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RESEARCH ARTICLE

Multipoint near-infrared spectrometry for real-time monitoring of protein conformational stability in powdered infant formula

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Abstract

Powdered infant formula (PIF) can be the sole source of nutrition for babies and infants. Monitoring conformational changes in protein during manufacture of PIF is critical in order to maintain its nutritional value. This study presents the development of a *calibration* model for monitoring conformational changes in PIF protein by applying a novel multipoint near-infrared (NIR) spectrometry. NIR spectra were collected for PIF and PIF proteins, casein and whey protein isolate, before and after heat treatment. Results show that principal component analysis showed discrimination between native protein at room temperature and protein conformational changes caused at elevated temperature. *Partial least squares regression analysis* showed good *calibration* models with correlation coefficients ranging between 87% and 99% for the prediction of protein quality. This novel multipoint NIR spectrometry could serve as a simple in-line tool to rapidly monitor protein quality during processing stages, contributing to product nutritional value.

Keywords

ATR-FTIR, near-infrared spectrometry, *partial least squares regression*, powdered infant formula, principal component analysis

History

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Introduction

Good nutrition is essential for the growth and development that occurs during an infant's first year of life. Due to our changing pace of life, the rising incidences of working mothers, increasing disposable incomes and other drivers, increasing number of infants are weaned off breast milk to infant formula. Thus, a very high percentage, >70% of European and indeed global babies and infants consume powdered infant formula (PIF). PIF is a milk-like food which is intended to supplement or replace the milk of the baby's mother. As PIF may be the sole source of nutrition for babies and infants, its nutritional profile and safety are critical for the health and well-being of the babies and infants that depend on it for their survival and growth.

Since milk products are an excellent media for multiplication of potentially pathogenic bacteria, appropriate heat treatment is applied during the production of PIF to ensure microbiological safety, and thus making it safe for the consumption to infants (Agostoni et al., 2004; Raikos, 2010; Rudloff & Lönnerdal, 1992; Sarriá et al., 2001). Ultrahigh temperature treatment (UHT; >130 °C, 3–5 s) has been commonly adopted as a process for the production of ready to feed formula, particularly in Europe (Ferrer et al., 2002; Rudloff & Lönnerdal, 1992).

However, proteins are sensitive to temperature changes that occur during processing stages in the food and pharmaceutical industries. Consequently, depending on the extent of heat treatment applied during the manufacture of PIF, the proteins in PIF can undergo conformational changes which often lead to protein aggregation. Aggregates formed can be reversible or irreversible and can have reduced activity or are inactive and may be immunogenic compared to the native molecule (Izutsu et al., 2006; Pabari et al., 2011). This can significantly affect the nutritional quality and safety of PIF. Thus, maintaining the native conformation of protein during the manufacture of PIF is critical. General bioanalytical benchtop and off-line methods such as the Kjeldahl procedure for protein measurement are time and resource consuming and require the operator to have extensive technical training.

Near-infrared (NIR) spectrometry have been increasingly evaluated as an in-line process monitoring tool due to growing economic and legislative demands on product quality, particularly with the evolution of Process Analytical Technology (PAT) in industries. NIR spectrometry offers many beneficial features including speed, non-invasiveness and often requires little or no sample preparation. Several studies have reported that NIR can be applied to study freeze-dried induced changes and temperature induced transitions in the secondary structural conformation of proteins (Bai et al., 2005; Izutsu et al., 2006; Navea et al., 2003; Pieters et al., 2011; Wu et al., 2000). NIR spectroscopy devices traditionally used for compositional and process analysis are usually confined to single-point analysis, consequently spatial information is not captured and the measurement may not be representative of the entire batch. Given the critical nature homogeneity plays in PIF manufacture, this single location value may be misleading to represent the bulk sample. This has been considered as one of the main barriers for NIR spectroscopy becoming widely used in PAT (Kauppinen et al., 2013). For quality assurance, spatial information of chemical constituents is paramount.

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The purpose of this present study is to develop a calibration model to facilitate rapid monitoring of protein conformational changes caused by elevated temperatures, commonly encountered during the processing stages in industries, by using a novel multipoint NIR spectrometry.

Moreover, the development of new technologies such as MultiEye® with Pérot interferometer (VTT, Espoo, Finland), as used in this study, offers potential for low cost, hand held, portable systems for the direct and sensitive multi-component analysis (Alvarez-Jubete et al., 2013).

