

Use of FTIR and mass spectrometry for characterization of glycated caseins

Christine M Oliver^{1*}, Ashwini Kher¹, Don McNaughton¹ and Mary Ann Augustin^{1,2,3}

¹ School of Chemistry, Monash University, Clayton, VIC 3800, Australia

² Food Science Australia, 671 Sneydes Road, Werribee, VIC 3030, Australia

³ CSIRO Food Futures Flagship

Received 26 October 2007; accepted for publication 14 August 2008; first published online 5 January 2009

This study investigates the potential of Fourier transform infrared spectroscopy (FTIR) to monitor glycation-induced changes in protein structure. Aqueous solutions of sodium caseinate and glucose (1:2 w/w, pH 6.7) were heated at 90 °C for 0, 10, 20, 40 and 60 min. Evidence for caseinate glycation was obtained by mass spectrometry techniques (electrospray (ESI) and matrix-assisted laser desorption ionisation (MALDI)). FTIR was able to discriminate between glycated and non-glycated sodium caseinate, when the data were analysed by multivariate statistical methods; principal component analysis (PCA) and soft independent modelling of class analogy (SIMCA). The techniques used were complementary and provided different levels of information about the glycated samples.

Keywords: Glycation, Maillard, casein, mass spectrometry, Fourier transform infrared spectroscopy.

Glycation involves the covalent attachment of reducing sugars to available $-NH_2$, essentially the lysine residues (although the $\alpha-NH_2$ and other amino acids can also take part) of a protein. This conjugation is the first step of a complex series of reactions, known collectively as the Maillard reaction (glycation) (Maillard, 1912). The reaction is accelerated by heat and can occur in both the solid state or in aqueous systems (Morgan et al. 1999). The conjugation of sugars to proteins results in changes to the functional properties of the protein (Oliver et al. 2006a). As the reaction occurs naturally, it is a promising approach to alter the functional properties of proteins for food purposes. Information on the extent of glycation and changes to protein structure are of interest as they may be related to the functional properties of glycated proteins.

Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) and electron spray ionization mass spectrometry (ESI-MS) have been successfully applied to analyse glycated proteins (Guy & Fenaille, 2006; Kislinger, 2004; Sanz et al. 2007). Use of other techniques such as electrophoresis (Steffan et al. 2006), nuclear magnetic resonance spectroscopy (Oliver et al.

2006b) and circular dichroism (Chevalier et al. 2002; Wooster & Augustin, 2007) have been used to examine structural and conformational changes to milk proteins on glycation. Fourier Transform Infra Red (FTIR), by virtue of its ability to provide information on the structure and environment of proteins, also has potential for investigating structural changes to proteins caused by glycation. The purpose of this work was to examine the extent of glycation in heated aqueous systems of sodium caseinate and glucose by mass spectrometry and to determine the usefulness of FTIR for probing changes to protein structure.

Materials and Methods

Preparation of Glycated Sodium Caseinate

Sodium caseinate (ALANATE 810 from New Zealand) and glucose (Merck, Germany) were used. Aqueous mixtures (20% total solids (TS), pH 6.7) of sodium caseinate (10% TS) and glucose (10% TS) were heated at 90 °C for 10, 20, 40 and 60 min. An unheated solution (0 min) was prepared as the control. Samples of protein and sugar alone were subjected to the same treatment. All samples were subsequently freeze-dried to obtain the products in a powder form. The powders were stored at $-18\text{ }^\circ\text{C}$ until analysis.

*For correspondence; e-mail: Christine1.Oliver@csiro.au or ollieincork@yahoo.co.nz

Fourier Transform Infra Red (FTIR) Spectroscopy and Data Analysis

Spectra were acquired using a Specac Ltd (Orphington, UK) Golden Gate Mk II attenuated total reflectance (ATR) accessory with Diamond Top-plate and ZnSe lenses coupled to a Bruker (Erlingen, Germany) Equinox 55. Twenty individual spectra of 64 scans were recorded for each sample at 4 cm^{-1} resolution. Second derivative spectra were calculated in Opus (Bruker) software using 13 point Savitsky-Golay smoothing. Principal Component Analysis (PCA) and Soft Independent Modelling of Class Analogy (SIMCA) was performed using the computer software program, The Unscrambler® V9.2 (Camo, Norway).

