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Raman Spectroscopic Study of Structural Changes in Hake (*Merluccius merluccius* L.) Muscle Proteins during Frozen Storage

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This paper examines changes in the structure and functionality of fish muscle proteins at frozen storage temperatures known to render very different practical storage lives (−10 and −30 °C). Apparent viscosity and dimethylamine (DMA) content showed drastic temperature-related differences during storage. Raman spectroscopy revealed the occurrence of some structural changes involving secondary and tertiary protein structures. The changes in secondary structure were quantified, showing an increase of β-sheet at the expense of α-helix structure. The νC−H stretching band near 2935 cm^{-1} increased in intensity, indicating denaturation of the muscle proteins through the exposure of aliphatic hydrophobic groups to the solvent. These structural changes were more pronounced at −10 °C but occurred at both storage temperatures, whereas changes in apparent viscosity and DMA only occurred in storage at −10 °C. The possible utility of these structural changes for quality assessment is discussed.

KEYWORDS: Hake muscle; frozen storage; structural changes; Raman spectroscopy; fish quality

INTRODUCTION

The myofibrillar proteins are of special interest as major components imparting textural attributes to fish muscle. During freezing and frozen storage unwanted changes in the texture of fish muscle have been reported to produce a hard, dry, and fibrous product (*1*, *2*). Evidence on textural changes in fish muscle during frozen storage is available in the literature (*3*). It has been found that textural attributes change significantly with time in fish muscle frozen stored after preprocessing in different ways, and depending on storage temperature. Freezing and frozen storage also affects functional properties of fish myofibrillar proteins. The extractability and apparent viscosity of fish muscle homogenates decrease with storage time or temperature (*4*−*9*).

Structural differences in proteins have been found between fresh and frozen stored hake muscle after 10 months at temperatures known to render very different practical storage lives (−10 and −30 °C) (*7*). Such structural changes mainly entailed an increase of β-sheets at the expense of α-helices and exposure of hydrophobic groups in these conditions. In myosin isolated from cod, loss of α-helical content has been observed by Raman spectroscopy after formaldehyde addition and frozen storage (*10*). Differential scanning calorimetry (DSC) showed some protein denaturation of fish muscle during frozen storage, mainly in the myosin molecule (*8*, *11*, *12*).

In prolonged frozen storage of fish muscle at −10 °C, the unextractable residue after extraction with NaCl, sodium dodecyl sulfate (SDS), and SDS plus mercaptoethanol (ME) was composed of a network of myosin from the residual sarcomere A-band, in the form of interconnected and/or aggregated thick filaments rich in β-sheet structures (*9*).

Changes occurring in textural attributes and functional properties of myofibrillar proteins have been used for the development of methods to assess the quality of frozen stored hake muscle (*4*, *13*). Alternatively, spectroscopy offers measurement procedures that can be used in situ without the need of sample pretreatment. Some spectroscopic procedures (involving visible, infrared, and NMR spectroscopies) have been used to assess the quality of frozen stored fish. Some of them studied in parallel spectroscopic changes with texture measurements (*14*). Others employed multivariate data analysis to correlate the spectral results with some commonly used quality parameters, such as dimethylamine (DMA) content (*15*, *16*), water holding capacity (WHC), or sensory analyses (*17*). In other works, the correlation is directly performed based on storage time (*18*). However, none of the above methods analyze and interpret the observed spectral results in terms of the molecular changes that occur in fish muscle during frozen storage.

Raman spectroscopy was therefore used in the present research with the aim of studying the evolution of structural changes occurring in hake muscle proteins during frozen storage, the comparison of these changes with functionality, and the possibility of using these structural changes to determine frozen fish quality.

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MATERIALS AND METHODS

Fish Source. Gutted hake (*Merluccius merluccius* L.) were obtained in June from the Galician shelf. The fish averaged 1.3 ± 0.25 kg in weight and 51.4 ± 2.4 cm in length. The fish were transported in ice to the Instituto del Frío in insulated containers with a perforated platform inside to prevent contact between fish and melted ice. The fish, in post rigor condition, were headed, filleted, and washed with iced water to remove blood, remains of viscera, etc. Some fillets were kept unfrozen for subsequent analysis. The rest were frozen in a blast freezer (Frigoscandia, Aga Frigoscandia, Freezer Division, Helsingborg, Sweden) until the thermal center reached −40 °C (within 1.3 h), and the resulting frozen fillets were vacuum packed in Cryobac BB-1 bags and stored at −10 and −30 °C for 38 weeks. Analyses were performed at 1, 7, 16, 24, and 38 weeks. Fillets from three individuals were used in each sampling day. For analysis, frozen fillets were thawed overnight in a chill room at 4 °C.

