Near infrared spectroscopic determination of casein content in the study of micelle interactions

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Introduction

Caseins are the predominant milk protein compounds of almost all mammalian species. Their importance is expressed from both a nutritional and a technological point of view. Their biological function is to provide a source of phosphate and calcium for tissue calcification, as well as amino acids and biologically active peptides.[1] Furthermore, their amount and quality have great influence on milk rennet properties and cheese yield.[2] Caseins constitute a heterogeneous group of phosphoproteins, phosphorylated to serine clusters, present as stable calcium phosphate protein complexes named micelles. Caseins from milk of ruminants have been extensively studied, and have shown to consist of four main components; α_{s1}-, α_{s2}-, β- and κ-casein. The α_s- and β-caseins are insoluble at the calcium ion concentration present in milk, while κ-casein is insensitive to calcium and thus plays a role in maintaining the stability and integrity of the whole micelle structure.[1] The nature of links between the different subunits has been deeply studied, but the exact structure of the casein micelle is still debated. Despite the many applications of near infrared (NIR) spectroscopy in the food and agricultural sectors, the relevance of this technique to study the secondary structure of proteins has received minor attention.

The aim of this work was to investigate the near infrared spectra of casein solutions in order to improve knowledge of the structure of casein aggregates and subunits.

Materials and methods

Samples

Raw bulk milk samples (n =58) were collected from different farms in the Asturias region of Spain during a one month period.

Analyses (in duplicates) of total protein (%TP) and non-caseinic nitrogen (%NCN) were performed by Kjeldahl's method, and casein content was calculated as the difference between TP and NCN.[3,4] Milk samples were split into two aliquots: one was ultra-centrifuged at 100 000 g for 1 hour at 4°C ±1°C in order to obtain the native casein by sedimentation; the other was acidified with 3 N HCl until pH = 4.6 and then centrifuged at 30 000 g for 30 min at 4°C ±1°C to get the acid casein. The two types of casein (n=116) were then reconstituted to their initial concentrations in milk by dilution in phosphate buffer (PBS 0.1 M, pH = 6.8).

Capillary zone electrophoresis (CZE) analyses were also carried out on reconstituted samples with a Beckman P/ACE MDQ apparatus (Beckman Instruments, Fullerton, CA, USA). Separations were performed under denaturant conditions at 38°C ± 1°C in a coated fused-silica capillary by applying a 25 kV voltage, as reported by Recio.[5]

Near infrared spectroscopy

Spectra of reconstituted casein were collected at 37°C ± 1°C with two spectrometers, an FT-NIR (Perkin-Elmer, Waltham, Massachusetts, USA) and a Foss-NIRSystem 6500 (Foss, El Leende, The Netherlands). FT-NIR analysis was performed in transflectance mode (1112–2500 nm; resolution = 4 cm^{-1}). Duplicate spectra from each sample were averaged. The Foss-NIRSystem 6500 instrument was equipped with a transport module, and spectra were collected from two subsamples between 400 and 2500 nm. The first subsample was placed in a 50 mm diameter gold transflectance cell, with a 0.1 mm sample thickness camlock cell, and scanned at 2 nm intervals. The second subsample was analysed in reflectance mode with an opaque liquid cell. Both subsamples were analysed in duplicate and each spectrum was averaged from 32 scans. Data were processed using The Unscrambler v.9.2 (Camo Inc., Oslo, Norway).

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Results and discussion

Milk is a very complex matrix, consisting of proteins in colloidal dispersion, fat in emulsion and minerals in solution. We worked with a simplified model and focused attention only on the caseinic portion, but maintained the same properties and proportions present in milk (solution state, pH and casein concentration on the basis of Kjeldahl's results). Under such conditions it was possible to evaluate the sensitivity of the spectroscopic technique when applied to a single matrix, using different instruments and procedures. Therefore, NIR and electrophoretic analysis were carried out.

The electrophoretic analysis performed on samples of reconstituted casein allowed the separation of different casein fractions. The obtained results expressed as normalised area (area ÷ migration time) are shown in Table 1.

Table 1. Content of casein fractions in reconstituted samples. Values are expressed as normalised area (area ÷ migration time).

	αs2	αs1	αs0	κ	βb	βa1	βa2
Min	766.86	7120.00	1880.48	421.26	587.66	979.86	5836.59
Max	6507.49	31340.95	11235.07	6279.85	7655.70	17999.70	34282.35
Mean	3062.36	19093.72	6259.92	2404.99	2066.77	10044.21	17229.53
SD	1317.33	6640.27	2417.29	1953.98	1899.03	4761.58	7540.38

PLS analyses were performed with NIR spectra collected from the three spectral methods and electrophoretic data. Table 2 shows the statistical parameters obtained, in terms of number of latent variables (LV), coefficient of correlation in calibration (Rcal) and in cross-validation (Rval), root mean square of standard error in cross-validation (RMSECV). The preprocessing procedures applied to spectra (SG: second derivative Savitzky-Golay, 5 points, polynomial order 2; SNV: Standard Normal Variate) are also reported (Table 2).