Mass Spectrometry

For MALDI-TOF-MS, the samples were desalted using C18 Zip tips (Millipore), according to the manufacturer's recommendations, and eluted with 50% acetonitrile containing 0.1% TFA. Analysis was performed on a Voyager-DE STR (Applied Biosystems, Framingham, MA) operating in linear mode. Sinapinic acid was used as matrix solution. Briefly, matrix was spotted on the sample plate and allowed to air-dry; sample in acetonitrile/water (1:1 v/v) containing 0.1% v/v formic acid was subsequently spotted onto the dried matrix and allowed to air-dry. Data from 500 laser shots (337 nm nitrogen laser) were collected, and the signal averaged and processed with the instrument manufacturer's data Explorer software. ESI-TOF-MS was performed on an Agilent LC/MSD TOF mass spectrometer (Agilent Technologies, Palo Alto, CA). Samples were injected orthogonally into the MS via auto sampler in 20% acetonitrile/0.1% formic acid at 0.25 ml/min. All data were acquired in positive ion mode and reference mass corrected via a dual-spray ESI source. Each data point on the total ion chromatograms (TIC) is an average of 10,000 transients, producing a scan every second. Spectra were created by averaging the scans across each peak. Scans were acquired over 100–2500 m/z.

Results

Fourier Transform Infra Red Spectroscopy

The FTIR spectra for sodium caseinate, glucose, unheated sodium caseinate/glucose mixture and a heated sodium caseinate/glucose mixture (glycated sample) are shown in Fig 1a–d, respectively. The sodium caseinate spectrum shows all the normal features of a protein spectrum with strong amide I and amide II features between $1700\text{--}1500\text{ cm}^{-1}$ and is identical to recent literature with the amide I of the powder form centred at 1660 cm^{-1} (Pereda et al. 2007). The glucose spectrum likewise shows the normal features of glucose as recently described (Heise, 2002). The spectra of the mixtures in Fig 1c & 1d appear at first glance to be simple linear combinations of the spectra