Apparent Viscosity. This was determined in a homogenate of thawed muscle (*4*) with slight modifications (*13*). Previously thawed samples were homogenized with NaCl (1:4), pH 7.0 (phosphate buffer, 50 mM); the homogenates were filtered through gauze and centrifuged for 10 min at 1500 rpm to remove air bubbles (*19*). Measurements were made at 12 rpm on a Brookfield model DV-III rotary viscometer (Stoughton, MA). Results were expressed in centipoises (cP).

Dimethylamine (DMA). The amine extracts were prepared from 50 g of thawed muscle (*20*). Results were expressed as mg of DMA-N/100 g of muscle.

Raman Spectroscopy. Portions from different parts of fillets were transferred to glass tubes (5 cm height and 5 mm i.d.; Wilmad Glass Co., Inc., Buena, NJ) to fill ∼1 cm length (*7*). Spectra were excited with the 1064-nm Nd:YAG laser line and recorded on a Bruker RFS 100/S FT spectrometer. The scattered radiation was collected at 180° to the source, and frequency-dependent scattering of the Raman spectra that occurs with this spectrometer was corrected by multiplying point by point with $(\nu_{laser}/\nu)^4$. Reported frequencies are accurate to ±0.5 cm^{-1}, as deduced from frequency standards measured in the spectrometer. Raman spectra were resolved at 4-cm^{-1} resolution with a liquid nitrogen-cooled Ge detector. The samples, thermostated at 15−20 °C, were illuminated by laser power at 300 mW. Two thousand scans were recorded for each of three samples from the same fillet, resulting in a total of 6000 scans per fish. Raman spectra were processed with Opus 2.2 (Bruker, Karlsruhe, Germany) and SpectraCalc (Galactic Industries, Salem, NH) software.

The Raman band near 1450 cm^{-1}, due to methylene and methyl bending modes and considered to be invariable during conformational changes of proteins, was used as an internal intensity standard for normalization of spectra according to literature (*21*−*24*).

Percentages of protein secondary structures were determined according to Alix (*25*). For that purpose, the water spectrum was first subtracted from the hake muscle spectra following the same criteria as described in other papers (*7*, *25*−*27*). The vibrational modes of peptide backbone or amino acid side chains as well as aqueous contribution were assigned according to the literature (*28*−*33*). The intensity values of Raman bands from various atomic groups were determined after spectral normalization.

Spectra in the C−H stretching region were deconvoluted with a resolution enhancement factor of 3.

Statistical Analyses. A first exploratory analysis of variance (ANOVA) was performed with storage time as covariate, to see the effect of storage temperature (−10 and −30 °C). Then, for each temperature, one-way analysis of variance was performed with storage time as the factor. The Levene test was used to check the equality of variances. Where variances were equal, the difference between means was analyzed by the F test. Where equality of variances could not be assumed, Welch & Brown-Forsythe's robust test for equality of means was used. Once the difference between means was assumed, multiple paired comparisons were used to determine which means differed from one another. The Bonferroni test was used where variances were presumed equal and the Tamhane T2 test was used where equality of variances could not be assumed. Results are presented in the tables and figures. The software used was SPSS 11.0 (SPSS Inc., Chicago, IL).

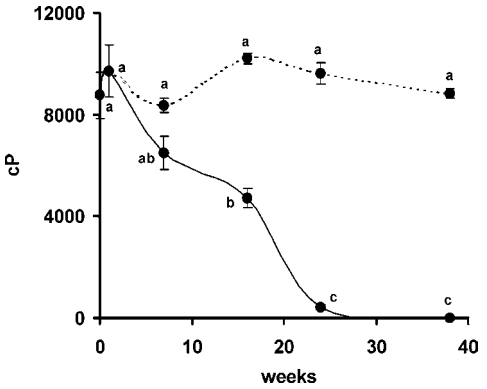

Figure 1. Apparent viscosity (cP) of homogenates from unfrozen hake fillets (0 weeks) and stored at –10 °C (solid lines) and –30 °C (dotted lines) (mean ± SEM). Different letters for the same temperature indicate significant differences ($P < 0.05$).

