



Analytical Methods

A proteomic approach to detect lactosylation and other chemical changes in stored milk protein concentrate

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ABSTRACT

Milk proteins undergo chemical changes such as lactosylation, deamidation and protein cross-linking during processing and storage of milk products. A proteomic technique combining two-dimensional gel electrophoresis and mass spectrometry was used to investigate chemical modifications to proteins, in milk protein concentrate (MPC80), during storage. Lactosylation, deamidation and protein cross-linking were observed on 2-DE gels. They were storage temperature-, humidity- and time-dependent. Lactosylated whey proteins were well separated on 2-DE in vertical stacks of spots. The masses of the spots varied by multiples of 324, indicating the attachment of lactose to lysine residues in the proteins. The trypsin-digested spots of α -lactalbumin were analysed by MALDI-TOF mass spectrometry, which indicated multiple lactosylation sites. The lactose adducts on gels were quantified by image analysis, allowing development of adducts over time to be monitored. The results show that proteomics can be used for the detection and quantification of chemical modifications to proteins in stored MPC80.

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1. Introduction

Milk protein concentrate (MPC) powder is used as an ingredient in dairy products, such as yoghurt and cheese. Although milk powders have greater stability than liquid milks, they can undergo chemical changes during storage under certain conditions, particularly high temperatures and humidities, which can cause deterioration of their functional properties and nutritional quality. The chemical reactions which can occur include Maillard reaction, lipid oxidation, lipolysis, proteolysis and protein cross-linking. Importantly, the Maillard reaction occurs in milk powder under relatively mild storage conditions (Thomas, Scher, Desobry-Banon, & Desobry, 2004).

In the Maillard reaction, lactose binds to protein at lysine residues, resulting in the formation of the first-stage Maillard product, lactulosyllysine. Consequently, the lactose molecules block the ϵ -amino groups of lysine residues, decreasing the digestibility of protein and the bioavailability of the essential amino acid, lysine (Erbersdobler & Somoza, 2007). Moreover, advanced Maillard products can lead to browning and the formation of high-molecular weight protein complexes. These cause discolouration and may be responsible for deterioration in functional properties (e.g., solubility, foaming and emulsifying) of the milk powder. The high-molecular

weight protein complexes could also be formed via dehydroalanine, a β -elimination product of the phosphoserine present in the casein fraction of milk. Dehydroalanine can react with lysine and other amino acids to produce protein cross-linking via, for example, lysinoalanine and histidinoalanine (Singh, 1991).

Therefore, analysis and quantification of these chemical reaction products in milk powder are important for assessing the effects of processing, such as heat treatment and prolonged storage. Furosine is often measured as an indicator of the first stage Maillard reaction product, lactulosyllysine (Bosch, Alegria, Farré, & Clemente, 2008; Resmini, Pellegrino, & Battelli, 1990), and shows a significant increase in milk protein concentrate, containing 80% protein (MPC80), during storage at relative humidities of 44–84% and temperatures of 25–40 °C (Le, Bhandari, & Deeth, 2011). Other methods, such as immunochemical techniques (Matsuda, Kato, Watanabe, & Nakamura, 1985) and capillary electrophoresis (Otte, Larsen, & Bouhallab, 1998), have also been used to study the Maillard reaction. Lactosylation can also be directly determined by mass spectrometry (MS) or MS in combination with liquid chromatography (Czerwenka, Maier, Pittner, & Lindner, 2006; Losito, Carbonara, Monaci, & Palmisano, 2007; Monaci & Van Hengel, 2007). Holland, Gupta, Deeth, and Alewood (2011) recently confirmed the presence of lactose adducts in α -lactalbumin (α -LA) and β -lactoglobulin (β -LG) 2-DE gel spots of UHT milk, stored at 28 °C for 2 months, by using MALDI-TOF-MS. They also observed deamidation in α _{S1}-casein (α _{S1}-CN) and β -LG in the stored UHT milk (Holland et al., 2011). By using proteomic techniques, individual milk proteins, their post-translational modifications, genetic variants

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(O'Donnell, Holland, Deeth, & Alewood, 2004), native (Holland, Deeth, & Alewood, 2008) and non-native disulphide bonds (Chevalier, Hirtz, Sommerer, & Kelly, 2009; Chevalier & Kelly, 2010) and also non-disulphide cross-links have been identified (Holland et al., 2011). In addition, isodi-peptides such as lysinoalanine and histidinoalanine obtained from acid hydrolysis of proteins, cross-linked via dehydroalanine, have been quantified by gas (Montilla, Gómez-Ruiz, Olano, & del Castillo, 2007) and liquid chromatography (D'Agostina, Boschini, Rinaldi & Arnoldi, 2003; Faist, Drusch, Kiesner, Elmadfa, & Erbersdobler, 2000). One-dimensional gel electrophoresis has also been used to identify protein cross-linking in UHT milk during storage at mild temperatures (Al-Saadi & Deeth, 2008).

The aim of this study was to use proteomic techniques to identify chemical changes, particularly lactosylation, in the proteins of MPC80 during storage at various temperatures and humidities.

2. Materials and methods

2.1. Materials

Milk protein concentrate (MPC80) was obtained from Murray Goulburn Co-op Ltd. (Melbourne, Victoria, Australia). Portions of the powder were then stored at a range of temperatures, 25–40 °C, and relative humidities, 44–84%, for up to 12 weeks, as previously described by Le et al. (2011). The control samples were freshly prepared MPC80 stored at –20 °C. Immobiline™ Dry Strips™ (pH 4–7, 24 cm) and IPG buffer (pH 4–7) were obtained from GE Healthcare (Sydney, Australia). Acrylamide/N,N'-methylene diacrylamide Solution 29:1, was obtained from Bio-Rad laboratories. Modified porcine trypsin (proteomics grade), apomyoglobin, sinapinic acid and 2,5-dihydroxybenzoic acid (DHB) were obtained from Sigma (Sydney, Australia). Other chemicals were analytical grade.

