Two-dimensional mid infrared and near infrared correlation spectroscopy: a useful tool for study and quantification of heat denaturation and aggregation of whey protein isolates

Marie-France Laporte, Muriel Subirade and Paul Paquin

Centre de Recherche en Sciences et Technologie du Lait, Département de Science et Technologie des Aliments et Nutrition, Faculté des Sciences de l'Agriculture et de l'Alimentation, Pavillon Paul Comtois, Université Laval, Ste-Foy, Québec, Canada G1K 7P4.

Introduction

In the food industry whey protein isolates (WPI) are used, among others, as functional ingredients, in dedicated partially, denaturated and aggregated forms (microparticulation). The type of applications depends on the level of denaturation and/or aggregation of WPI. Unfortunately, quantification of partial denaturation and aggregation of whey protein is still a problem. Although not completely elucidated yet, mechanisms of denaturation and aggregation of protein have been widely studied and successfully characterised in mid-infrared (mid-IR) spectroscopy.

Near infrared (NIR) spectroscopy appears to be a promising tool for monitoring protein denaturation. NIR is widely used in the food industry, especially in the dairy sector where it is offering the possibility of on-line monitoring of major components.[1] However there are some problems with NIR, for example, the difficulties in interpreting spectral variations, as well as in assigning specific wavelengths to the complex PLS model developed for quantification. However, in two-dimensional spectroscopy, mid-IR can become a useful tool for the establishment of a relationship between NIR spectral variations and changes in the secondary structure of proteins under heating perturbation.

As a first step toward the complete NIR calibration for WPI, the objective of this study is to use bi-dimensional correlation spectroscopy as developed by Noda[2,3] and applied by several authors,[4–7] in order to investigate whether denaturation and aggregation phenomenon, described in mid-IR, can also be observed in NIR. This interpretative spectroscopy will provide the scientific basis for the selection of NIR wavelengths for protein denaturation and aggregation quantification.

Material and methods

Solutions and heat treatment

For 2-D correlation:

- 5% (w / v) WPI (94% proteins) solution pH 8.0 (Davisco Foods International, Inc., Le Sueur, USA).

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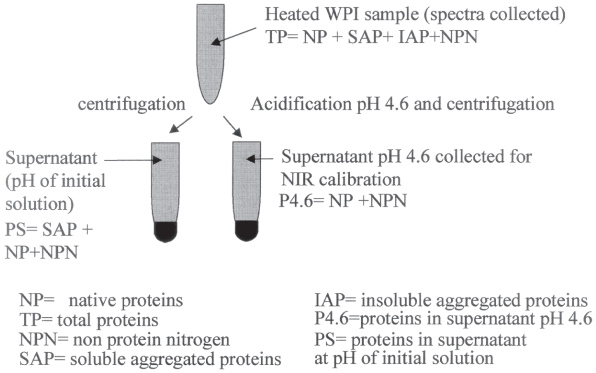


Figure 1. Method for obtaining WPI samples with different levels of denaturation and aggregation (modified Sanchez *et al*. 1997).

For calibration:

- Samples at pH 6.0 and 8.0, acidified and non-acidified centrifuged supernatants (Figure 1) were also collected.
- In order to study the same WPI sample in both IR apparatus, the thermal treatment was completed prior to spectroscopic analysis.
- Solutions were heated, according to treatments 23, 40, 50, 60, 70, 80 and 90°C, for 30 min in a water bath.

Spectroscopy

- Infrared spectra of thermally treated WPI solutions were recorded at room temperature.

Mid-IR

Nicolet Magna 560 Fourier transform infrared spectrometer, running Omnic software for data collection, cooled mercury-cadmium-telluride detector, transmission, CaF_2 windows, 6 µm spacer, 2 cm^{-1}, 128 scans.

NIR

Bomem MB-Series spectrophotometer, 0.5 mm quartz cell, 16 cm^{-1}, 128 scans. Data collection with a water spectrum background.

Data pretreatment

Mid-IR

Subtraction of water reference spectrum and elimination of residual water vapour were performed with Spectra Calc (Galactic Industries Corp. Salem, New Hampshire, USA). Second derivative was applied using the Savitzky–Golay algorithm (polynomial 2 and point 15) under Grams/32 (Galactic Industries Corp.).