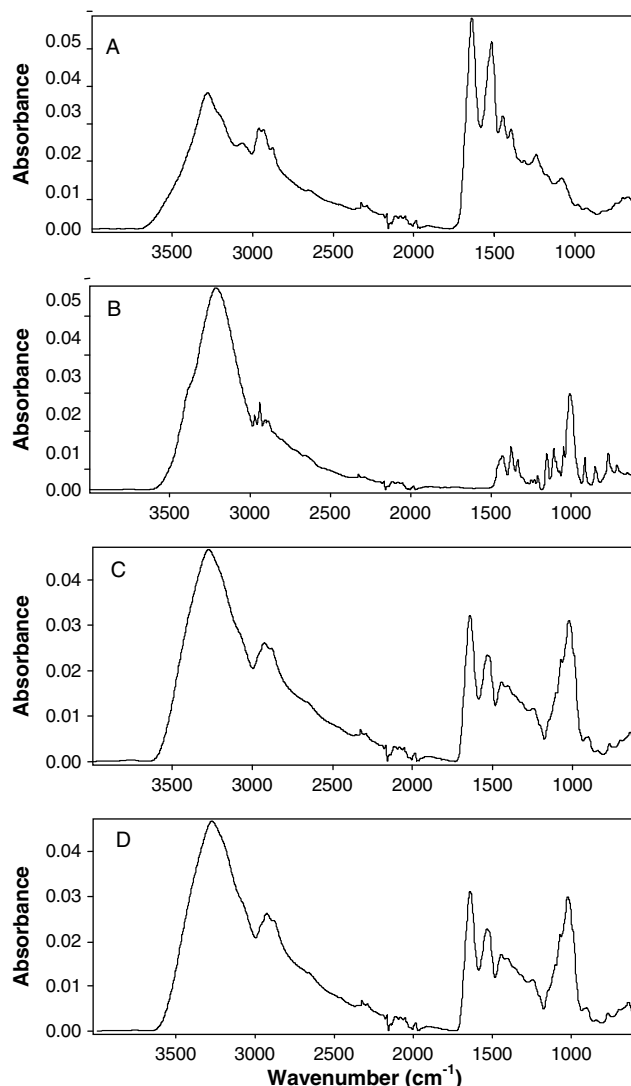


Fig. 1. Average vector-normalised baseline corrected ATR spectra of freeze-dried A, sodium caseinate; B, Glucose; C, Sodium caseinate/glucose, unheated (0 min); D, Sodium caseinate/glucose following heat treatment at $90\text{ }^{\circ}\text{C}$, 60 min. NB. The region $2300\text{--}1900\text{ cm}^{-1}$ shows features due to diamond absorbance.

of caseinate and glucose and the minute changes that occur from molecular interactions require careful analysis. The changes in the FTIR spectra of the mixtures subjected to heat treatment were examined initially using visual peak picking of spectral second derivatives of the averaged individual spectra. This derivative approach, which removes baseline effects and highlights small changes in band shape and slope, proved that the changes to spectral bands were too small to be easily discerned and that analysis required a multivariate statistical approach to extract the spectral changes. PCA of the entire data set of 120 spectra was performed on the unheated and glycated samples (heated $90\text{ }^{\circ}\text{C}$ for 10, 20, 40 and 60 min) and a protein

Table 1. Class differences between unheated mixture and glycosylated sodium caseinate powders determined by SIMCA/PCA of FTIR data (1800–1400 cm^{-1}). Twenty independent spectra per sample were collected for each model

Heat treated samples compared with unheated blend	Model distance from unheated mixture model
Unheated blend	1.0
90 °C for 10 min	10.46
90 °C for 20 min	14.21
90 °C for 40 min	23.64
90 °C for 60 min	44.23
90 °C for 60 min (protein alone)	13.17

heated sample using the spectral region where the major amide and carbohydrate bands appear (600–1800 cm^{-1}). PCA essentially breaks the dataset down into orthogonal components that describe the variance in the dataset and allow one to examine only the spectral differences (Esbensen, 2001). The PCA scores plots showed that the spectra from the different samples clustered individually and the loadings plots showed that the largest differences between glycosylated and non-glycosylated samples were due to changes in the amide I and II region, hence subsequent PCA analysis used only the region 1400–1800 cm^{-1} . Following PCA, a supervised pattern recognition technique SIMCA, (Esbensen, 2001) was used to classify the 6 sample types (one untreated mixture, 4 mixtures treated for different times and a protein only sample heated for 60 mins) into class models. Cooman's plots, which show the orthogonal distances from all objects to the class models were examined and it was apparent that the models for the unheated and heated for 10, 20, 40 min, whilst forming defined groups (clusters) in the Cooman's plots did overlap each other, whilst the sample heated for 60 min was clearly separated. This is most likely due to the state of glycosylation varying through the course of the heat treatment. Further, class model distances were determined in order to measure the degree of separation between the classes into which the samples fell. The model distance is found by fitting objects from different classes to their own model and then all other models in turn and the distance measures calculated from the pooled residual standard differences. The distance of a model to itself is 1 whilst a large inter-model distance indicates a clear separation between models with a model distance of >3 an indicator of significant difference (Esbensen, 2001). Table 1 summarises the results of this analysis where the model distance (distance between the model centres) of each class from the unheated sample mixture class is listed. It is apparent that the model classes, based on bands due to protein, are distinctly separated from the untreated blend. The model for the protein only sample heated for 60 min is also significantly different from the 40 & 60 min heated