2.2. Methods

2.2.1. 2-DE

Milk powder samples were analysed by two-dimensional gel electrophoresis (2-DE) under reducing conditions. This method

separates milk proteins by their isoelectric point and molecular weight. MPC80 (10 mg) containing approximately 8 mg of protein was dissolved in 1 ml of solubilisation solution (8 M urea, 4% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate, 100 mM dithiothreitol). A 150 µl aliquot of the solution was diluted with solubilisation solution to a final volume of 450 µl. The solubilised samples were focused and electrophoresed by the method of Holland et al. (2011). Gels were stained with colloidal Coomassie Blue G-250. Gel images were obtained on an ImageScanner III (GE Healthcare) in transmission mode at a resolution of 300 dpi.

2.2.2. Extraction of intact proteins

The extraction of the intact proteins from gel pieces was by the method of Galvani, Bordini, Piubelli, and Hamdan (2000). Briefly, spots of interest were excised from gels, crushed and treated with 10 µl of a solution containing 50% formic acid, 25% acetonitrile (ACN), 15% isopropanol and 10% water in an ultrasonic bath for 5 min. The samples were centrifuged at 9300g for 2 min and the supernatant was collected for MS analysis (see below).

2.2.3. In-gel tryptic digestion

The method for in-gel tryptic digestion was described previously (Holland et al., 2004). The spots of interest were cut from the gels as pieces of about 1 mm². The gel pieces were washed with Milli-Q water and destained twice with 100 µl of 40 mM triethylammonium bicarbonate (TEAB) in 50% ACN, for 30 min each time. The gel pieces were dehydrated with 200 µl of 100% ACN for 10 min and then rehydrated with 20 µl of trypsin solution (10 µg/ml in 40 mM TEAB, pH 8) and incubated overnight at 37 °C. The digested samples were extracted twice with 20 µl of 5% formic acid in 50% ACN, followed by speed-vacuum drying.

2.2.4. Acid precipitation

MPC80 (50 mg) was dissolved in 1 ml of Milli-Q water and mixed thoroughly with a Vortex mixer. For the precipitation of caseins, 42 µl of 1 N HCl was added into 1 ml of the reconstituted powder to achieve a final pH of 4.6. After 30 min at room temperature, samples were centrifuged at 16,100g at 25 °C for 5 min. The supernatant (acid whey) was analysed by MS.

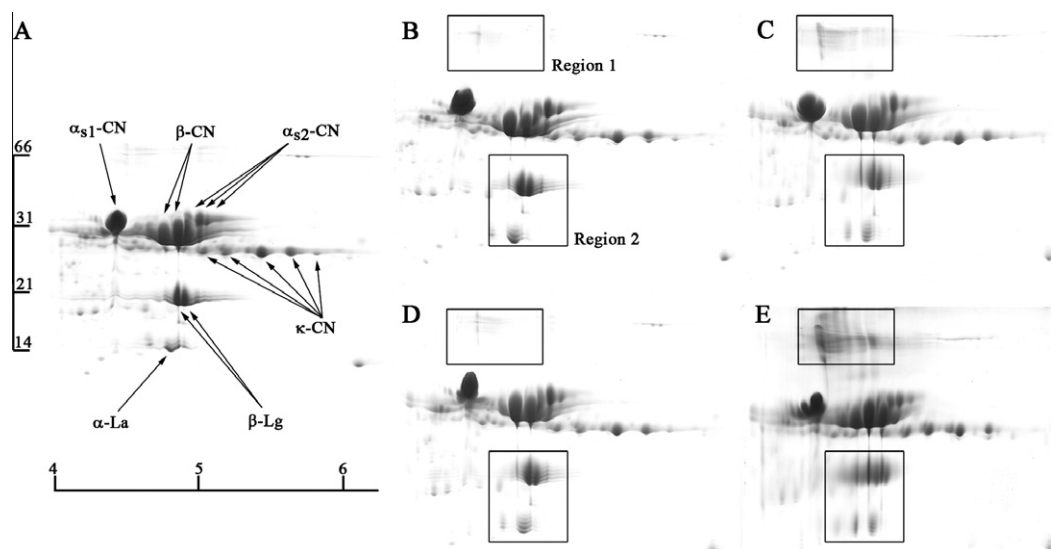


Fig. 1. Reducing 2-DE of MPC80 stored at different temperatures and relative humidities for 4 weeks. The samples were separated by using IPG strips (pH 4–7) in the first dimension and 25 × 20 cm 14% polyacrylamide gels in the second dimension. (A) Control; (B) 25 °C/44% RH; (C) 40 °C/44% RH; (D) 25 °C/84% RH; (E) 40 °C/84% RH. The major milk proteins are labelled in panel A. The vertical scale shows apparent molecular mass in kDa, and the horizontal scale shows pI. Regions showing major changes are designated by the boxes in panels B–E.