NIR

Second derivative was applied using the Savitzky–Golay algorithm (polynomial 2 and point 13) under Grams/32 (Galactic Industries Corp.).

For easier observation, both mid-IR and NIR derivative spectra were multiplied by –1.

2-D correlation

Synchronous and asynchronous correlation between mid (1600–1700 cm^{-1}, amide I band) and near (4200–4900 cm^{-1}) infrared regions were calculated on second derivative spectra with KG2D software developed by Dr.Wang. The first spectrum, which corresponds to the less denaturated, was used as reference (Figure 3 = 23°C and Figure 4 = 70°C).

Chemical

In order to investigate the quantification of the levels of denaturation and aggregation in heated samples, reference analysis on proteins (Kjeldahl) were performed as schematised below.

Calibration

Calibration was performed with PLS under Grams/32 on a total of 33 samples which include: pH 8.0 and pH 6.0 heated WPI solutions, pH 8.0 and pH 6.0 non-acidified centrifuged supernatants, and finally pH 8.0 and pH 6.0 acidified centrifuged supernatants.

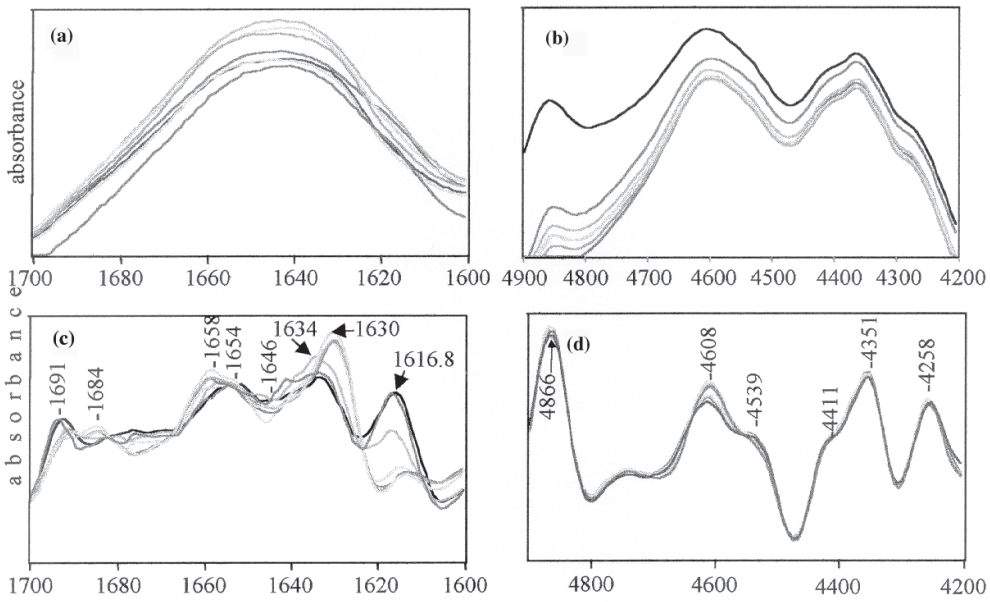
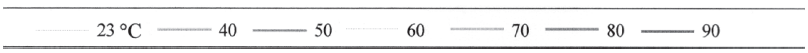


Figure 2. Original and corresponding second derivative spectra of WPI solutions (5%, pH 8.0) heated from 23°C to 90°C and recorderd at room temperature. (a) Original mid-IR spectra of the amide I region (1700–1600 cm^{-1}). Spectral contribution of water and residual water vapour were subtracted as described in materials and methods; (b) original NIR (4900–4200 cm^{-1}) spectra; (c) corresponding second derivative amide I mid-IR spectra; (d) corresponding second derivative NIR spectra.



Spectral regions selected for calibration correspond to those (10) identified from 2-D correlation in part I as potentials for monitoring protein denaturation and aggregation: 4196–4230 cm^{-1} (4220 cm^{-1}); 4250–4310 cm^{-1}; 4339–4359 cm^{-1} (4347 cm^{-1}); 4383–4402 cm^{-1} (4392 cm^{-1}); 4454–4474 cm^{-1} (4462 cm^{-1}); 4509–4529 cm^{-1} (4519 cm^{-1}); 4590–4610 cm^{-1} (4602 cm^{-1}); 4654–4674 cm^{-1} (4664 cm^{-1}); 4777–4797 cm^{-1} (4787 cm^{-1}) and 4853–4873 cm^{-1} (4863 cm^{-1}).