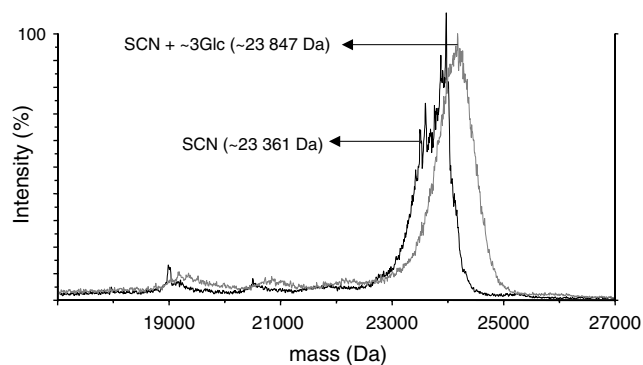


Fig. 2. MALDI-TOF-MS spectra (17000–27000 m/z) of non-glycosylated (0 min) and glycosylated sodium caseinate (60 min). (SCN=sodium caseinate).

protein-glucose samples showing that the observed differences are due to both glycosylation and heating.

Mass Spectrometry

Further analysis of the samples was performed by MS. The MALDI-TOF-MS of sodium caseinate alone, both unheated (0 min) and heated (60 min), were superimposable (results not shown). When heated in the presence of glucose, however, glycosylation occurred, as evident by a marked increase in the average molecular mass of sodium caseinate, which intensified with heating time. After 60 min, an increase in the average molecular mass of sodium caseinate from $\sim 23\,361$ to $\sim 23\,847$ Da was observed, corresponding to an average degree of glycosylation of 3 glucose moieties ($162 \times 3 = 486$ Da) (Fig. 2). The increase in average molecular weight of the protein was due, at least in part to the attachment of glucose residues on low molecular weight peptides present in the sodium caseinate sample (results not shown). ESI-TOF-MS, which allows a more accurate determination of the protein molecular weight, showed a heterogeneous mixture of various glycoform species was produced. Such heterogeneity increased with heating time. After 60 min glycoforms were observed having 0, 1, 2, 3 and a maximum of 4 glucose moieties attached (Fig. 3).

Discussion

This work shows the ability of PCA and SIMCA to classify sodium caseinate samples based on time of heat treatment in the presence of reducing sugar i.e. extent of glycosylation, using FTIR. In particular, the samples could be discriminated based on spectral differences in their amide I and II regions. It is interesting to note that the model distances increased with heating time, indicating that the more extensive the heat treatment (i.e. extent of glycosylation) the greater the dissimilarity in the amide I and II structural regions of the samples. In particular, the class distances for