Table 1. Protein Content in the Homogenate (mg of protein per g of homogenate) Used for Apparent Viscosity Analysis from unfrozen Hake Fillets (0 weeks) and Stored at –30 and –10 °C[a] (Mean ± SEM)

weeks	–30 °C	–10 °C
0	34.2 ± 1.0 (a)	34.2 ± 1.0 (a)
1	33.9 ± 0,9 (a)	33.9 ± 0.9 (a)
7	34.6 ± 0.6 (ad)	33.2 ± 0.8 (a)
16	38.6 ± 0.5 (bd)	36.0 ± 0.8 (a)
24	34.3 ± 0.8 (a)	33.2 ± 1.0 (a)
38	27.8 ± 1.2 (c)	14.9 ± 0.6 (b)

[a] Different letters in the same column indicate significant differences ($P < 0.05$).

RESULTS

Apparent Viscosity. There was a significant and pronounced time- and temperature-dependent change of apparent viscosity during frozen storage (**Figure 1**). No significant differences were observed with storage time at −30 °C. Apparent viscosity decreased steadily in samples stored at −10 °C and after 24 weeks, this functional property reached values below the sensitivity of the viscometer. However, protein content in the homogenate at this temperature only decreased significantly at 38 weeks (**Table 1**).

Dimethylamine (DMA). Significant and time−temperature differences were also found for DMA. Throughout storage, the levels of this compound remained relatively low in hake fillets stored at −30 °C but increased significantly in fillets stored at −10 °C (**Figure 2**).

Secondary Structure. Figure 3 shows the amide I Raman region of hake fillets stored at −10 and −30 °C. The character of this amide I profile can be described in terms of νC═O, νC−N, in-plane δNH, and δC_α−C−N vibrations. The strongest intensity in the spectra profile of unfrozen muscle (1655 cm^{-1}) is attributed to proteins with high α-helix content, on the basis of well-known spectrum−structure correlations (*7*, *28*, *34*).

At −10 °C (**Figure 3a**), the intensity maximum of this band shifted to higher frequencies as from the 16th week, while at −30 °C (**Figure 3b**) such an upshift was observed only during the final week of storage. This frequency change is generated by a decrease in α-helix content resulting from frozen storage (*7*). Estimates of secondary structure percentages can be made from the amide I spectral profile, since this is composed of contributions from α-helix, β-sheet, turns, and unordered

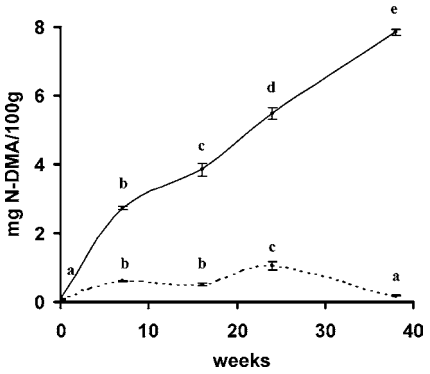

Figure 2. Dimethylamine (mg of DMA-N/100 g) from unfrozen hake fillets (0 weeks) and stored at −10 °C (solid lines) and −30 °C (dotted lines) (mean ± SEM). Different letters for the same temperature indicate significant differences ($P < 0.05$).

structures (*25*, *34*, *35*). The values of these percentages (**Figure 4**) confirm that the most pronounced changes occurred in the hake muscle stored at −10 °C, mainly involving an increase of β-sheet at the expense of α-helices.

On the other hand, we observed increasing intensity in the range 1225−1240 cm^{-1} from samples stored at both −10 and −30 °C from 24 weeks (**Figure 5**). This spectral change could be caused by β-sheet formation (*7*), which is consistent with the quantitative estimates from amide I band analysis. However, the assignments in the amide III spectral profile are complicated by the fact that vibrational spectroscopy of proteins produces a complex pattern of bands in the range 1225−1350 cm^{-1}. This is due to the fact that frequency ranges that are characteristic of β-sheets (1230−1245 cm^{-1}) and random coil (1240−1255 cm^{-1}) overlap to some extent (*31*).