Materials and methods

Sample preparation

Powdered infant formula (SMA nutrition, Berkshire, UK) and PIF proteins [casein (Sigma–Aldrich, Dublin, Ireland) and whey protein isolate (WPI) (Davisco foods, Le Sueur, MN) were subjected to elevated temperatures commonly encountered during processing stages such as spray drying. Elevated temperatures examined were 80 °C (Memmert oven, Fisher Scientific, Dublin, Ireland) and 105 °C (Gallenkamp BS Oven 250 Size 2, AGB Scientific, Dublin, Ireland), for 10 min and 24 h.

MultiEye® NIR spectrometry

The multipoint NIR spectrometry assessed in this study is the MultiEye® (Innopharma labs, Dublin, Ireland), which utilizes a Fabry-Pérot interferometer (VTT, Espoo, Finland) (Figure 1). The Fabry-Pérot interferometer offer advantages of small dimensions, speed of wavelength tuning and high optical throughput, compared to tunable filters such as Acousto-Optic and Liquid Crystal Tunable Filters.

The MultiEye® has four-measurement channels with four reflectance probes attached, and a wavelength ranging from 1515 to 2200 nm. Each reflectance probe consists of two arms; one arm attached to the spectrometry and other arm to the illumination source. The tip of each probe consists of seven fibres; six bundled with a circular arrangement attached to the arm connected to the illumination source and the seventh fibre (central) attached to the arm connected to the detection source.

Measurements were performed with the four probes arranged in a square shape, set perpendicular with the samples and at 1 mm distance. Known amounts of sample 5–12 g were placed in a one-use aluminium dish and its surface was slightly flattened. Samples were scanned at the full wavelength range using a 5-nm scanning interval. For each sample, measurements were taken at five different regions of the sample surface, acquiring 20 measurements (four probes by five different regions). The five different regions measured by each probe were averaged. Dark and white references were recorded before sample measurements.

Attenuated total reflectance-FTIR spectrometry

Attenuated total reflectance (ATR)-FTIR spectra were recorded by a PerkinElmer Spectrum 400 spectrometry (Waltham, MA) equipped with a universal ATR sampling accessory (with diamond crystal) in the 650–4000 cm^{-1} range. Spectra were obtained at 25 °C with 16 scans at a spectral resolution of 1 cm^{-1}, using air as the reference spectrum.

Data analysis

Raw data were pre-processed by standard normal variate transformation and Savitzky–Golay smoothing using the *R* statistical computing package. Principal component analysis (PCA) was used to analyze variation in the spectra of samples before and after heat treatment. Spectral data from each of the four probes per sample were averaged for the PCA analysis. The information from data sets is described as principal components (PCs) (Grewal et al., 2014; Jiang et al., 2015). Partial least squares regression (PLSR) was used to generate calibration models to correlate NIR data with the intensity of the bands in amide I or amide A regions of the ATR-FTIR spectra. The intensity of ATR-FTIR bands in heat-treated samples were converted to percent protein denaturation by taking intensity of ATR-FTIR bands at room temperature (RT) as 0% denaturation. Leave-one-out cross-validation was used to assess the predictive ability of the calibration model. The model performance was also evaluated using the root mean-squared error of prediction (RMSEP) and the determination coefficient (R^2). The PCA and PLSR were performed using the *R* statistical computing package.

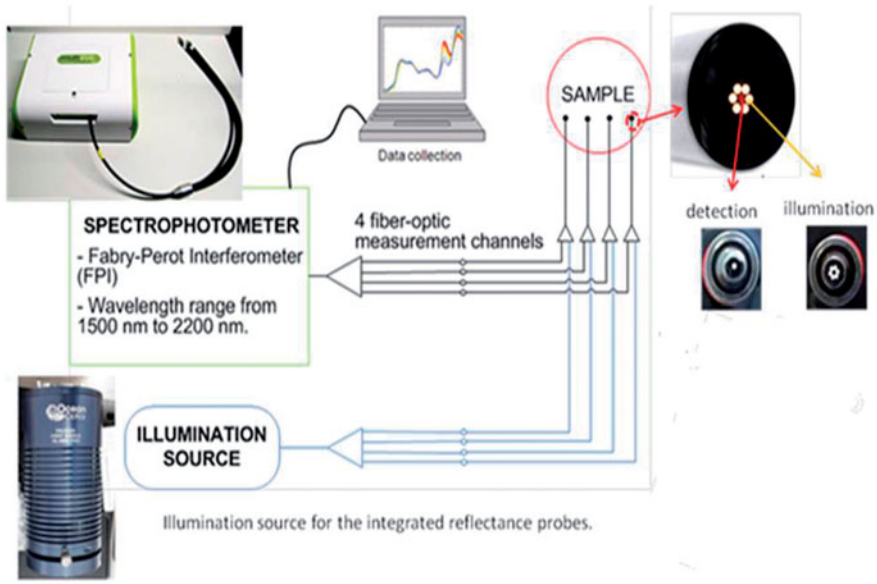

Figure 1. MultiEye® NIR spectrophotometer system showing spectrophotometer with four channels attached to four reflectance probe fibre. Each probe fibre attached to a detection and illumination source.