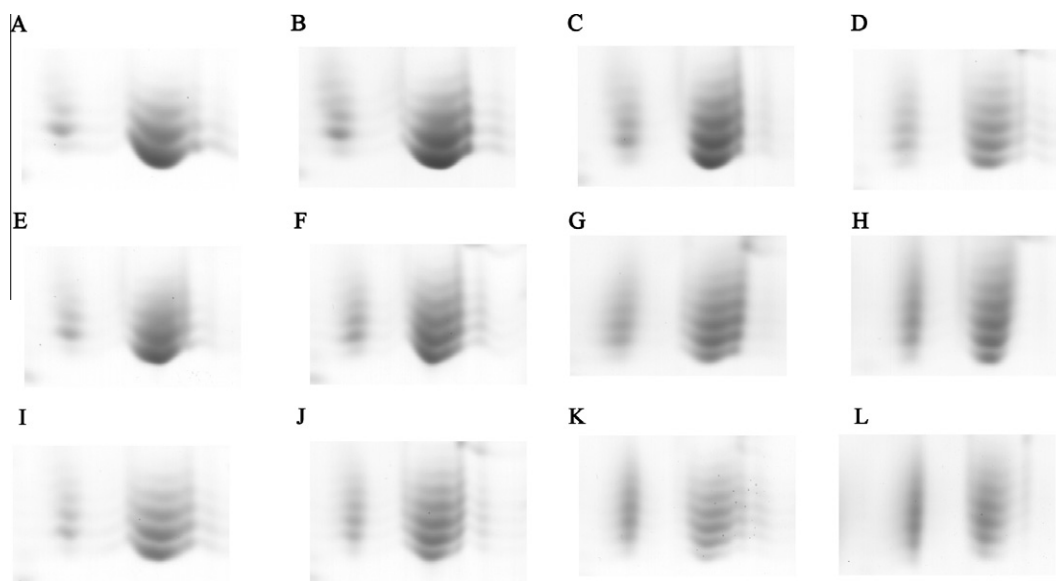


Fig. 2. Storage temperature and humidity effects on lactosylation of α -LA. α -LA spots were enlarged from 2-DE gels of MPC80 stored for 4 week at: (A–D) 25, 30, 35 and 40 °C, 44% RH; (E–H) 25, 30, 35 and 40 °C, 66% RH; (I–L) 25, 30, 35 and 40 °C, 84% RH.

2.2.5. Mass spectrometry

2.2.5.1. Protein analysis. After casein was precipitated from reconstituted MPC80, 1 μ l of the supernatant (acid whey) was mixed with 1 μ l of a matrix consisting of 10 mg of sinapinic acid in 1 ml of 1% formic acid/50% ACN. An aliquot of 2 μ l was spotted onto a stainless steel MALDI target. For intact α -LA spots, the extract was mixed with the matrix in the ratio 1:1 and 2 μ l was loaded onto the MALDI target. The acid whey, or intact α -LA, was analysed in positive linear mode, using a Voyager-DE STR MALDI-TOF mass spectrometer (ABSciex) with an accelerating voltage of 25 kV, grid voltage at 93% and a delay time of 975 ns. Apomyoglobin was used as a calibration standard.

2.2.5.2. Peptide analysis. Generally, peptides from tryptic digests were redissolved with 1% formic acid in 50% ACN, mixed with an equal volume of matrix solution (20 mg of DHB in 1 ml of 1% formic/50% ACN) and 1 μ l was spotted onto the MALDI target. Tryptic digests were analysed in positive ion reflector mode of a 4700 MALDI-TOF/TOF mass spectrometer (ABSciex), with a source voltage of 20 kV, grid voltage of 12 kV, mass range of 800–5000 Da and a focus mass of 1300 Da. A minimum of 500 laser shots was collected for each spectrum. Instrument calibration was performed using the mass standard kit for the 4700 Proteomics Analyser (ABSciex). Spectra were internally calibrated, if required, with trypsin autolysis peaks or known peaks from the proteins of interest.

2.2.6. Quantification by image analysis

Spots of α -LA in the vertical stacks were manually outlined and quantified using ImageMaster 2D Platinum (Version 5, Amersham Biosciences), which incorporates the Melanie software (Appel et al., 1997). The software calculates a spot volume essentially by summing the intensities of all pixels within the spot boundary following background subtraction. For relative quantification, the volume of an individual spot was expressed as a percentage of the volume of all spots within the stack. To calculate lactosylation level the spot volume was multiplied by the number of lactose adducts for that spot. 2-DE gels for the indicated samples were run 3 times to account for the effects of gel to gel variation. Values are expressed as mean of the three runs.

3. Results and discussion

3.1. 2-DE analysis of MPC80 during storage under different conditions

2-DE was used to investigate the effects of storage temperature, humidity and time on the proteins in MPC80. Samples were analysed by 2-DE, under reducing conditions, in order to observe non-disulphide cross-links. Fig. 1 shows 2-DE gels of the control sample of MPC80 and samples stored at 25 or 40 °C and 44 or 84% RH for 4 weeks. The major proteins α_{S1} -CN, α_{S2} -casein (α_{S2} -CN), β -casein (β -CN), κ -casein (κ -CN), β -LG A and B variants and α -LA in MPC80 powder were clearly observed (Fig. 1A). The three distinct spots of α_{S2} -CN represent different levels of phosphorylation. Each phosphate group attached to amino acids of protein shifts the position of the protein closer to the acidic side of the gel. More than 10 different κ -CN spots are present showing a complex of glycosylated, phosphorylated genetic variants of κ -CN (Holland et al., 2004).

There was little change between control MPC80 (Fig. 1A) and samples stored at 25 °C and 44% RH for 4 weeks (Fig. 1B). However, an increase in temperature induced changes in the protein profile, particularly in regions 1 and 2 where the high-molecular weight protein complexes, and α -LA and β -LG spot stacks, respectively, appeared (Fig. 1C). Similarly, higher humidity enhanced the extent of chemical modifications of the milk proteins (Fig. 1D and E).

The effects of temperature and time of storage on milk proteins in UHT milk were investigated by Holland et al. (2011) using 2-DE. The study showed that major changes occur, particularly in whey proteins and α_{S1} -CN. They include deamidation, lactosylation and protein cross-linking. Similar changes were observed in the current study of MPC80 during storage. The number of lactose adducts increased with increasing storage temperature, humidity and time (discussed below). Moreover, the formation of high-molecular weight protein complexes appeared to be temperature- and humidity-dependent. Fig. 1 shows more high-molecular weight protein complexes occurring in MPC80 samples stored at 40 °C compared to those at 25 °C, in the same humidity and time conditions (44% RH and 4 weeks). Similarly, high humidity accelerated the formation of non-disulphide cross-linked proteins in MPC80 samples. The effect of humidity was more obvious in samples

stored at high temperature (40 °C); the maximum effect was observed at 84% RH (Fig. 1E).