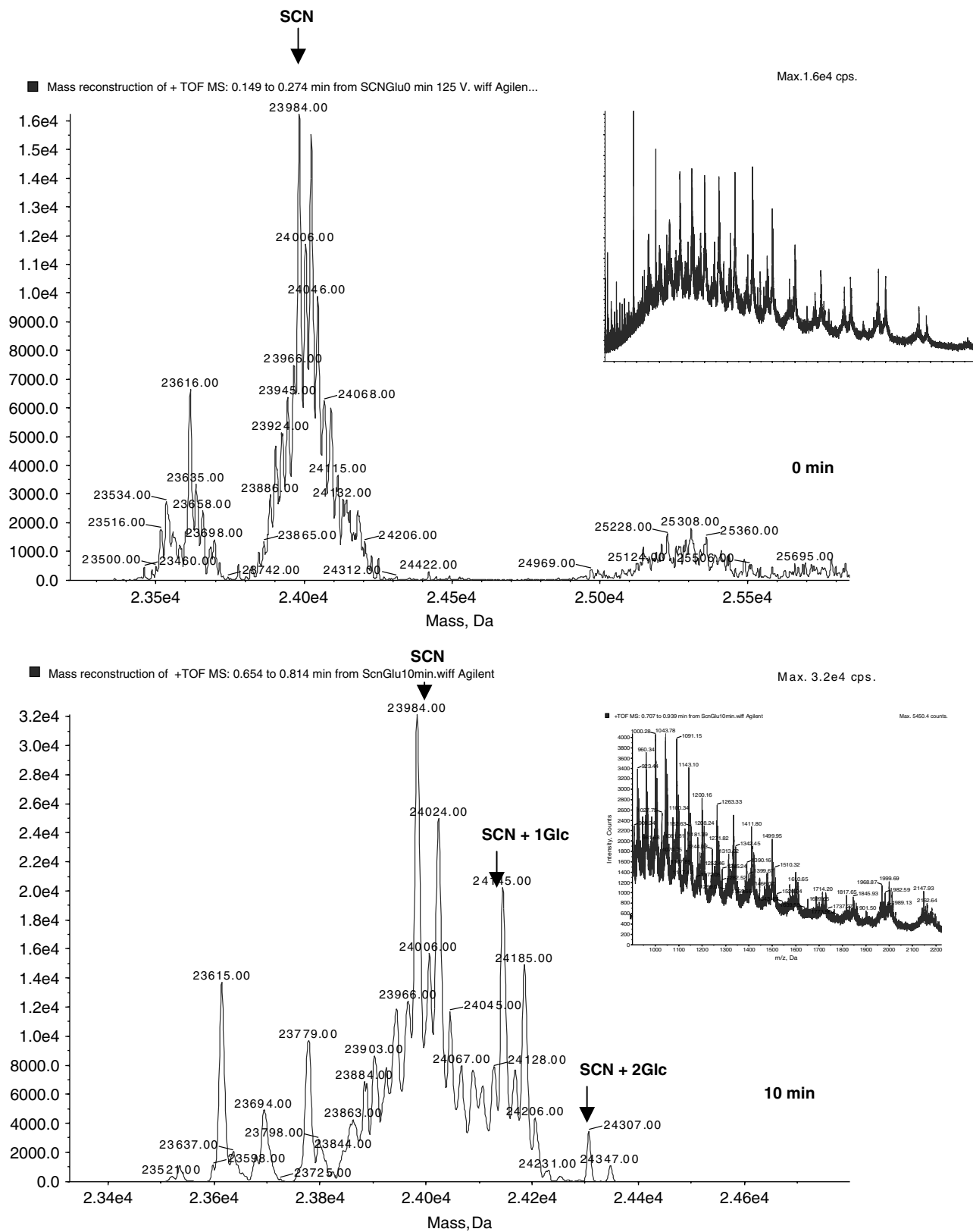


Fig. 3. Continued.