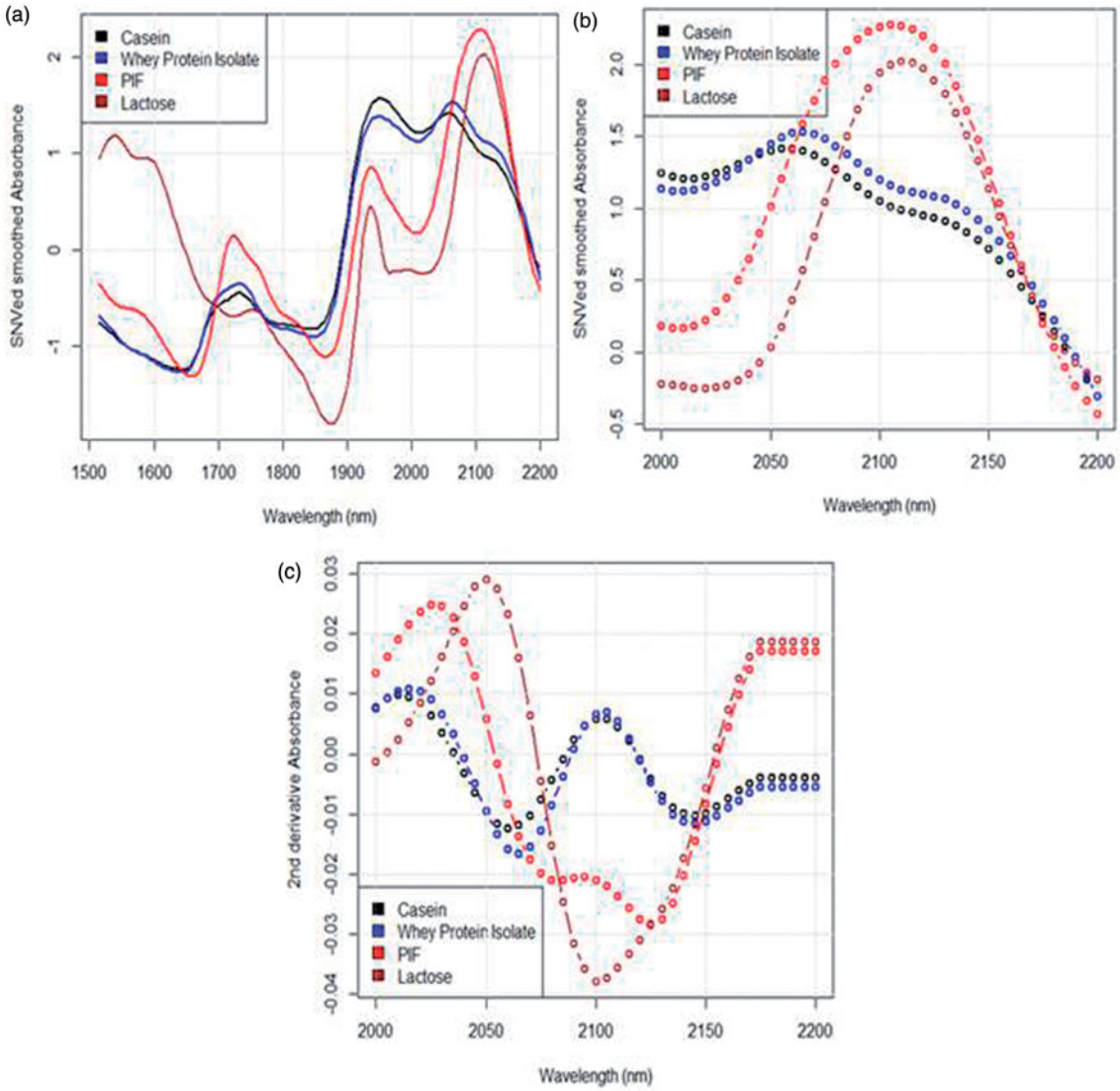

Figure 2. NIR spectra of PIF, PIF proteins, casein and WPI, and lactose (a) raw spectral data, (b) protein region between 2000 and 2200 nm and (c) second derivative spectra.