Two mechanisms are possible for the protein cross-linking: (I) via Maillard reaction products and/or (II) via dehydroalanine. Advanced Maillard reaction products, such as glyoxal and methylglyoxal, may react with lysine or other amino acids with nucleophilic groups (e.g., arginine) to form cross-linked proteins such as glyoxal lysine dimer (GOLD) and methylglyoxal lysine arginine dimer (MOLD) (Singh, 1991). Moreover, high-heat treatment, alkali treatment and/or storage of milk under severe conditions can lead to the β -elimination of phosphate groups from phosphoserine (Van Boekel, 1999). An active compound, dehydroalanine, is formed which reacts with lysine via its double bond producing lysinoalanine. Lysinoalanine has been detected by liquid and gas chromatography in dairy products; for example, cheese and UHT milk before and after storage (Al-Saadi & Deeth, 2008; Montilla et al., 2007). In addition, the high-molecular weight protein complexes in this study were mainly located above α_{S1} -CN in the 2-DE gel, i.e. they had similar pIs to α_{S1} -CN. The presence of a mixture of α_{S1} -CN, α_{S2} -CN and β -CN in the cross-linked spots was confirmed in stored UHT milk (Holland et al., 2011). However, the existence of cross-linked peptides could not be identified. We were not able to detect cross-linked peptides in MPC80. Further investigation will be required on model systems to gain a thorough understanding of the cross-linking mechanism.

3.2. Lactosylation of α -LA observed by 2-DE and MALDI-TOF-MS

The rate of lactosylation, the first stage of the Maillard reaction, of MPC80 depends on storage temperature, humidity and time. This has been shown by measurement of furosine, a product of hydrolysis of lactulosyllysine (Le et al., 2011). 2-DE was used to examine lactosylation in more detail. Fig. 2 shows the pattern of α -LA spots observed following storage of MPC80 at temperatures of 25, 30, 35 or 40 °C and relative humidity of 44%, 66% or 84%, for 4 weeks. There was an increase in the number of spots in the vertical stacks of α -LA with increasing storage temperature and increasing humidity. For example, in Fig. 2A, there are 3 distinct elongated spots following storage at the lowest temperature and

relative humidity (25 °C and 44% RH). Increasing the storage temperature to 40 °C resulted in 5 spots (Fig. 2D), while increasing the relative humidity to 84% also produced 5 spots (Fig. 2I). Based on previous results (Holland et al., 2011) each additional spot in the stack was assumed to be the result of an additional lactose adduct on the protein. Up to 6 spots (i.e. 5 lactose adducts) were observed at the higher temperatures and humidities.

MALDI-TOF-MS has been used successfully for detection of lactosylation of whey proteins in milk models (Carulli, Calvano, Palmisano, & Pischetsrieder, 2011; Meltretter, Seiber, Humeny, Becker, & Pischetsrieder, 2007), heat-treated milk (Fenaille, Parisod, Visani, Populaire, Tabet & Guy, 2006), stored UHT milk (Holland et al., 2011), milk powder and infant formula (Galvani, Hamdan, & Righetti, 2000; Meltretter, Birlouez Aragon, Becker, & Pischetsrieder, 2009; Scaloni, Perillo, Franco, Fedele, Froio, Ferrara, et al., 2002). This technique is faster and easier than LC-ESI-MS/MS as milk samples can be directly loaded onto MALDI targets (Meltretter et al., 2009). We used MALDI-TOF-MS to confirm the lactosylation pattern observed using 2-DE. The pattern of α -LA spots on 2-DE gels following storage of MPC80 at 25 °C, 84% RH for 0, 4, 8 and 12 weeks, was compared with the MALDI-TOF spectra obtained from acid whey of the same samples (Fig. 3). As expected, the number of spots in the α -LA stacks increased with increasing storage time (Fig. 3A–D). The number of spots in vertical stacks on 2-DE was also matched by the number of α -LA peaks in the MALDI-TOF-MS spectra (Fig. 3E–H). The separation of the lactosylated α -LA peaks was clearly seen by the mass increment of 324 Da, compared with that of native α -LA. In addition, a decrease in relative intensity of the native α -LA and increases in di-, tri- and tetra-lactosylated α -LA, with increasing time of storage, can be seen in Fig. 3. In fact, the signal intensity of the native α -LA peak decreased from approximately 95% to 55%, with increasing storage time from 0 to 12 weeks (Fig. 3E and H). Moreover, the difference in relative intensity of the native α -LA from MALDI-TOF-MS was also apparent in 2-DE. For example, the size of lowest α -LA spot, which corresponds to native α -LA with no attached lactose, was noticeably smaller for MPC80 stored at 40 °C and 84% RH compared to that of control sample and samples stored at milder conditions (Figs. 2 and 3).