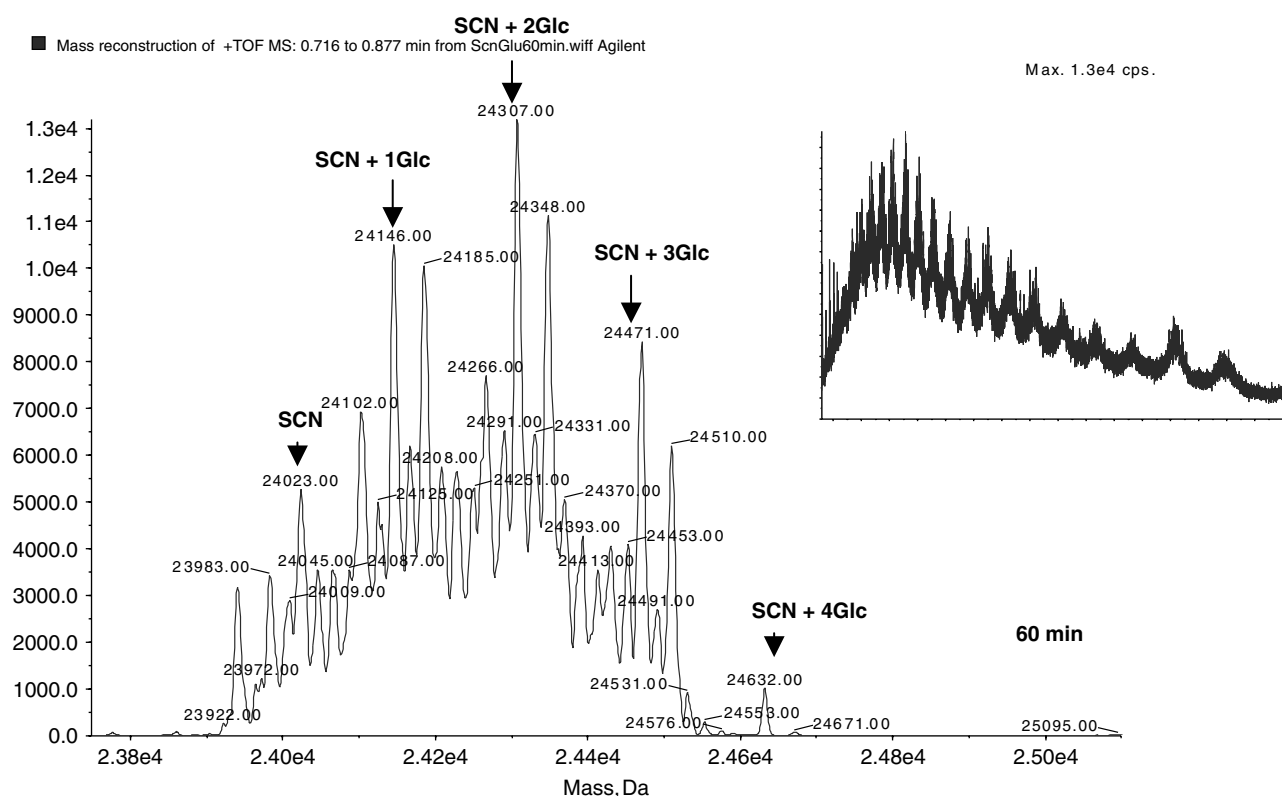


Fig. 3. Deconvoluted ESI-TOF-MS spectra (17000–27000 Da) of non-glycated and glycosylated sodium caseinate (10 & 60 min). Insets are total ion chromatograms (SCN=sodium caseinate).

samples heated at 60 min were considerably greater than that of the physical blend or for those samples exposed to shorter heating times. According to ESI-MS the longer heating time corresponded to a glycation of up to 4 glucose moieties attached. Based on the relative intensities of the different glycoforms in the ESI spectrum a 1:2:2:2:6:1:6:0:2 ratio of adducts having 0, 1, 2, 3 and 4 glucose moieties attached, respectively, was present.

Overall, the results reported herein show that FTIR can be used to discriminate between glycosylated and non-glycosylated sodium caseinate, when the data are analysed by multivariate statistical methods such as PCA and SIMCA. This is in contrast to others who found that simple FTIR spectral comparison could not distinguish between glycosylated and non-glycosylated ovalbumin (Sun et al. 2004). The differences in protein and conditions for glycation used may also however, have contributed to the lack of discrimination by FTIR for ovalbumin samples. For example, ovalbumin is a globular protein which is relatively heat-labile, and hence it may be more difficult to differentiate structural changes due to glycation versus those due to heat-treatment alone. The use of PCA and SIMCA in our work allows improved discrimination between spectra, at least for the non-globular protein sodium caseinate. Although ESI-TOF and MALDI-TOF mass spectrometry are a more accurate and sensitive means of screening complex protein

mixtures for quantification of degree of glycation, qualitative differences in protein structure due to glycation can be readily revealed by FTIR when used in conjunction with PCA and SIMCA. Hence FTIR may be an appropriate alternative to MS, depending on the detail of information required.

