JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

ARTICLE

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Rapid Fingerprinting of Milk Thermal Processing History by Intact Protein Mass Spectrometry with Nondenaturing Chromatography

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ABSTRACT: Thermal processing of foods results in proteins undergoing conformational changes, aggregation, and chemical modification notably with sugars via the Maillard reaction. This can impact their functional, nutritional, and allergenic properties. Native size-exclusion chromatography with online electrospray mass spectrometry (SEC-ESI-MS) was used to characterize processing-induced changes in milk proteins in a range of milk products. Milk products could be readily grouped into either pasteurized liquid milks, heavily processed milks, or milk powders by SEC behavior, particularly by aggregation of whey proteins by thermal processing. Maillard modification of all major milk proteins by lactose was observed by MS and was primarily present in milk powders. The method developed is a rapid tool for fingerprinting the processing history of milk and has potential as a quality control method for food ingredient manufacture. The method described here can profile milk protein oligomeric state, aggregation, and Maillard modification in a single shot, rapid analysis.

KEYWORDS: Milk proteins, β-lactoglobulin, casein, α-lactalbumin, electrospray, Maillard, processing, lactose

INTRODUCTION

The protein components of milk are of significant nutritional and functional importance and are used in a variety of food products as whole milk or various economically important value-added ingredients. The proteome of cow's milk is dominated by the caseins, β-lactoglobulins(β-Lg), and α-lactalbumin (α-La), the latter two being found in the whey fraction of milk. All three major proteins of milk have also been characterized as food allergens.[1] In whole milk, the caseins are assembled into very large micellar structures, comprising α- and β-caseins and the less abundant κ-casein. Caseins are heterogeneous and undergo a variety of post-translational modifications including proteolysis, phosphorylation, and glycosylation. Highly mobile, these proteins have been termed rheomorphic as they do not undergo a clear denaturation on heating, unlike the globular whey proteins. The whey fraction is less complex, comprising primarily the 14200 Da calcium-binding protein α-La and the lipocalin retinol-binding protein β-lactoglobulin, together with minor constituents such as bovine serum albumin and immunoglobulin.[2] At low pH or moderately elevated temperatures, α-La exists in a partially- folded or "molten globule" state and may undergo hydrolysis following extensive heating.[3] β-Lg is present as a mixture of monomers and dimers at neutral pH, the proportion of monomers increasing on heating to 70 °C,[4] and it had been suggested that it forms a partially folded intermediate following thermal and alkaline pH-induced denaturation.[5] By virtue of its free cysteine residue,[6] β-Lg can participate in disulfide exchange with α-La and the caseins to form complex aggregated structures.[7]

Thermal processing of food and food ingredients can be difficult to control (particularly in the context of an extended food supply network) and can result in changes in functional and organoleptic properties. Modern analytical techniques such as mass spectrometry (MS) make it possible to profile complex mixtures of proteins in unprecedented detail. This provides opportunities to develop new tools for quality control and authentication of food ingredients, such as milk and derived products, which were not previously possible. Cow's milk proteins have been extensively characterized by biochemical, molecular, and more recently MS techniques. As a less complex mixture of proteins, milk is amenable to not only conventional "bottom-up" proteomics approaches[8] but also "top-down" approaches where intact protein is directly characterized and can be fragmented for identification.[9] Typically,

Table 1. Milk Samples Used in the SEC-ESI MS Analysis[a]

milk no.	description	processing group
1	supermarket own-brand whole milk	pasteurized
2	supermarket own-brand whole organic milk	pasteurized
3	supermarket own-brand pet milk	processed liquid
4	branded filtered whole milk	pasteurized
5	branded dairy whole (Jersey/Guernsey)	pasteurized
6	branded lactose free whole milk	pasteurized
7	branded baby milk	processed liquid
8	supermarket own-brand UHT	processed liquid
9	supermarket own-brand evaporated	processed liquid
10	supermarket own-brand skimmed milk powder	SMP
11	supermarket own-brand full fat milk	pasteurized
12	commercial SMP	SMP
13	NIST SRM1549	SMP
14	Nortons dairy raw milk	unpasteurised

[a] Products were sourced from local retailers with the exception of milk 14, which was a kind donation from Nortons dairy (Norfolk, United Kingdom). NIST SRM1549 was obtained from NIST. SMP, skimmed milk powder.