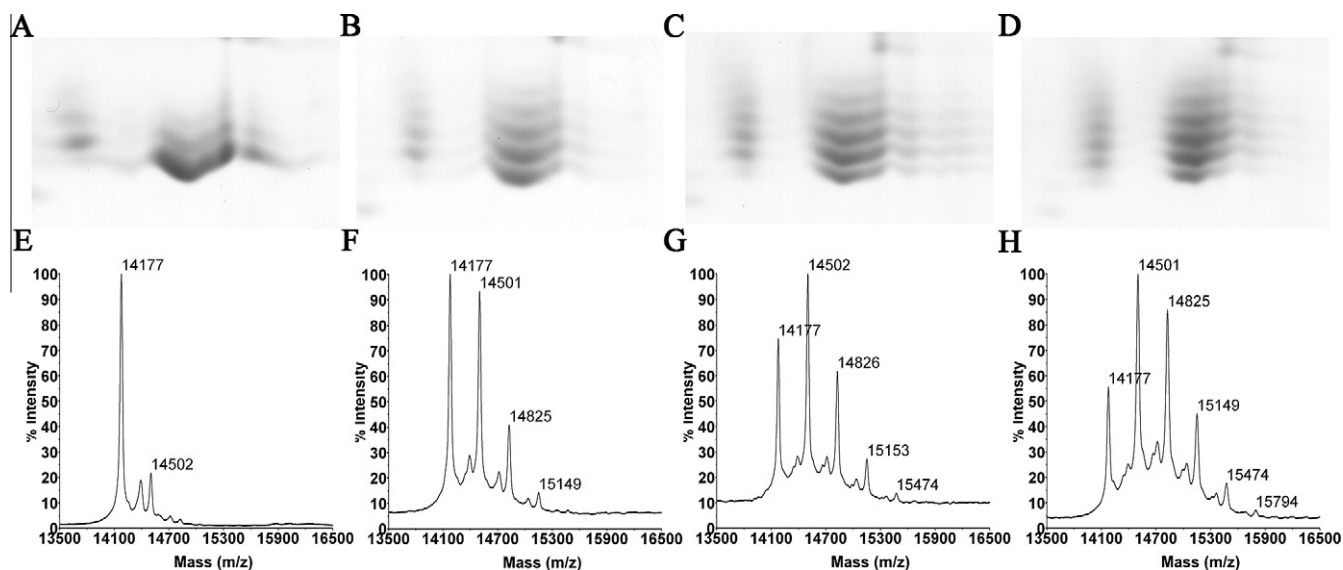


Fig. 3. Comparison of 2-DE and MALDI-TOF MS showing the effect of storage time on lactosylation of α -LA. α -LA spots were enlarged from 2-DE gels (A–D) and the MALDI-TOF-MS spectra were obtained from acid whey prepared from the same MPC80 samples (E–H) before (A and E) and after storage at 25 °C and 84% RH for: (B and F) 4 weeks; (C and G) 8 weeks; and (D and H) 12 weeks. Lactose adducts were observed by the mass increment of 324 Da from the MS spectra, assigned as 14,502 Da (1 lactose); 14,825 Da (2 lactoses); 15,149 Da (3 lactoses); 15,474 Da (4 lactoses); and 15,797 Da (5 lactoses).

3.3. MALDI-TOF-MS analysis of 2-DE gel spots of α -LA

Galvani et al. (2000) used MALDI-TOF-MS of intact proteins to demonstrate the presence of lactose adducts in 2-DE gel spots of casein and whey protein, by an increment in the mass of 324 Da. However, the different lactosylated forms of the proteins were not resolved on the 2-D gels. Holland et al. (2011), using IPG strips, with narrower pH ranges and larger format gels, were able to resolve multiple α -LA and β -LG spots from stored UHT milk samples. Moreover, MALDI-TOF-MS was used to confirm the presence of lactose adducts in tryptic digests of the stacked spots. We examined the intact protein extracted from each spot (Fig. 4A–D) and

in-gel tryptic digests of each spot (Fig. 4E–H) by MALDI-TOF-MS to confirm the lactosylation status.

For the intact protein, the molecular mass of the lowest spot (Spot 1) was observed at 14,887.8 Da (Fig. 4A). This value is higher than the theoretical molecular mass of native α -LA (14,177.34 Da). After alkylation of the thiol groups of cysteine with iodoacetamide, 8 cysteine molecules in α -LA were irreversibly transformed to the stable S-carboxyamidomethylcysteine (CAM; adduct: $-\text{CH}_2-\text{CO}-\text{NH}_2$), which leads to an M_w increase of 456.4 (8×57.05). Thus, the expected mass of modified α -LA increased to 14,633.7. The additional mass of 254 Da probably arises from the effect of concentrated formic acid (50%) during protein extraction from the