Table 2. Statistical descriptors for NIR calibrations of reconstituted casein fractions.

Instrument	Spectral range	Variable	LV	Rcal	Rval	RMSECV	Preprocessing
FT NIR transflectance	1112–2500 nm	αs_2		poor	poor		
		αs_1	4	0.918	0.864	3206.36	none
		αs_0	1	0.837	0.813	1346.24	none
		κ	10	0.999	0.801	1207.78	SG
		βb		poor	poor		
		βa_1	3	0.991	0.750	3414.98	SG
		βa_2		poor	poor		
NIR transflectance	400–2500 nm	αs_2	6	0.847	0.667	1085.12	none
		αs_1	3	0.888	0.861	3504.54	SNV
		αs_0	3	0.88	0.862	1260.51	SNV+SG
		κ	7	0.963	0.800	1261.66	SNV
		βb		poor	poor		
		βa_1	7	0.915	0.823	3262.47	None
		βa_2	2	0.895	0.858	4426.92	None
NIR reflectance	400–2500 nm	αs_2		poor	poor		
		αs_1	3	0.905	0.863	3311.70	SNV+SG
		αs_0	3	0.938	0.898	978.78	SNV+SG
		κ	5	0.949	0.920	759.72	SNV
		βb	9	0.983	0.675	716.48	SNV+SG
		βa_1	4	0.842	0.810	3213.80	None
		βa_2	6	0.933	0.810	4631.04	None

The best performances were obtained with reflectance measurements. This is due to the fact that reconstituted samples are similar to opaque solutions and the reflectance mode exhibits dependency on the light scattering phenomena. NIR reflectance measurements allowed the use of longer pathlengths compared with transflectance mode, with a different depth of light penetration for each wavelength. This fact can explain the great variation when measuring heterogeneous samples.[6]

Satisfactory results were obtained during calibration, with the exception of αs_2 casein, while statistics in validation could be improved by increasing the sample set and creating a reference dataset. However, these results were consistent with previous studies on milk matrices. [2, 7]

To assess the effect of spectral region on different calibrations, a comparison was made using only spectral data between 1112 and 2500 nm. These new models showed performed poorly (data not shown), and it therefore seemed that in this case the Vis-NIR spectral region was essential to create good predictive models.

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In order to assess the ability of NIR to discriminate between the application of physical and chemical treatments, principal component analysis (PCA) was applied on the second derivative of reflectance spectra.

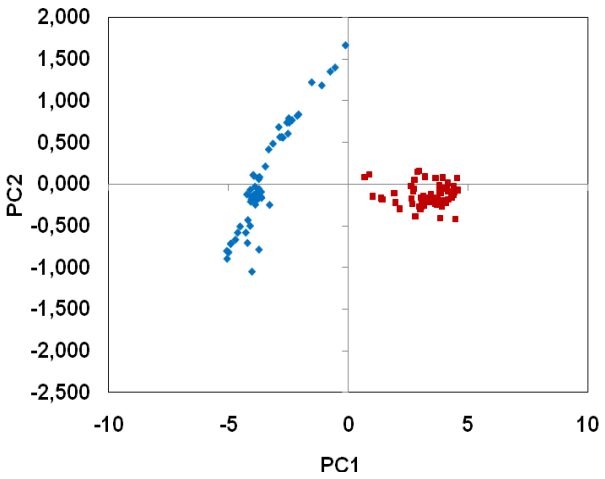

Figure 1. Scores plot obtained from the PCA analysis performed on the second derivative of reflectance NIR spectra of native (blue dots) and acid (red dots) casein reconstituted solutions.

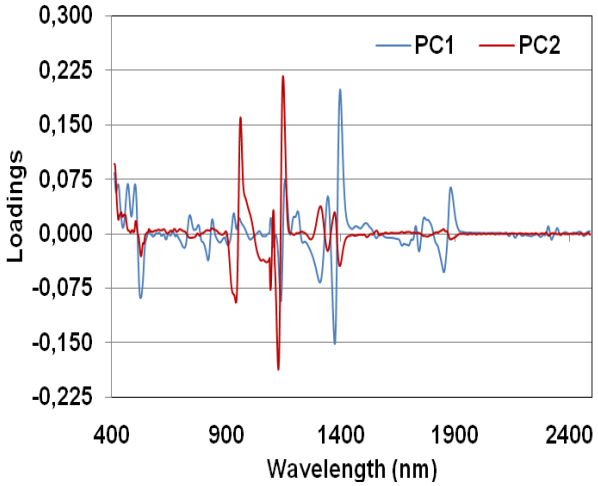

Figure 2. Loadings plot on PC1 and PC2 obtained from the PCA analysis.