Results and discussion

Two-dimensional mid–near IR spectroscopy and study of protein denaturation and aggregation

As presented in Figure 2(a and b), characterisation of thermal denaturation (unfolding and aggregation) of protein is nearly impossible from underivative spectra. On the other hand corresponding second derivative spectra [Figure 2(c and d)] show characteristic bands arising from proteins. The same information, in much more detail, is seen in synchronous correlation maps presented in Figures 3 and 4.

Standard convention for analysis is used: solid and dashed lines in the contour maps represent positive and negative correlation peaks, respectively. A positive cross-peak indicates that the intensity variations observed at the two spectral coordinates are changing in the same direction, while a negative cross-peak indicates that the variations occur in an opposite direction.

Figure 3 presents the synchronous correlation map of five mid-IR and NIR spectra of WPI solutions (pH 8.0) heated at 23, 40, 50, 60 and 70°C. Figure 4 shows the same correlation but with solutions heated at 70, 80 and 90° C. Amide I bands of WPI samples, heated up to 70°C, present few changes.[10] Above this critical temperature the authors observed the disappearance of bands characteristic of native protein and bands associated with the formation of intermolecular hydrogen bonds (aggregation). Thus, it can be said that Figure 3 represents denaturation, particulary unfolding of protein, while Figure 4 represents further unfolding events, mainly at the aggregation stage.[10,11]

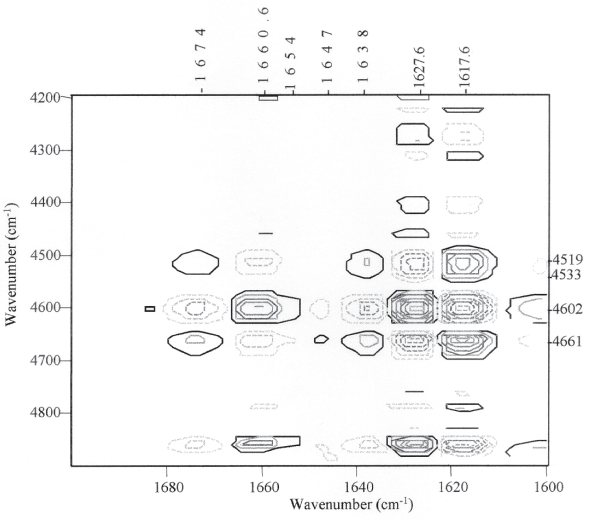


Figure 3. Synchronous 2-D mid-IR and NIR correlation contour map of heated (23, 50, 60 and 70°C) WPI spectra in the 1700–1600 cm^{-1} (mid-IR) and 4900–4200 cm^{-1} (NIR) regions.

Table 1. Attribution of mid-IR wavelengths to the secondary structure of proteins.

Wavelength cm^{-1}	Secondary structure of protein
1618	β-sheet (↑ with denaturation and ↑ ↑ with aggregation)[11]
1628–1632	β-sheet (↑ denaturation; aggregation)[9,11,13]
1647	α-helix[9] or unordered[14] (↓aggregation)
1652	Unordered[13] or α-helix and random[9]
1658	α-helix[12,13]
1661	Turns[9,13]
1676	β-sheet or turns (↓denaturation and aggregation)[9]
1684	β-sheet (↑ aggregation)[11]
1694	Turns[14] or β-sheet[13]

Mid-IR information

Major mid-IR bands arising in 2-D analysis from thermally pertubated mid-IR spectra are consistent with both derivative spectra [Figure 2(c)] and with the literature, as shown in Table 1. Figure 3, which represents protein denaturation, presents bands at 1617, 1627, 1638, 1647, 1653, 1660 and 1674 cm^{-1}. According to several authors,[9–12] those bands can be related to unfolding and denaturation phenomenon of β-lactoglobulin and α-lactalbumin. Figure 4 shows intensification of the 1618 cm^{-1} band, a decrease of 1676 cm^{-1}, broadening of 1629–1646 cm^{-1} and, the most important feature, growth of new bands at 1684 and 1694 cm^{-1}. All this information indicates further denaturation and aggregation processes. It should be emphasised that observed bands are often not exactly the same as those