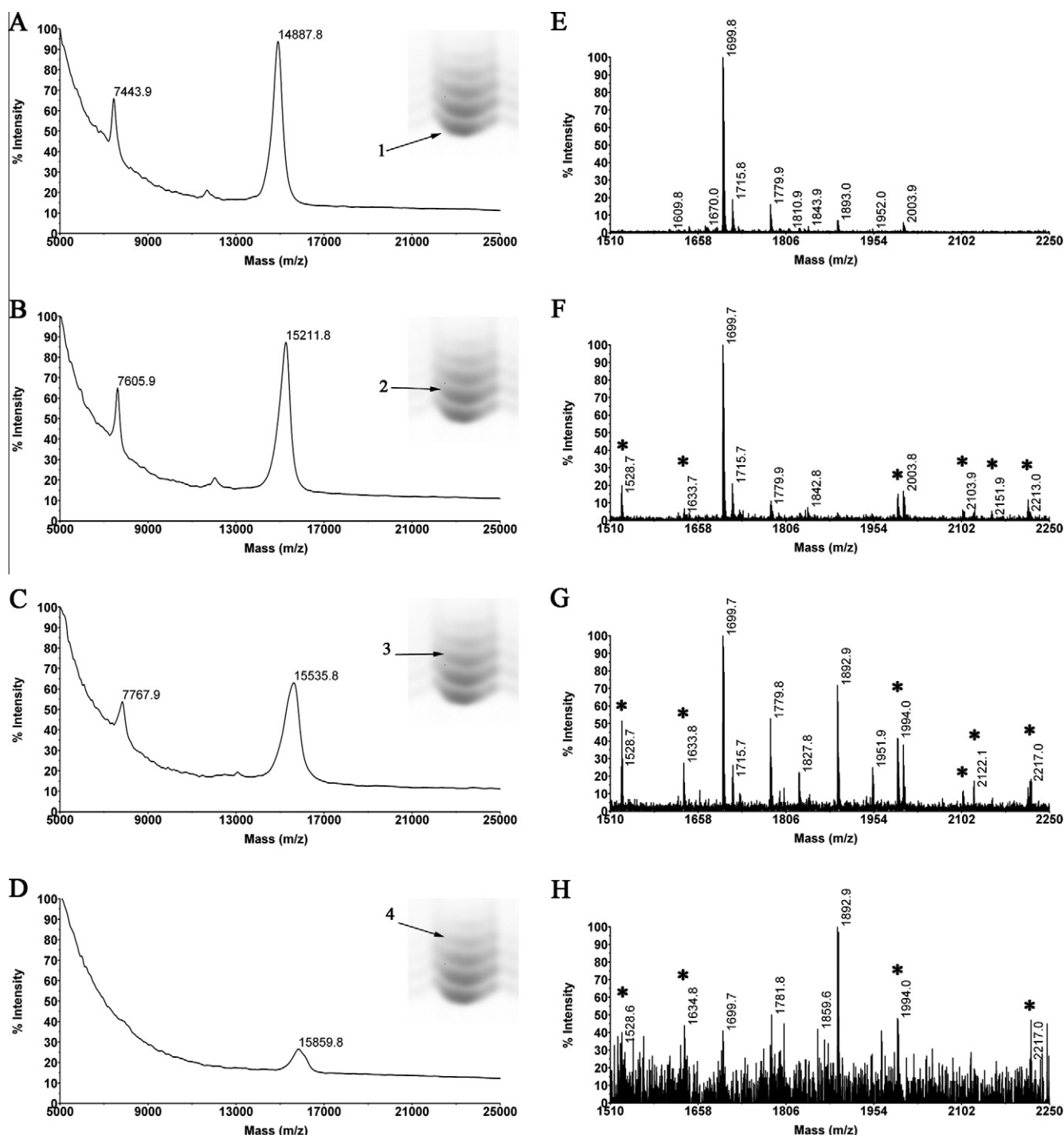


Fig. 4. Lactosylation of α -LA. MALDI-TOF spectra of intact α -LA extracted from the 2-DE gel spots (A–D) and in-gel tryptic digests of the same spots excised from gel (E–H). Insets show the relevant spots on a gel of MPC80 stored at 25 °C, 84% RH for 4 weeks. Peaks corresponding to lactose adducts on peptides are marked with an asterisk *.

Table 1
Lactosylated peptides identified in α -LA from stored MPC80.

Mass	Residues	Sequence	Lactosylation site
1528.71	134–142	LDQWLCEKL	K141
1633.76	20–29	EQLTKCEVFR	K24
1994.05	114–127	ILDKVGINYWLAHK	K117
2103.95	128–141	ALCSEKLDQWLCEK	K133
2122.14	113–127	KILDKVGINYWLAHK	K117
2151.96	99–113	FLDDDLTDDIMCVKK	K112
2213.08	118–133	VGINYWLAHKALCSEK	K127
2217.03	128–142	ALCSEKLDQWLCEKL	K133 or K141

Table 2
Percentage volume of stacked α -LA spots on 2-DE gels of MPC80 quantified by image analysis. Samples were stored at 44% RH for 4 weeks at -20 (control), 25, 30, 35, and 40 °C. Value are means $\pm$ SD ($n = 3$).

Temperature (°C)	Spot 1 (%)	Spot 2 (%)	Spot 3 (%)	Spot 4 (%)	Spot 5 (%)
Control	81.1 $\pm$ 0.9	18.9 $\pm$ 0.9	0	0	0
25	51.8 $\pm$ 1.8	33.4 $\pm$ 1.8	14.8 $\pm$ 0.8	0	0
30	46.3 $\pm$ 2.6	32.2 $\pm$ 1.8	21.5 $\pm$ 0.9	0	0
35	30.3 $\pm$ 3.4	28.6 $\pm$ 2.2	23.6 $\pm$ 1.2	17.4 $\pm$ 1.1	0
40	27.5 $\pm$ 1.9	27.5 $\pm$ 3.4	20.9 $\pm$ 1.7	15.3 $\pm$ 0.9	8.8 $\pm$ 1.0

gel. Ultrasonification enhances the chance of formic acid reacting with serine or threonine residues (seven of each in α -LA) that could explain a mass increase of 28 Da (Galvani et al., 2000). Multiple formylation could explain the mass increase of α -LA. Moreover, iodoacetamide could react with free amines during alkylation of the cysteines if pH is too high or alkylation time is too long (e.g., 20 min). Regardless of the source of the additional mass, the increment in mass of 324 of the second bottom spot (Spot 2), over the native α -LA spot (Spot 1) indicated one lactose molecule attached

to the protein (Fig. 4A and B). Similarly, the separation of the multiple lactosylated adducts on 2D gels was based on differences of 324 Da in mass between adjacent spots (Fig. 4).

In the tryptic digests, the peptides modified by lactose were identified by mass increases of 324 Da and were observed in all spots except the lowest one, corresponding to native α -LA (Fig. 4E–H). The lactosylated peptides identified in spots 2, 3 and 4 are listed in Table 1. The lactosylation sites identified were K²⁴, K¹¹², K¹¹⁷, K¹²⁷, K¹³³ and K¹⁴¹ (based on UniProt database entry P00711), similar to those observed in stored UHT milk (Holland et al., 2011). Marvin, Parisod, Fay, and Guy (2002) found 5 lactosylation sites of α -LA (from infant formula powders) at K¹¹³, K³², K¹²², K²⁴, and K¹²⁷ by using nano-ESI-MS/MS (Marvin et al., 2002), while Arena et al. (2010) identified 12 different lactosylation sites (K²⁴, K³², K³⁵, K⁷⁷, K⁸¹, K⁹⁸, K¹¹², K¹¹³, K¹¹⁷, K¹²⁷, K¹³³ and K¹⁴¹) of α -LA, from powdered milk for infant nutrition, by enriching lactosylated peptides with phenylboronic acid (Arena et al., 2010). Generally, lactose can attach to any of 12 lysine positions of α -LA resulting in lactosylated lysines that are no longer recognised by proteolytic enzymes. However, even at the highest temperature and humidity we saw a maximum of six lactose adducts on α -LA. It may be that steric factors prevent further lactosylation of individual α -LA molecules. The reduction in digestibility of lactosylated milk proteins has been reported (Henle, 2007; Sebekova & Somoza, 2007); lactosylation may also increase the allergenicity of individual proteins (Arena et al., 2010).