As shown in Figure 1, the two groups of samples are perfectly separated along the first principal component (PC1), which was able to explain 98% of the total variance. The second principal component (PC2) explained the remaining 2% of the total variance and seemed to be able to weakly separate inside the group of reconstituted samples of native casein.

The loadings plot (Fig. 2) indicated that the separation of the two groups was mainly based on wavelengths related to P-OH stretching (1300, 1876 nm), P-H stretching (1370, 1394 nm) and some related to C-H bond stretching.[6]

These results showed the capacity for NIR spectroscopy to distinguish changes in the mineral equilibrium induced by acidification. In fact, caseins are phosphorylated aggregated proteins present in milk in a micellar state which may be separated by acidification, ultracentrifugation, and enzymatic coagulation. Casein separated by acidification is called acid, demineralised or isoelectric, because it's obtained by acidifying the skimmed milk to a pH of 4.6, corresponding to the isoelectric point of casein.[8] Acid casein is also known as demineralised casein because, as a result of the pH change, it loses part of the calcium and phosphate responsible of the micellar state. Casein obtained by ultracentrifugation is called 'native casein' and maintains the structure that it has in milk.[8] Thus, although it is the same protein on the basis of its primary structure, the two types of casein present variations in micelle interactions and links between subunits.

To better understand the molecular differences between native and acid caseins, the second derivative of mean reflectance spectra of two types of reconstituted samples was compared. In particular, it was possible to distinguish between the specific absorption bands exclusively related to nitrogen compounds and those related to the bonds involved in the stabilisation of the micelle structure.

As shown in Figure 3, the portion of the spectrum between 2000 and 2300 nm appeared to be each type of casein superimposed. Indeed in this area it's possible to recognise the absorption bands related to the stretching and bending of the N-H bond, in particular: 2120 nm N-H stretch + C=O stretch of amino acids, 2148 and 2176 nm combination bands of amide I and III; 2248 nm N-H stretch + NH_3 deformation of amino acids; 2290 nm N-H stretch + C-H deformation of amino acids.[6]

The absorptions at 2440 and 2482 nm are related to the free P=O group.[6] Casein contains both colloidal organic phosphorus in the form of phosphoserine, and colloidal inorganic phosphorus salified to amino-terminal or carboxylic groups of proteins through calcium. In both cases, the free P=O groups are present.[8] These groups were therefore detectable in both the native and the acid casein. In the region around 1050 nm the second overtone of the NH group stretching and the combination of the free P=O with the amide I could be recognised.[6] Another common area of the spectra was identified between 1500 and 1580 nm, where there are bands related to the first overtone of the NH stretching.[6] Otherwise, the two types of spectra were very different in the area between 1200 and 1400 and between 1800 nm and 2000 nm, ascribable to changes in the phosphate bonds and water.[6]

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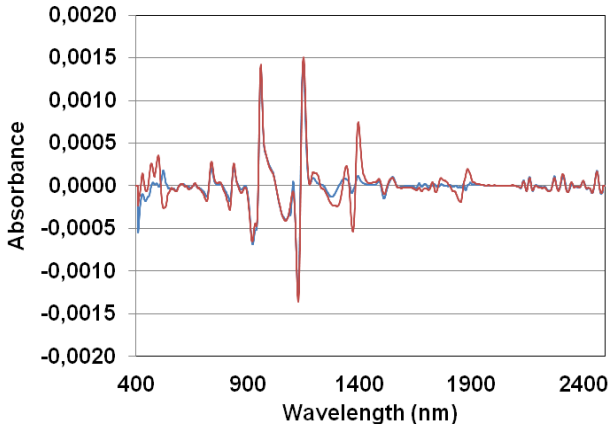

Figure 3. Second derivative mean spectra of native (blue line) and acid (red line) casein reconstituted samples.

Bands at 1234 and 1294 nm in the reconstituted acid casein were noticeable. These wavelengths are assigned to P-OH stretching.[6] When phosphate is bound to serine, two acid functions are free for any salification with cations, especially calcium. It was evident that these bands should be found in reconstituted acid casein: in this case the two acid groups remain free since the calcium phosphate bond is lost.[8] The same phenomenon occurred more markedly in the region between 1850 and 1950 nm. In fact, the spectrum of acid casein reconstituted samples showed stronger absorption bands relative to OH and POH stretching.[6]

Conclusion

In this work a comparison of performance in calibration of different NIR apparatus and sample presentation modes was made. In the case of casein spectra, the spectral range was found to be more influential than the type of instrument. These preliminary results confirmed the applicability of NIR spectroscopy to investigate the structure of casein micelles. Further studies coupled with investigation in the mid-infrared region could help to better characterise the secondary structure of this protein complex. Electrophoretic analyses using a dedicated separation system (Rotofor® system-Biorad, Hercules, California, USA) are in progress to recover and purify each single casein fraction with the aim to identify their spectral contribution to the spectra of the whole casein micelle.

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