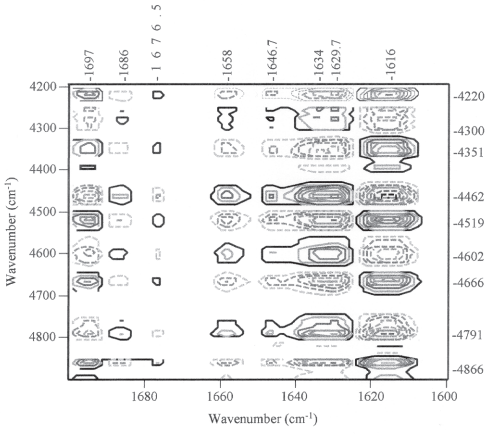


Figure 4. Synchronous 2-D mid-IR and NIR correlation contour map of heated (70, 80 and 90°C) WPI spectra in the 1700–1600 cm^{-1} (mid-IR) and 4900–4200 cm^{-1} (NIR) regions.

Table 2. Chemical assignment of near infrared wavelengths.

Wavelengths cm^{-1}	Chemical assignment
4234	N–H combination bands[6]
4255	CH stretch /CH deformation[15]
4280	C–H combination band[6]
4347	C–H combination band (β-lact)[15]
4368	C–H combination band[6,7]
4411	C–H combination band (β-lact)[15]
4460	Amino acids?[16]
4539	C–H combination band (β-lact)[15]
4608	N–H combination bands amideB/amide II[6]
4666	combination band amide I/amide III ?[16]

from the literature. This is because WPI is not a purified protein, but rather a blend of β-lactoglobulin, α-lactalbumin, BSA, fat, lactose and minerals.

According to these IR data, two-dimensional spectroscopy appears to be a valuable tool for studying the changes in the secondary structure of proteins under heating perturbation.

NIR information

In Figures 3 and 4 the most important feature relies on the fact that only three major NIR bands arose during denaturation while ten were clearly visible during aggregation.

Major bands arising in both synchronous correlation maps are related to those from the literature, as shown in Table 2, except for the peak at 4663 cm^{-1} which cannot be precisely assigned to any protein

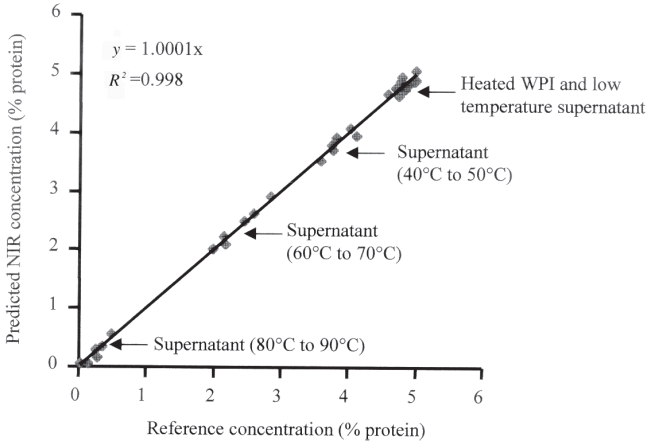


Figure 5. Predicted NIR v. Kjeldahl protein content in WPI solutions presenting different levels of denaturation and aggregation. PLS calibration performed with spectral regions identified in Figure 4.

structure (combination band amide I/amide III ?). The peak at 4791 has also been tentatively assigned to combination bands amide A/II and second overtone of OH bending of water.[4] As previously reported by some authors,[4,5] thermal unfolding processes can therefore be studied either by using the amide I band in mid-IR or the N–H or C–H combination bands in NIR.

Results (Figure 5) seem to indicate the real potential of near infrared as a valuable method for the determination of levels of denaturation and aggregation in whey proteins.

Conclusions

- Two-dimensional correlation spectroscopy provides a new insight the study of thermal denaturation and aggregation phenomenon of whey protein isolates.
- Two-dimensional spectroscopy led to the assignment of NIR regions highly correlated with well-characterised mid-IR denaturation and aggregation bands.
- Furthermore, the inclusion of these NIR wavelengths into a PLS calibration model allowed the successful quantification of protein in samples with different levels of denaturation and aggregation.
- NIR spectroscopy therefore appears to be a promising tool for monitoring and quantifying complex phenomenon such as denaturation and aggregation of proteins.

Acknowledgements

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