In conclusion, this preliminary investigation confirmed the usefulness of FTIR with PCA/SIMCA analysis and MS techniques for characterisation of whole glycosylated proteins. The techniques used were complementary and provided different levels of information about the glycosylated samples: FTIR providing a means of discriminating samples based on differences in protein structure as a result of glycation whereas mass spectrometry enabling the exact degree of glycation to be established based on protein MW differences. While MS techniques are now used for routine analysis of glycosylated proteins, the potential of FTIR needs to be further explored for it to be usefully exploited for quality control or as a means of probing changes to protein structure, and the extent to which it can be used to reflect changes in protein functionality. Taken together, the results confirm that an increase in the degree of glycation leads to an increase in structural change to the protein.

This research has been supported by DIAL (Dairy Innovation Australia Ltd), Food Science Australia and the CSIRO Food

Futures Flagship. Thanks are due to P. Udabage and A. Puvanenthiran, Food Science Australia for providing samples and useful discussions. MALDI-TOF-MS was carried out by Simon Harris, Monash University. We would also like to thank Bio21 Australia Ltd for access and use of their ESI-TOF-MS.

References

- Chevalier F, Chobert J-M, Dalgalarondo M, Choiset Y & Haertlé T** 2002 Maillard glycation of beta-lactoglobulin induces conformation changes. *Nahrung* **46** 58–63
- Esbensen KH** 2001 Multivariate Data Analysis in Practice, 5th edition, Oslo, Norway: CAMO Process AS
- Guy PA & Fenaile F** 2006 Contribution of mass spectrometry to assess quality of milk-based products. *Mass Spectrometry Reviews* **25** 290–326
- Heise HM** 2002 Glucose Measurements by Vibrational Spectroscopy. In *Handbook of Vibrational Spectroscopy (Applications of Vibrational Spectroscopy in Life, Pharmaceutical and Natural Sciences)* Vol 5, pp. 3280–3294 (Eds JM Chalmers & PR Griffiths). Chichester, UK: John Wiley & Sons Ltd
- Kislinger T** 2004 Analysis of protein glycation products by matrix-assisted laser desorption ionization time-of-flight mass spectrometry. *Current Medicinal Chemistry* **11** 2185–2193
- Maillard L-C** 1912 Action des acides amines sur les sucres. Formation des melanoidins par voie methodique. *Comptes rendus hebdomadaires des séances de l'Académie des Sciences* **154** 66–68
- Morgan F, Léonil J, Mollé D & Bouhallab S** 1999 Modification of bovine β -lactoglobulin by glycation in a powdered state or in an aqueous solution: effect on association behavior and protein conformation. *Journal of Agricultural and Food Chemistry* **47** 83–91
- Oliver CM, Melton LD & Stanley RA** 2006a Creating proteins with novel functionality via the Maillard reaction: a review. *Critical Reviews in Food Science and Nutrition* **46** 337–350
- Oliver CM, Melton LD & Stanley RA** 2006b Glycation of caseinate by fructose and fructo-oligosaccharides by controlled heat treatment in the 'dry' state. *Journal of the Science of Food and Agriculture* **86** 722–731
- Pereda M, Aranguren MI & Marcovich NE** 2007 Characterization of chitosan/caseinate films. *Journal of Applied Polymer Science* **107** 1080–1090
- Sanz ML, Corzo-Martinez M, Rastall RA, Olano A & Moreno FJ** 2007 Characterization and in vitro digestibility of bovine β -lactoglobulin glycated with galactooligosaccharides. *Journal of the Science of Food and Agriculture* **55** 7916–7925
- Steffan W, Balzer HH, Lippert F, Sambor BC, Bradbury AGW & Henle T** 2006 Characterization of casein lactosylation by capillary electrophoresis and mass spectrometry. *European Food Research and Technology* **222** 467–471
- Sun Y, Hayakawa S & Izumori K** 2004 Modification of ovalbumin with a rare ketohexose through the Maillard reaction: effect on protein structure and gel properties. *Journal of Agricultural and Food Chemistry* **52** 1293–1299
- Wooster TJ & Augustin MA** 2007 Rheology of whey protein-dextran conjugate films at the air/water interface. *Food Hydrocolloids* **21** 1072–1080