3.4. Quantification of lactosylation

The level of lactosylation of α -LA was quantified by image analysis of the gel spots. Each spot within the vertical stack was manually outlined and the spot volume was determined. Gels of samples stored at a range of temperatures (25–40 °C), humidities (44–84% RH) and times (0–12 weeks) were analysed. Due to the

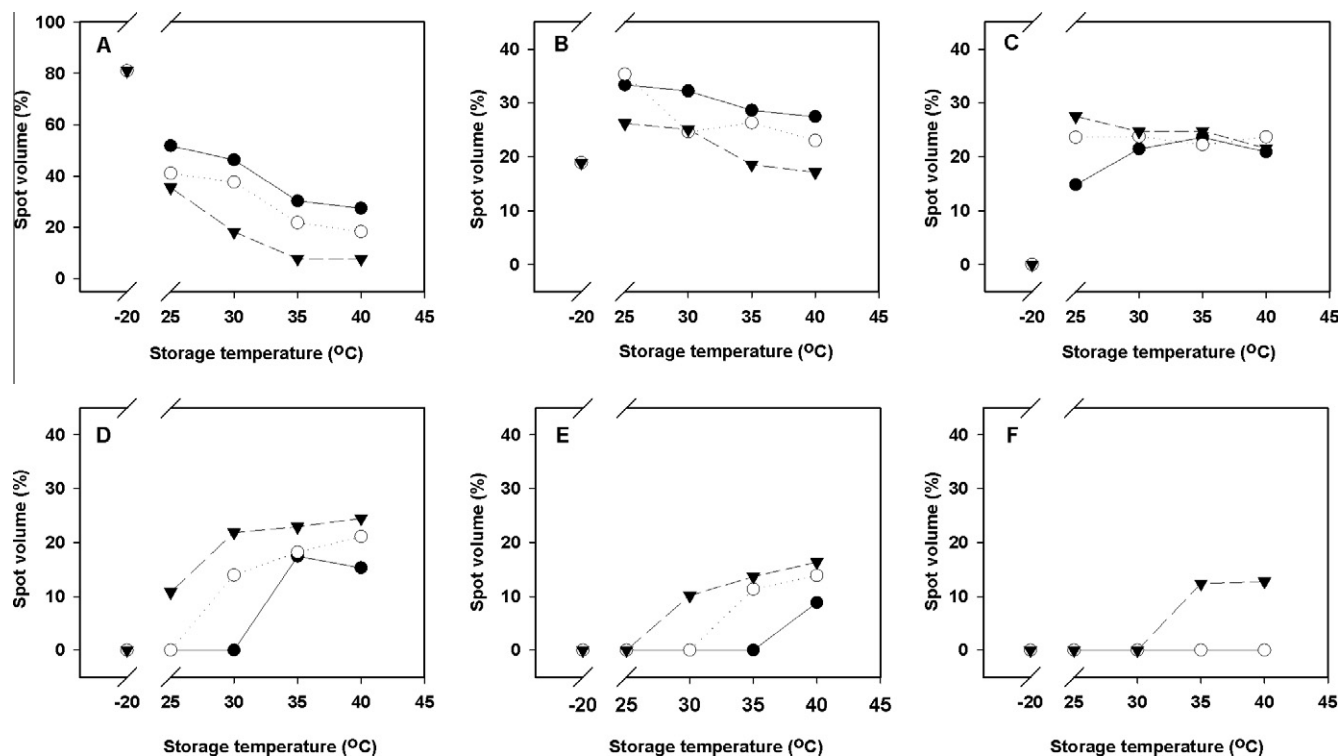


Fig. 5. Distribution of lactosylated forms of α -LA. Spots corresponding to α -LA on 2-DE gels of MPC80 stored at different temperatures (20–40 °C) and relative humidities: ●, 44%; ○, 66%; ▼, 84%, for 4 weeks were quantified according to the numbers of lactose adducts: (A) no lactose adduct; (B) 1 lactose adduct; (C) 2 lactose adducts; (D) 3 lactose adducts; (E) 4 lactose adducts; (F) 5 lactose adducts (Note: The Y-axis scale in figure A differs from that of figures B to F).