Received: April 13, 2011
Accepted: October 18, 2011
Revised: October 12, 2011
Published: October 18, 2011

ACS Publications © 2011 American Chemical Society

dx.doi.org/10.1021/jf203151e | J. Agric. Food Chem. 2011, 59, 12420–12427

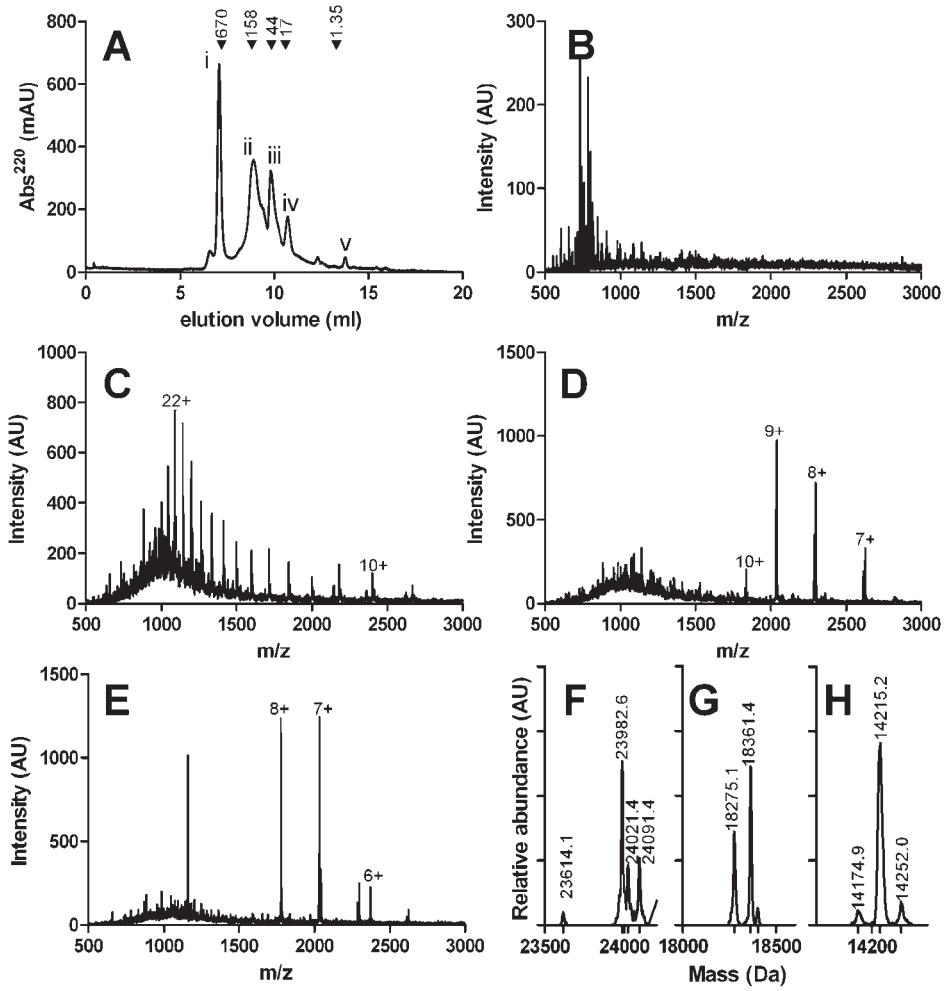

Figure 1. Analysis of a typical pasteurized milk (milk 5) by SEC-ESI-MS. Panel A shows the elution of milk components from the column as detected by UV absorbance at 220 nm [molecular mass (kDa) and elution of marker proteins are shown across the top]. Particular regions of interest are marked as follows: (i) aggregate material, (ii) caseins, (iii) β-Lg, (iv) α-La, and (v) unknown. Mass spectra of these regions of interest are shown in subsequent panels: (B) aggregate material, (C) caseins, (D) β-Lg, and (E) α-La. Deconvoluted spectra of these regions are shown as follows: (F) caseins, (G) β-Lg, and (H) α-La.

reverse phase chromatography has been applied as a preseparation for milk proteins, allowing analysis of intact proteins by electrospray ionization ESI-MS.[10] Matrix-assisted laser desorption/ionization time-of-flight MALDI-ToF-MS has also been applied to unfractionated milk for charactization of milk-processing history with some success.[11,12]

Perhaps unsurprisingly, lactosylation has been identified as a dominant modification occurring in proteins of thermally processed milk.[13] As lactosylation appears to be strongly linked to thermal processing, especially spray-drying,[14] it has been suggested that analysis of protein modification could be used as an indicator of processing history.[10] Many different thermal treatments are used in milk products, typically for the improvement of shelf life. Among the most common are high-temperature short-time (HTST) pasteurization, UHT evaporation, and spray drying. Such thermal processes can, however, potentially impact nutritional and functional qualities of milk proteins. Structural (primarily tertiary) changes, aggregation, thermally induced hydrolysis, and lactosylation can all occur as a result of thermal treatment. Although covalent modification of proteins and heat-induced hydrolysis can be detected using both bottom-up and top-down MS techniques, structural changes present difficulties to conventional analysis. Attempts have been made to assess structural changes in milk proteins by changes in behavior during reverse-phase high-performance liquid chromatography (HPLC)[15] and in charge-states generated during ESI.[16] Structurally sensitive techniques must rely on the detection of intact proteins by MS. However, the use of denaturing chromatographic conditions typically limits such techniques.

