and to decrease of their dynamics (Fraile, Patron-Gallardo, Lopez-Rodriguez, & Carmona, 1999). This greater lipid chain order is expected from the fact that the transition from intrachain C=C to C–C lipid bonds involves more packing and subsequent greater intermolecular ordering (Applegate & Glomset, 1991). Also, aldehydes derived from intrachain C=C bonds comprise straight hydrocarbon chains which are capable of greater intermolecular order than that of the original lipid molecules. On the other hand, the broadening of the 2927 cm^{-1} band toward lower frequencies is caused by methine C–H stretching absorption near 2900 cm^{-1} (Socrates, 2001, Chap. 2), which results from oxidation of C=C groups to hydroxymethine groups. It should be noticed that some of the above alterations may be visible, even during the second day of storage, particularly intensity changes of the 3055 and 3015 cm^{-1} bands (Fig. 2). One-way analysis of variance (ANOVA) and the resulting bar diagram for the

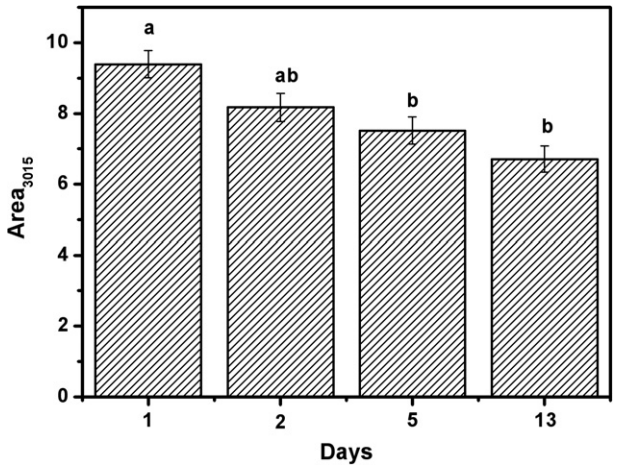

Fig. 3. One-way analysis of variance (ANOVA) for the area of the 3015 cm^{-1} band. Results are shown as the mean ± SE. Different letters indicate significant differences ($P < 0.05$).

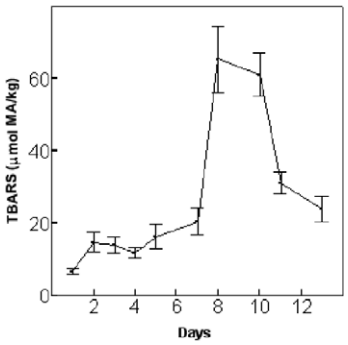

Fig. 5. Changes in thiobarbituric acid-reactive substances (TBARS) of sardines stored in ice. Results are expressed in μmol MA/kg muscle. Results are shown as the mean ± SEM.

3015 cm^{-1} band area (Fig. 3) shows that the absorption at this frequency decreases with the iced storage time. These results, hence, suggest the use of the DRIFT spectroscopic technique as a useful tool for studying the postmortem changes occurring in this species.

As to the 1800–1500 cm^{-1} spectral region, it is dominated mainly by the C=O stretching absorption band of ester carbonyl in triglycerides which appears at around 1745 cm^{-1} (Fig. 4). A gradual decrease in the frequency of this band is visible during iced storage. This can be attributed to the appearance of lipid oxidation products, such as aldehydes and ketones, which appear in the 1735–1710 cm^{-1} range (Socrates, 2001). This is consistent with the evolution of thiobarbituric acid-reactive substances (Fig. 5). The spectral change may also be associated with an increase in infrared absorption of free fatty acids (FFA) in the 1730–1700 cm^{-1} range, this FFA accumulation being due to the hydrolysis of lipids caused by lipase and phospholipase (Chaijan et al., 2006). On the other hand, the amide I band (mainly amide C=O stretching frequencies of protein backbones), observed between 1700 and 1600 cm^{-1}, is conformation-sensitive and can be used to determine protein secondary structure contents, such as β-sheets, a feature which was already employed to investigate changes of hake muscle under different frozen storage conditions by vibrational spectroscopy (Careche et al., 1999). However, due to the influence of the water bending band near 1645 cm^{-1}, we cannot use the protein amide I band with this structural aim. Nevertheless, this is not the case for the amide II band, which can be described in terms of coupled N–H bending and C–N stretching vibrations falling in the 1560–1515 cm^{-1} range (Krimm & Bandekar, 1986). The spectral profile for the amide II band (Fig. 6) shows two bands near 1550 and

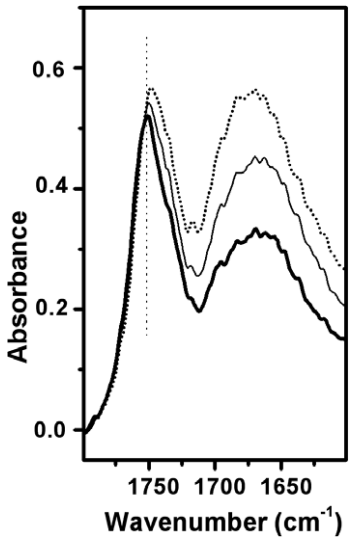

Fig. 4. The averaged DRIFT spectra of sardine muscle samples on the first (bold line), second (thin line) and thirteenth (dotted line) day of iced storage in the 1800–1600 cm^{-1} region. The spectra were normalized with respect to the CH_2 asymmetric stretching band.