The high α-helix content of hake muscle proteins is further supported by the prominent Raman band at 940 cm^{-1}, which originates from a coupled main chain and side chain νC−C mode common to α-helical proteins (*21*, *34*−*36*). The intensity of this band decreased significantly as a function of storage time (**Figure 6**) but no significant changes were observed as a function of temperature. However, the effect was slightly more intense at −10 °C in the last weeks.

Other Structural Features. In addition to the changes in protein secondary structures, aromatic amino acid chains presented several characteristic Raman bands (**Table 2**). A decrease of intensity in the 759-cm^{-1} band could be attributed to a change in the amphipathic environment of the average Trp side chain, in the sense of a more hydrophilic indolyl environment or solvent exposure (*37*−*39*). Although nonsignificant differences (at 95%) were found in the variations of intensity of this band during frozen storage at both temperatures, this band was slightly less intense in frozen hake muscle stored for 38 weeks than in fresh muscle.

The ratio of the tyrosyl doublet markers at 855 and 828 cm^{-1}, which is a Raman indicator of hydrogen bonding by tyrosine phenoxyls (*40*), has been used to determine whether the Tyr residues in proteins are exposed (0.9−1.45) or buried (0.7−1.0), even reaching values as low as 0.3 for strong hydrogen-bond acceptors (*36*). This ratio did not vary with frozen storage (**Table 2**).

In the 2800−3100-cm^{-1} region of the Raman spectrum, aliphatic amino acids, peptides, and proteins exhibit C−H stretching vibrations (*41*, *42*). **Figure 7** shows the intensity values of the strongest νC−H band located near 2935 cm^{-1}. The intensity of this νC−H band increased at both −10 and −30 °C after the 16th week, and this increase was significantly higher at −10 °C. A band with relatively low νC−H frequency emerged near 2847 cm^{-1}, this band being more prominent at 38 weeks for samples stored at −10 °C (**Figure 8**). This may be assigned to methylene CH_2 groups bound to certain atoms that are more electronegative than carbon (*43*, *44*).

Another spectroscopic parameter considered was the intensity of the O−H stretching band relative to the C−H stretching intensity. This intensity ratio, measured through the Raman signals near 3220 and 2935 cm^{-1}, $I_{3220\ cm^{-1}}/I_{2935\ cm^{-1}}$ (**Figure 9**), decreased gradually during frozen storage, particularly at −10 °C.

DISCUSSION

The apparent viscosity values and the DMA content in hake muscle frozen stored at −10 and −30 °C were as expected and consistent with the literature (*6*, *45*−*48*).

Conversion of α-helices into β-sheets, a slight diminishment of tryptophan band (759 cm^{-1}) at 38 weeks, and the absence of changes in the intensity ratio of the tyrosyl doublet at both temperatures were in agreement with previous data (*7*). The present work shows additionally that these changes are not linear in respect of time.

In the 2800−3100-cm^{-1} region, the intensity increasing with storage time and temperature of the νC−H band near 2935 cm^{-1} can reasonably be attributed to protein denaturation involving changes in tertiary structure with solvent exposure of hydrophobic groups, as previously shown in denaturation of other proteins (*41*, *49*, *50*). These effects run in parallel to the