Results and discussion

Spectral investigation

MultiEye® NIR

Near-infrared spectra of PIF and PIF proteins, casein and WPI, in wavelength ranged between 1515 and 2200 nm is outlined in Figure 2(a). The spectra showed various absorption bands between 1900 and 2200 nm. The absorption band between 1900 and 2000 nm is associated with the water molecule (Corredor et al., 2011). While the absorption bands between 2000 and 2200 nm (Figure 2b) arise mainly from proteins and have been reported to be important for the study of the structure of proteins (Bai et al., 2005). PIF and PIF proteins (casein and WPI) showed a broad band in the region 2000–2200 nm. In order to achieve a better band resolution and to distinguish the band positions more clearly, the second derivative of each NIR spectrum was generated and is outlined in Figure 2(c).

Second derivative NIR spectrum of PIF showed a broad band at 2075 nm which was merged with another broad band at 2125 nm. These integrated broad bands could be related to absorption bands of proteins in PIF, also observed in the second derivative NIR spectra of casein and WPI (Figure 2c). Second derivative NIR spectra of casein and WPI showed absorption bands at ~2065 nm, which are related to amide A–amide II combination bands in the protein. A band at 2145 nm was also observed, related to amide B–amide II combination bands in the protein (Bai et al., 2005). In the second derivative NIR spectrum of PIF, broad bands for proteins were further integrated with the absorption band of lactose at 2100 nm (Gombás et al., 2003; Siesler et al., 2002).

Attenuated total reflectance-FTIR

ATR-FTIR spectrum of PIF showed a high-intensity absorption band for lactose in the region 800–1220 cm^{-1} (12 500–8196 nm) (Figure 3a) (Solís-Oba et al., 2011). However, unlike in NIR, this absorption band for lactose was beyond the amide I region, 1600–1700 cm^{-1} (6250–5882 nm) in the ATR-FTIR spectrum.

In the ATR-FTIR spectrum of PIF, the second derivative of amide I region showed bands at 1629, 1639, 1656 and 1693 cm^{-1} (6138, 6101, 6038 and 5906 nm), similar to that observed in casein and WPI, as expected (Figure 3b and c). ATR-FTIR spectra of casein and WPI were found to be similar. The second derivative of the amide I region, 1600–1700 cm^{-1} (6250–5882 nm), showed that casein and WPI are the most structured protein with multiple components corresponding to different secondary structures.