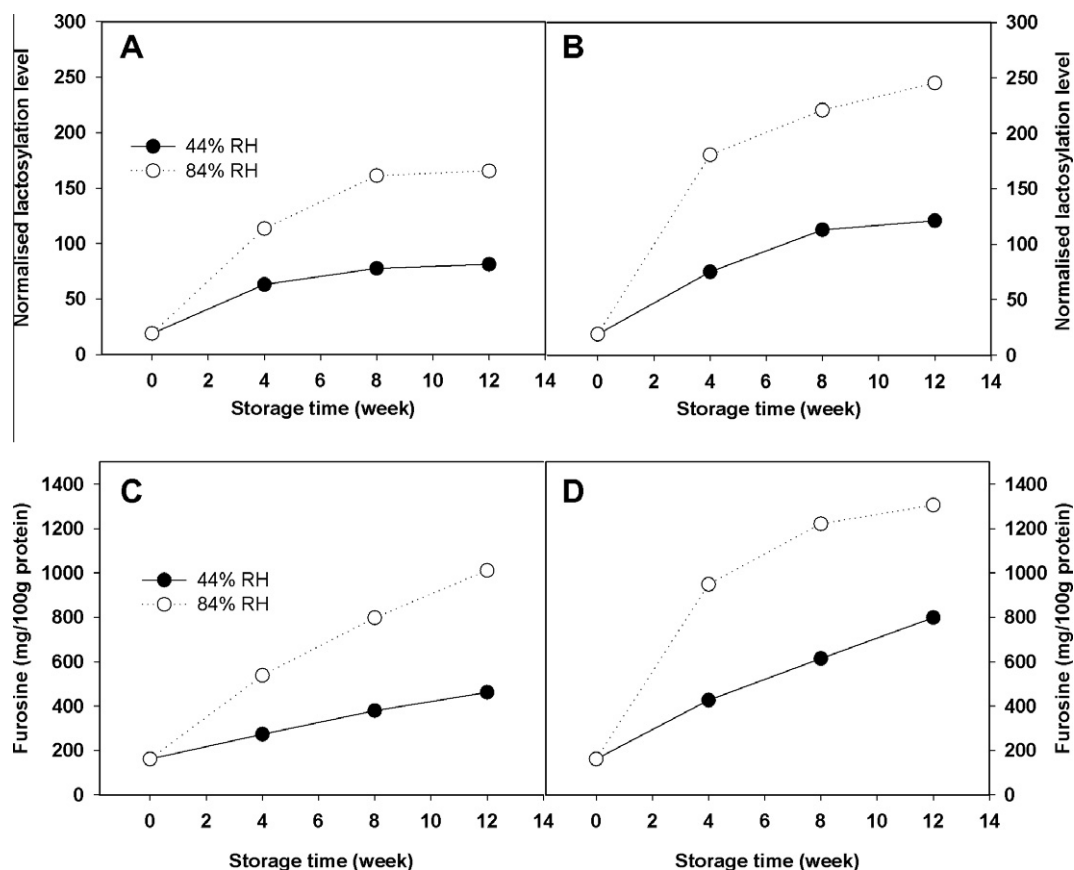


Fig. 6. Correlation between normalised lactosylation level and furosine content (reported in Le et al., 2011). The relative spot volume of each α -LA spot was multiplied by the number of lactoses attached and expressed as normalised lactosylation level of MPC80 samples stored at (A) 25 °C and (B) 30 °C (●, 44% RH and ○, 84% RH) for 0, 4, 8 and 12 weeks. The furosine contents of corresponding samples are shown in (C) and (D).

large number of stored MPC80 samples, only samples which were stored at 25, 30, 35 and 40 °C (44% RH, 4 weeks) were analysed by 2-DE in triplicate. Faint bands that could not be clearly defined were not included in the quantification. The results are expressed as means $\pm$ standard deviation in Table 2. Notably, almost 19% of the α -LA in the control MPC80 had a single lactose adduct, presumably as a result of the powder production process. This increased to 33% after storage at 25 °C for 4 weeks but then decreased at higher temperatures as more highly lactosylated forms were produced. Native, or non-lactosylated α -LA, decreased from 81% to less than 30% of the total after 4 weeks at 40 °C and almost 10% was found with 4 lactose adducts per molecule.

Fig. 5 shows the distribution of lactosylated forms obtained from 2-DE of MPC80 samples stored at 25–40 °C and 44–84% RH for 4 weeks. This clearly shows the decrease in native and increase in different lactosylated forms of α -LA during storage, as well as the compounding effect of the relative humidity during storage. For example, no triple lactosylated forms of α -LA were observed after 4 weeks at 30 °C when the relative humidity was 44%, but over 20% was triple lactosylated when the relative humidity was increased to 84%. The most highly lactosylated form that was quantified (5 lactose) was only observed at 84% relative humidity.

The degree of lactosylation is often determined from measurement of furosine, a by-product of the first stage Maillard reaction product, lactulosyllysine, that is produced during acid hydrolysis. 2-DE provides an alternative way of looking at the lactosylation level and we were interested to determine if the amount of lactose adducts from the 2-DE gels would correlate with furosine measurements. A normalised lactosylation level was calculated for

each sample by multiplying the percentage spot volume by the number of lactose adducts on that spot and then summing the values for all the spots within the stack. The normalised lactosylation levels of samples stored at 25 or 30 °C and 44 or 84% relative humidity, for up to 12 weeks, were compared to furosine contents of the same samples published earlier (Le et al., 2011) in Fig. 6. The trend of the normalised lactosylation level was similar to the furosine content. However, it appeared to flatten out after 8 weeks of storage whereas the furosine content was still increasing. The normalised lactosylation level calculated here was based solely on α -LA. While it may be more sensitive to the Maillard reaction because of its small size and the surface location of lysine residues, the limited number of available lysines may be rapidly exhausted. In contrast, the furosine level is a measure of the total amount of lactulosyllysine formed in all proteins in the sample, including the caseins, where lactosylation may proceed more slowly due to constraints imposed by the micellar structure, but continue for longer due to the larger pool of lysine residues.

Image analysis of spots from 2-DE gels also allows quantification of α -LA species containing different numbers of lactoses, providing an additional level of information on the lactosylation process in milk powders. However, as indicated in the gel of MPC80 stored at 40 °C and 84% RH for 4 weeks, shown in Fig. 2L, the spots of α -LA may become ill-defined if samples are stored at high temperature, high humidity and for prolonged time. This is caused by the increased complexity of the mixture of lactose adducts and other chemical reactions occurring along with the Maillard reaction during storage. Thus, the method may be most suitable for protein quantification in samples stored under mild conditions or for short times.

4. Conclusion

2-DE is an extremely useful tool for detecting chemical changes such as lactosylation and protein cross-linking occurring in milk powder (MPC80) during storage. In conjunction with image analysis, protein quantification was also possible with this technique. Maillard reaction products, particularly lactose adducts of whey proteins were clearly separated on 2-DE. MALDI-TOF-MS of both the intact proteins and tryptic digests facilitated the identification of lactose adducts of α -LA and the position of the lysines with attached lactose. Maillard reaction and protein cross-linking are major causes of changes in the proteins of MPC80 during storage. The changes are enhanced by temperature, humidity and time of storage and hence, the conditions of milk powder storage should be carefully considered as these changes can cause deterioration of both the powder's functional properties and nutritional quality.

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