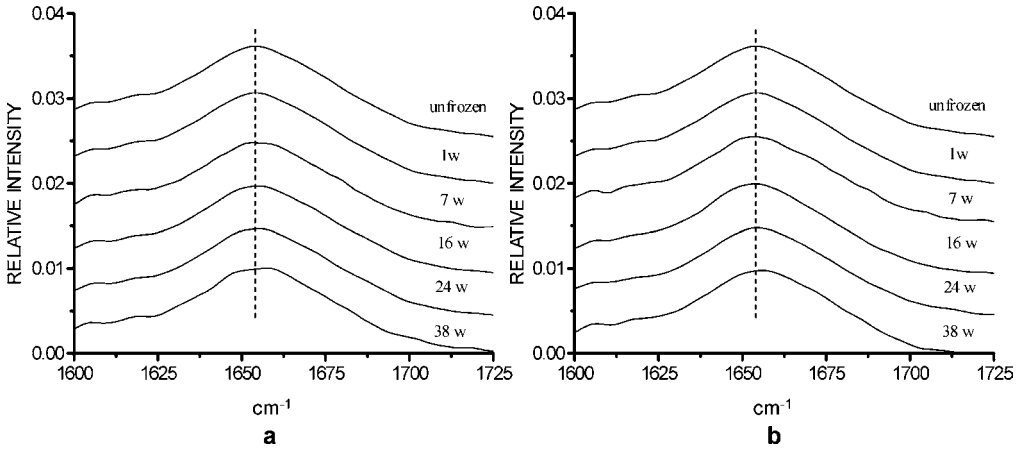

Figure 3. Raman spectra in the amide I region of hake fillets: (from top to bottom) unfrozen and frozen samples stored for 1, 7, 16, 24, and 38 weeks (w) stored at −10 (a) and −30 °C (b).

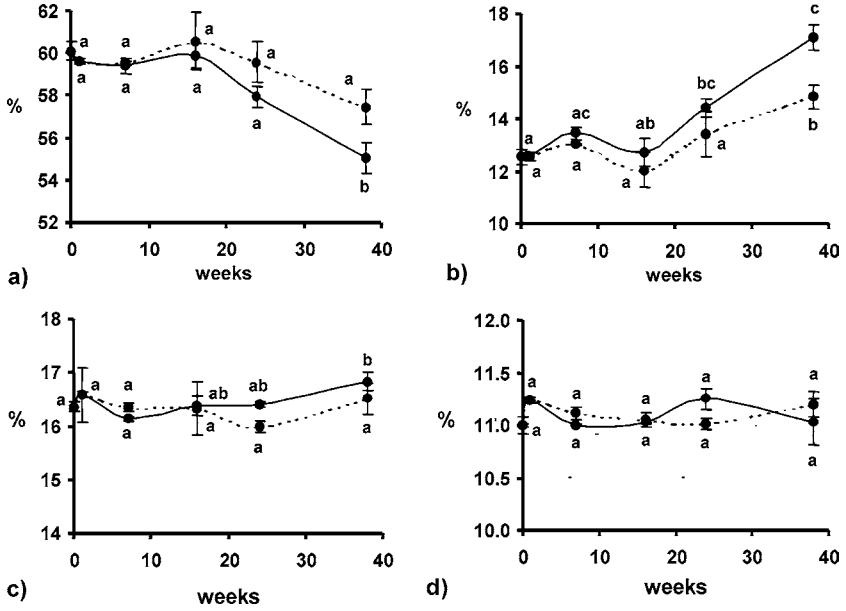

Figure 4. Protein secondary structures (percentage): (a) α-helix, (b) β- sheet, (c) turns, and (d) unordered, in unfrozen hake fillets (0 weeks) and stored at –10 (solid lines) and –30 °C (dotted lines) determined from Raman amide I spectra (mean ± SEM). Different letters for the same temperature indicate significant differences ($P < 0.05$).