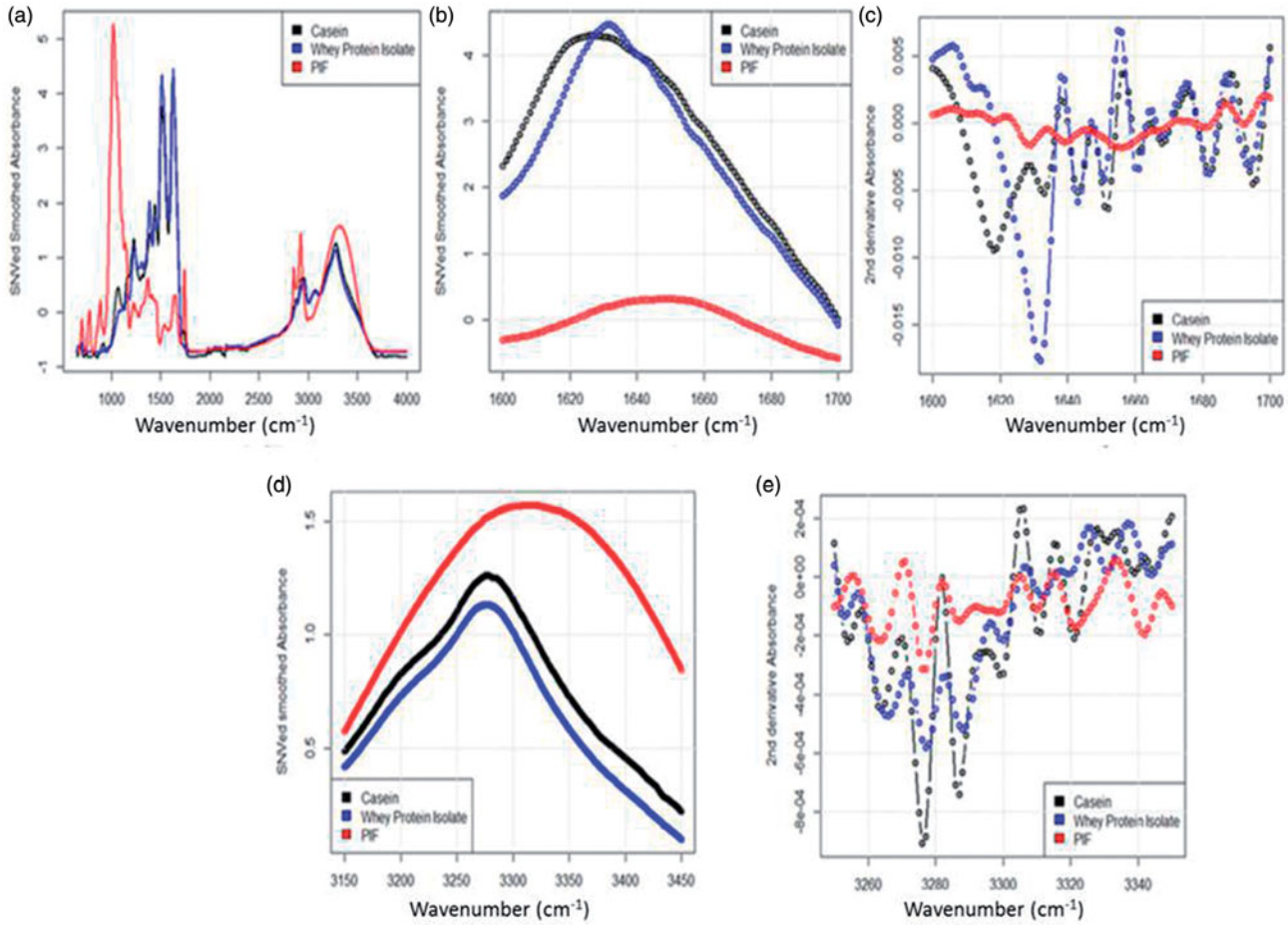

Figure 3. ATR-FTIR spectra of PIF and PIF proteins, casein and WPI: (a) raw spectral data, (b) amide I region, (c) second derivative spectra of amide I, (d) amide A region and (e) second derivative of amide A region.

For casein, the amide group of the peptide backbone consists of β-sheet at 1615, 1633 and 1643 cm^{-1} (6191, 6123 and 6086 nm) while the band at 1655 cm^{-1} (6042 nm) could be related to the α-helix or random coil structures (Paris et al., 2005; Wang et al., 2013). Similarly, in the ATR-FTIR spectrum of WPI, the bands at 1631, 1643 and 1694 cm^{-1} (6131, 6086 and 5903 nm) are due to β-sheet, while the band at 1650 cm^{-1} (6060 nm) is related to α-helical structures (Figure 3b and c) (Lee et al., 2007).

Likewise, the bands observed in the amide A and B regions of the ATR-FTIR spectrum of PIF were similar to that observed in the ATR-FTIR spectra of casein and WPI (Figure 3d and e) (Barth & Zscherp, 2002).

Investigation of temperature related changes in protein structure: PCA analysis

Figure 4 shows four spectra for each sample, obtained from each of the four probes arranged in a square shape. At RT, these four spectra were found to be similar, as expected.

PIF and PIF proteins, casein and WPI, were subjected to elevated temperatures commonly encountered during processing in the food and pharmaceutical industry; 80 °C and 105 °C for 10 min and 24 h. NIR spectra of these samples showed that absorption bands between 2000 and 2200 nm, related to proteins, shifted with an increase in temperature (80 °C and 105 °C) and heating time (10 min and 24 h).

In order to find associated spectral changes of protein due to a change in temperature, PCA was applied to NIR spectral intervals that contain information related to protein structure, i.e. 2000–2200 nm. Two principal components, PC1 and PC2 were found to be sufficient to describe 93% of variance in the conformational changes of protein in PIF and 97% of variance in the conformational changes of PIF proteins, casein and WPI, due to a temperature change. The distribution of the scores of the two principal components, PC1 and PC2, show that the NIR absorbance spectra were able to discriminate between the native protein conformation in samples at RT and conformational changes in samples subjected to elevated temperature (Figure 5a–c).

PIF and PIF proteins, casein and WPI, showed similar trends on the PCA plot. Samples at RT were separated away from samples treated at various temperatures, thus increase in temperature causes some degree of conformational changes in protein structure. Samples that are relatively close to each other are an indication of showing resemblance in protein conformation. Figure 5(a–c) shows that samples treated at 80 °C for 10 min and 24 h and at 105 °C for 10 min were close to each other, indicating similar changes in protein conformation. Sample treated at 105 °C for 24 h was farthest from these samples and the sample at RT, indicating a different degree of conformational change.