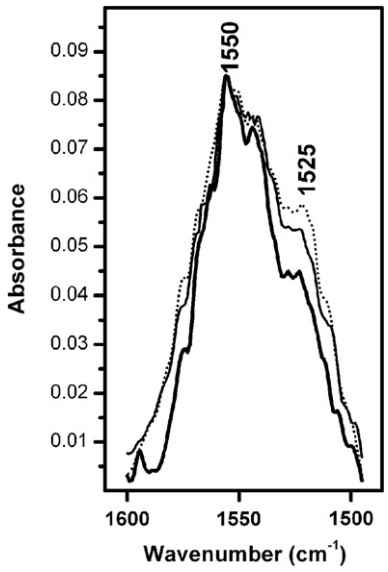

Fig. 6. Detailed averaged DRIFT spectra in the 1600–1500 cm^{-1} region of sardine muscle sampled on the first (bold line), second (thin line) and thirteenth (dotted line) day of ice storage.

1525 cm^{-1} which can be assigned to α-helices and β-sheets, respectively, according to well established spectrum-structure correlations (Goormaghtigh, Ruysschaert, & Raussens, 2006; Krimm & Bandekar, 1986; Oberg, Ruysschaert, & Goormaghtigh, 2004). Normalization of the amide II spectral profile with the 1550 cm^{-1} reveals that there is a significant increase in β-sheet content relative to α-helices upon storage. As occurs for the lipid bands above 3000 cm^{-1}, significant changes are visible in going from the first storage time to the second (Fig. 6).

From the spectra included in Fig. 7, it can be seen that the frequency of the band near 1395 cm^{-1}, which is attributable to the symmetric stretching of carboxylate group COO^-, upshifts over storage. This can be interpreted in terms of salt bridge disruption, because it is well established that this vibrational mode shifts to higher frequencies as the interactions of COO^- groups with their oppositely charged partners weaken (Carmona, Molina, & Rodriguez-Casado, 2003; Nakamoto, 1997, Chap. 4). This is consistent with the increase in pH that occurs during postmortem storage of sardines (Fig. 8). The spectral region below 1300 cm^{-1} is dominated by C–C, C–O and P=O stretching bands of lipids which mask the protein amide III band. No significant changes are observed in this range upon iced storage; therefore it has been omitted.

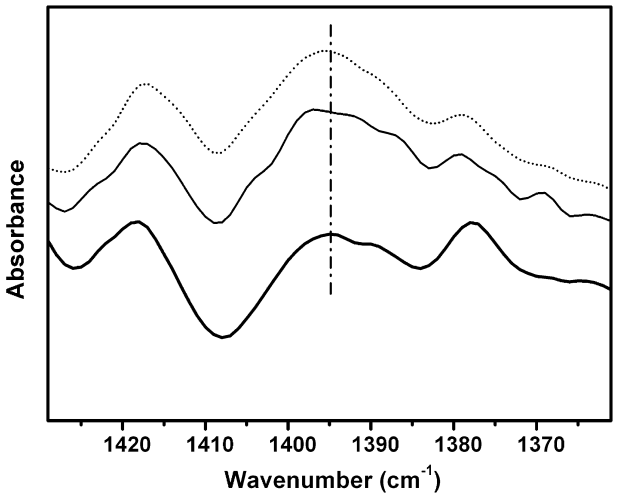

Fig. 7. Detailed averaged DRIFT spectra in the 1430–1360 cm^{-1} region of sardine muscle sampled on the first (bold line), second (thin line) and thirteenth (dotted line) day of iced storage.

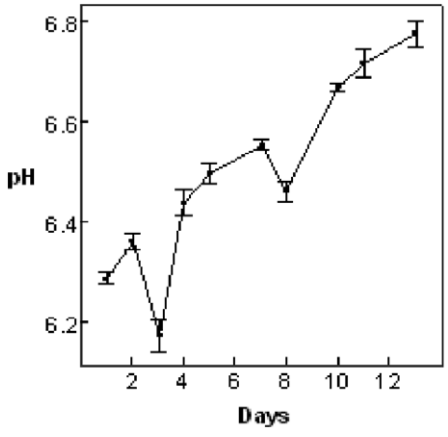

Fig. 8. Changes in muscle pH of sardines stored in ice. Results are shown as the mean ± SEM.

4. Conclusions

The main objective here has been to investigate the potential of DRIFT spectroscopy for the elucidation of postmortem changes in fish. In this connection we show for the first time that this spectroscopic technique is useful for checking the freshness and quality of sardine species throughout iced storage. The abrasive sample holders prepared here generate DRIFT spectra with characteristic lipid and protein bands that change over the iced storage, these bands being related to lipid oxidation and protein denaturation (secondary and tertiary structural alterations). Particularly, the most sensitive bands to iced storage are located near 3055 and 3015 cm^{-1} (lipid unsaturations), 1745 cm^{-1} (lipid carbonyl), 1525 cm^{-1} (β-sheet protein structure) and 1395 cm^{-1} (salt bridges). The fact that some of the changes are consistent with the evolution of well known physicochemical indicators of quality loss and also that the above spectral changes are visible even in early stages of iced storage, makes DRIFT spectroscopy promising in the field of fish quality evaluation. Finally, the above results also promise the use of a non destructive version of DRIFT spectroscopy with optical fibres.

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