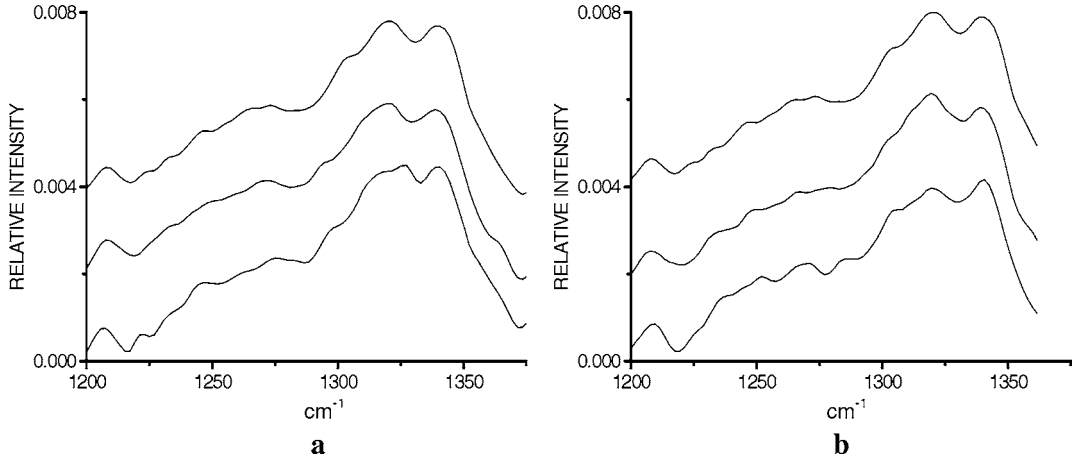

Figure 5. Raman spectra in the amide III region of hake fillets: unfrozen (upper) and frozen samples for 16 (middle) and 38 (lower) weeks stored at –10 (a) and –30 °C (b).

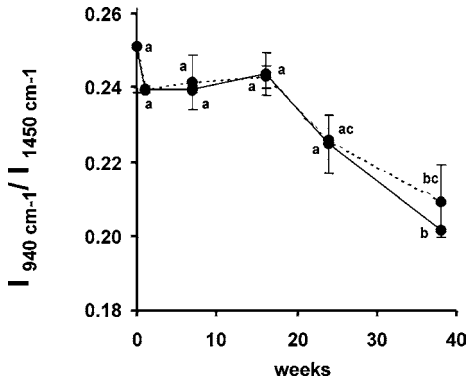

Figure 6. Relative intensity of the C–C stretching band from unfrozen hake fillets (0 weeks) and stored at –10 (solid lines) and –30 °C (dotted lines) (mean ± SEM). Different letters for the same temperature indicate significant differences ($P < 0.05$).

characteristic secondary structure bands, but temperature differences are more pronounced in this 2935-cm^{-1} band. In the same region, a shoulder band emerging near 2847 cm^{-1} has been observed, which was more visible only in the last 38th week of storage at −10 °C. This shoulder was previously observed in the nonextractable residue after NaCl, SDS, and ME from fish muscle stored at −10 °C for prolonged periods of time (*9*). It was suggested that this could be interpreted in terms of interaction of lysine or arginine side chains with FA. Changes in the O−H/C−H stretching band ratio were previously found to be sensitive to differences in freezing and frozen

Table 2. Changes in the Normalized Intensities of the 759-cm^{-1} (tryptophan) Band and the Tyrosyl Doublet at 855/828 cm^{-1} from Frozen Hake Muscle Stored at –10 and –30 °C[a] (Mean ± SEM)

	Trp band $I_{759\ cm^{-1}}/I_{1450\ cm^{-1}}$ (×10^{-1})		Tyr doublet $I_{855\ cm^{-1}}/I_{828\ cm^{-1}}$	
weeks	–10 °C	–30 °C	–10 °C	–30 °C
0	1.75 ± 0.03	1.75 ± 0.03	0.61 ± 0.01	0.61 ± 0.01
1	1.71 ± 0.18	1.71 ± 0.18	0.55 ± 0.07	0.55 ± 0.04
7	1.82 ± 0.13	1.85 ± 0.13	0.63 ± 0.03	0.53 ± 0.10
16	1.75 ± 0.05	1.89 ± 0.10	0.61 ± 0.17	0.65 ± 0.04
24	1.46 ± 0.01	1.53 ± 0.08	0.64 ± 0.04	0.66 ± 0.09
38	1.51 ± 0.12	1.57 ± 0.06	0.61 ± 0.04	0.57 ± 0.08

[a] Nonsignificant differences were found.

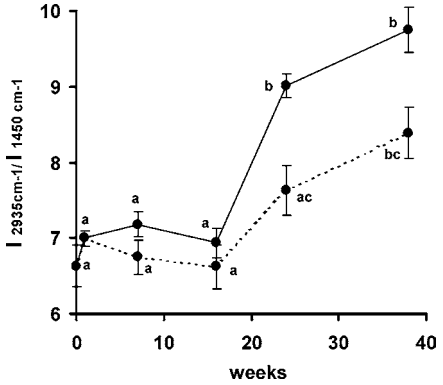

Figure 7. Relative intensity of the C–H stretching band from hake fillets stored at –10 (solid lines) and –30 °C (dotted lines) (mean ± SEM). Different letters for the same temperature indicate significant differences ($P < 0.05$).