The corresponding loadings plot in Figure 5(d–f) show the maximum absorbance change around the absorbance of proteins, thus confirming that the variance in the data was related to the conformational changes in the protein when subjected to elevated temperatures.

Thus, it can be said that the NIR spectrometry examined in this study is able to detect conformational changes in protein that arises from a change in temperature.

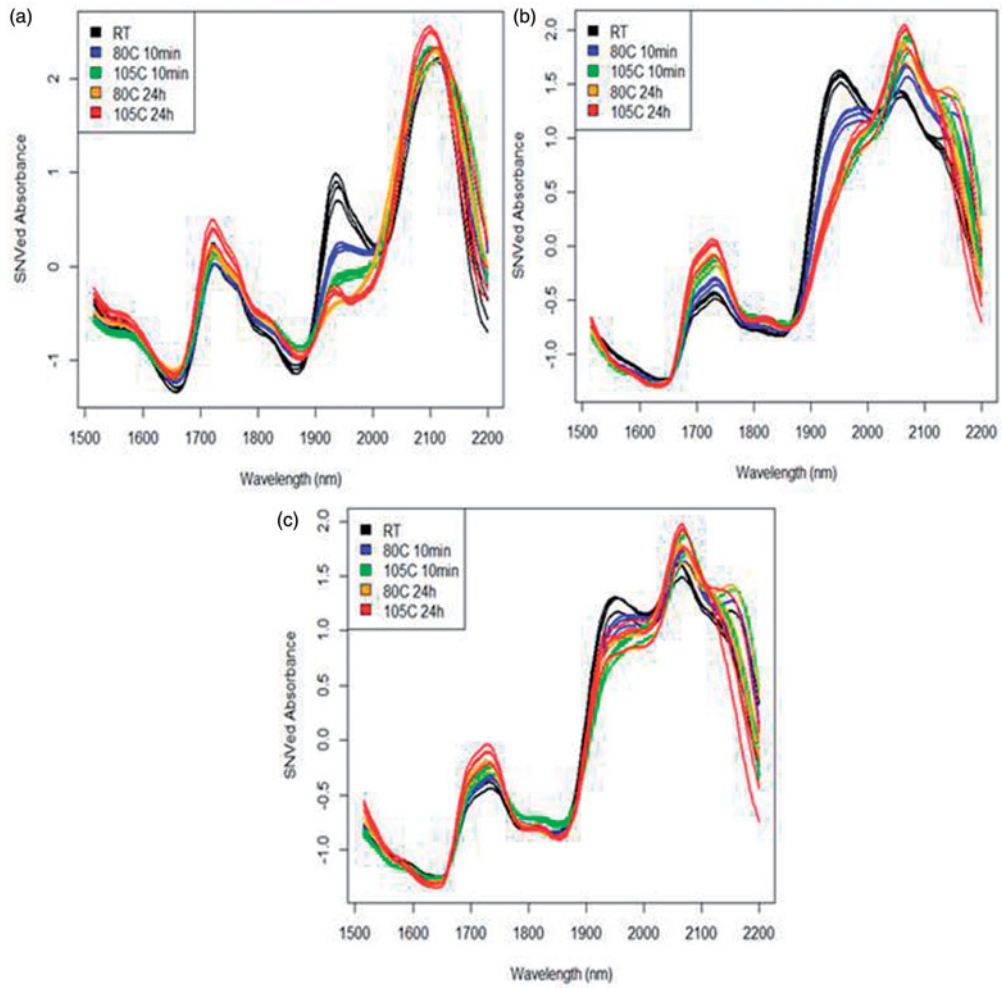

Figure 4. NIR spectra as a function of heating condition for (a) PIF and PIF proteins, (b) casein and (c) WPI.

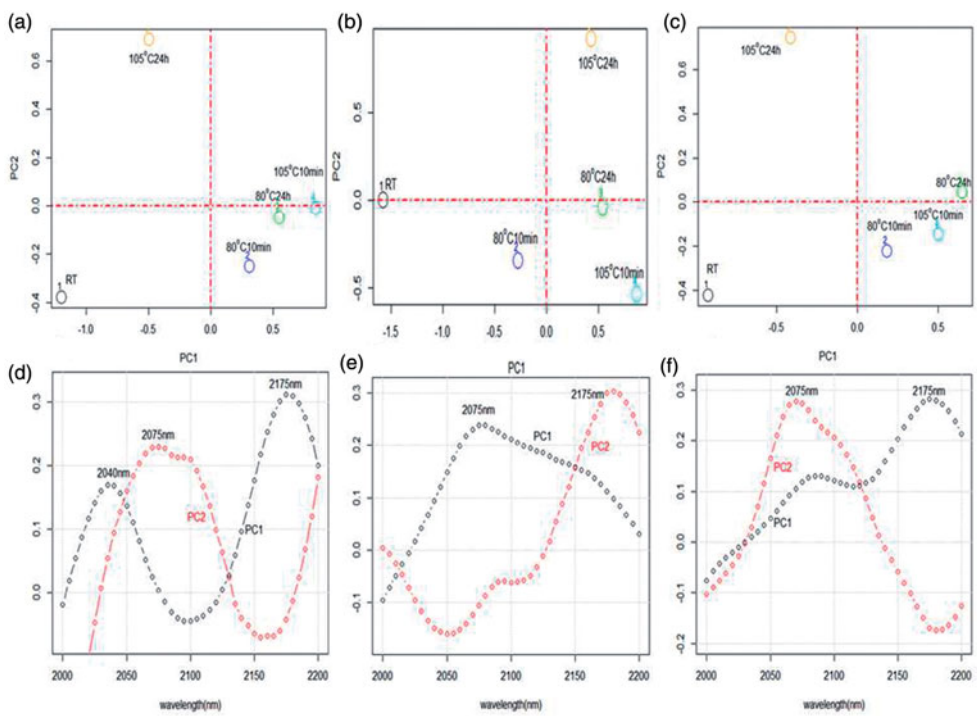

Figure 5. PCA results and loadings plot for (a and d) PIF and PIF proteins, (b and e) casein and (c and f) WPI.

Temperature related changes in protein structure: PLSR analysis

Partial least square regression was applied on the full NIR spectral data of PIF and PIF proteins, casein and WPI, before and after heat treatment, while using ATR-FTIR as a reference method, to build a model to predict the level of protein conformational changes. The PLSR models were evaluated using the cross-validated RMSEP and the determination coefficient (R^2), which was dependent on the number of components used for the calibration model. In order to avoid overfitting, a minimum number of components was chosen for the construction of the model.

Results generated by cross-validation with the leave-one-out option for PIF and PIF proteins, casein and WPI is outlined in Table 1 and Figure 6. In Figure 6, the points for each sample point correspond to four spectra analyzed by each of the four probes. For PIF and PIF proteins, casein and WPI, four, two and four components, respectively, were required in order to construct a calibration model with a high determination coefficient (R^2) of >87%, and low RMSEP of <2.7% (Table 1). Moreover, the models generated could explain >97% of variance observed in the protein conformation, resulting from a change in temperature (Table 1). The models outlined in Figure 6(a–c) show 0% protein denaturation at RT for PIF and PIF proteins (casein and WPI), while, an increase in temperature to 80 °C and 105 °C and incubation time of 10 min and 24 h resulted in conformational changes in protein. PIF showed higher percent of protein conformational changes at elevated temperatures which was <25% (Figure 6c). A corresponding colour change in PIF was also observed from cream colour at RT to dark brown, particularly, at 105 °C and incubation time of 24 h. This could be related to Maillard reaction occurring between carbonyl-groups of reducing carbohydrates, such as lactose or lipids with free amino-groups of proteins. This is reported to affect the milk protein quality and gives rise to compounds responsible for colour changes (Birlouez-Aragon et al., 2005; Ferrer et al., 2005). PIF proteins, casein and whey showed <6% of protein conformational changes at elevated temperature (Figure 6a and b).

The corresponding loadings plot in Figure 6(d–f) show the maximum absorbance change around the absorbance of proteins, thus confirming that the calibration model generated was related to the conformational changes in proteins, occurring due to a change in temperature.

Table 1. Results for the calibration model from PLS analysis.

Sample	Components	Determination coefficient (R^2)	RMSEP (%)	Percent variance explained (%)
PIF	4	0.8784	2.64	98.98
Casein	2	0.9905	0.21	97.77
WPI	4	0.8653	0.68	98.11

Conclusion

This study shows that a novel multipoint NIR coupled with PCA can discriminate between protein conformational changes in samples, before and after heat treatment. The PLSR calibration model shows good correlation of the NIR spectral data with certain bands of amide I or amide A of ATR-FTIR spectra for protein conformational changes. However, in the present study, PLSR analysis was carried out as a proof-of-concept. Hence the