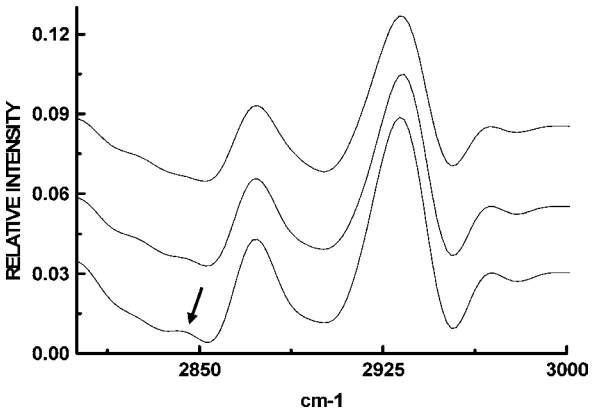

Figure 8. Deconvolved Raman spectra in the 2800–3000 cm^{-1} stretching region of unfrozen hake fillets (upper), stored at –30 (middle) and –10 °C for 38 weeks (lower). The arrow indicates a shoulder at 2847 cm^{-1}.

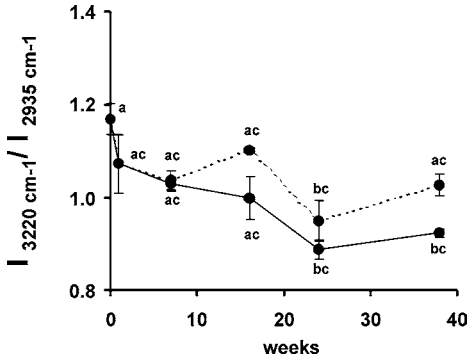

Figure 9. νO–H/νC–H intensity ratio from unfrozen hake fillets (0 weeks) and stored at –10 (solid lines) and –30 °C (dotted lines) (mean ± SEM). Different letters for the same temperature indicate significant differences ($P < 0.05$).

storage conditions for hake muscle (*7*). The results of the present study, based on a quantification of the νO−H/νC−H intensity ratio, confirm the existence of a relation with storage conditions and time in frozen hake muscle.

Observed changes in secondary and tertiary structure of proteins were not accompanied by loss of apparent viscosity in samples stored at −30 °C, which confirmed previous results (*7*). Other functional properties, such as extractability, likewise remained unchanged in the course of low-temperature frozen storage, in conditions where some protein denaturation was occurring (*8*). The reason could be that apparent viscosity and extractability are functional properties that depend on a number of factors (*51*−*53*), so that the resulting measurements show an average of the effects of these factors on the protein functionality. On the other hand, some of the protein changes may be events occurring during frozen storage, but may not be directly related to functionality loss. We cannot therefore yet attribute functional properties losses to specific protein structural changes.

This work shows that some Raman regions, which are characteristic of protein structure, can in practice be used as an indicator of quality, mainly in those cases where functional properties are not sensitive enough. Actually, although the maximum allowed storage temperature is −18 °C, most industries store fish at temperatures below −25 °C, where the loss of functionality is minimized. The maximum storage time applied commercially is, in some european countries, 2 years. If functionality remains virtually unchanged or it changes very little, both Administration and Industries have no means of assessing the storage time, with possible room for fraud. The availability of methodologies for evaluating the previous history of the fish would permit and encourage governments to require companies to provide consumers with better information, not only on ingredients and nutritional values, but also in processing methods.

The fact that there are Raman bands more prone to changes due to storage temperature and other bands more insensitive to temperature, but still presenting changes with storage time, shows the potential for Raman spectroscopy as a tool for establishing the time/temperature history of frozen fish muscle. Partial least squares regression (PLSR) models obtained in the 600−1800-cm^{-1} range support the above suggestion (manuscript in preparation). The calibration regression coefficients of the models (R) range from 0.99 to 0.97 and the prediction from 0.95 to 0.89, depending on the temperature considered and on whether this parameter is neglected. Similarly, the root mean square errors (RMSE) for calibration (C) range between 0.2 and 0.8 and for prediction (P) between 1 and 1.5 months.

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