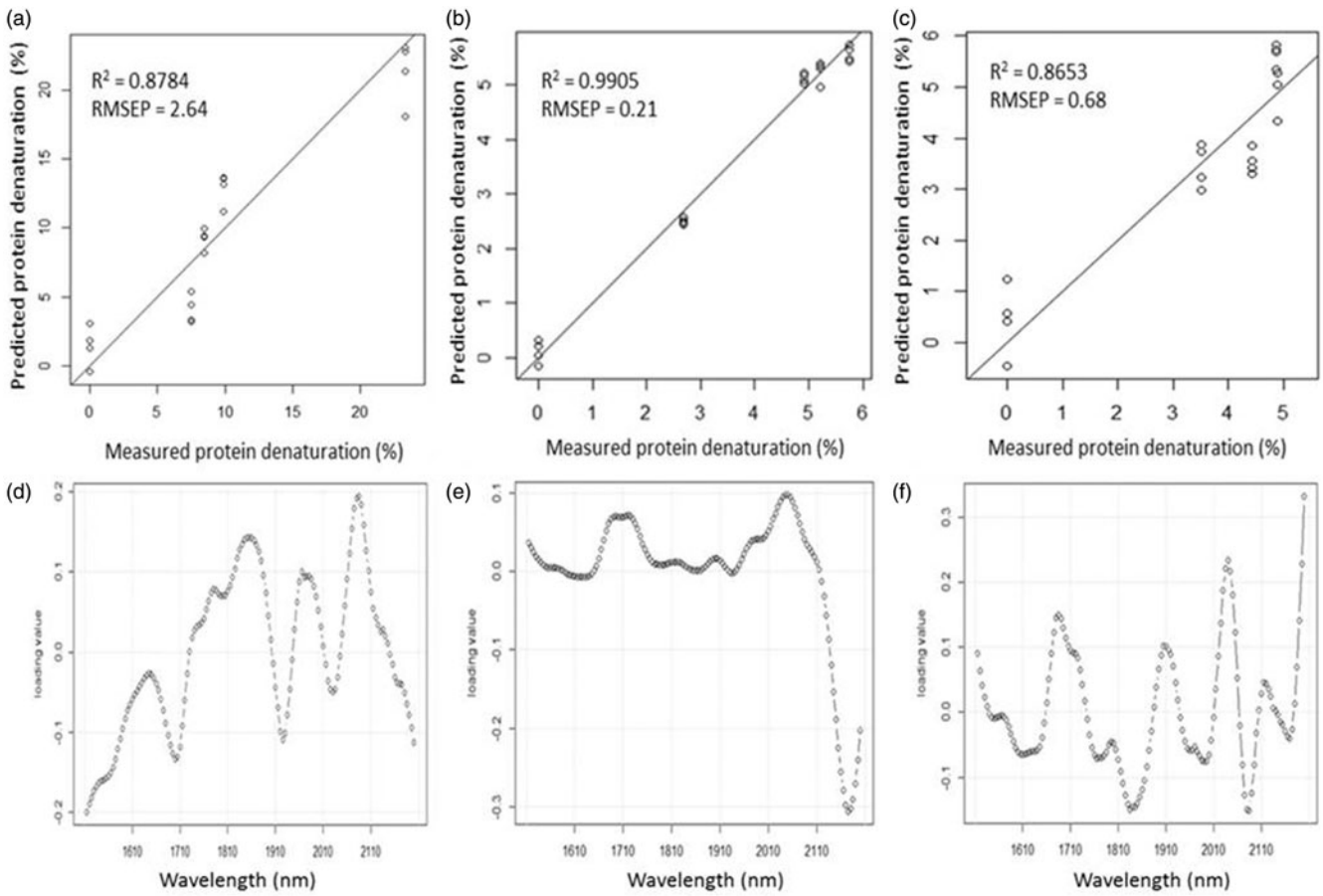

Figure 6. PLS model and loadings plot for (a and d) PIF and PIF proteins, (b and e) casein and (c and f) WPI.

feasibility of PLSR calibration model to determine protein conformational stability warrants further exploration, particularly with future studies involving more data points for PLSR analysis.

The approach could be possibly used effectively as an in-line monitoring tool to rapidly determine protein conformational stability in PIF in real-time from a number of locations. The multipoint probes can be placed at various points in the manufacturing process, thus allowing spatial analysis, and thereby enhancing process understanding and control during the in-line process monitoring. Moreover, the NIR spectrometry data can be analyzed by various chemometric methods and stored, thus allowing real-time measurements to verify product quality and release for subsequent processing without delay, in a manufacturing environment, hence contributing to cost effectiveness.

Declaration of interest

The authors have no conflicts of interest to declare. The research leading to these results has received funding from the European Community Seventh Framework Program (FP7/2207-2013) under grant agreement number 605134.

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