Here, we demonstrate a rapid and simple method for the characterization of thermal processing of milk products using nondenaturing size-exclusion chromatography (SEC) with online ESI-MS. In addition to information on protein presence and modification, this method also provides structural information on milk proteins. Using this method, we have analyzed a variety of commercial milk products with different thermal-processing histories (unpasteurized milks, HTST pasteurized milks, shelf-stable liquid milks, and skimmed milk powders).

MATERIALS AND METHODS

Sample Preparation. All milks and milk powders with the exception of NIST SRM1549 were obtained from local retailers (see Table 1). NIST nonfat milk powder reference material SRM1549 was obtained

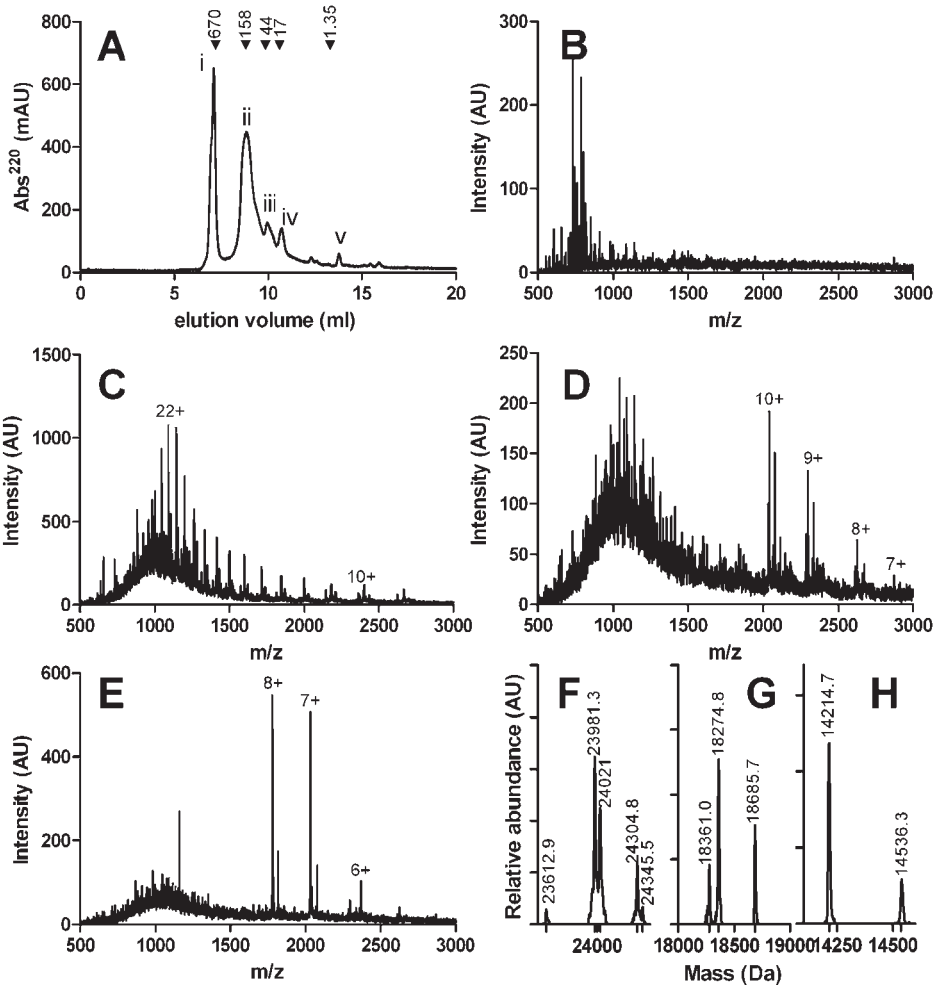

Figure 2. Analysis of a typical skimmed milk powder (milk 12). Panel A shows the elution of milk components from the column as detected by UV absorbance at 220 nm [molecular mass (kDa) and elution of marker proteins are shown across the top]. Particular regions of interest are marked as follows: (i) aggregate material, (ii) caseins, (iii) β-Lg, (iv) α-La, and (v) unknown. Mass spectra of these regions of interest are shown in subsequent panels: (B) aggregate material, (C) caseins, (D) β-Lg, and (E) α-La. Deconvoluted spectra of these regions are shown as follows: (F) caseins, (G) β-Lg, and (H) α-La.

directly from NIST (United States). Milk powders were reconstituted in water (1 in 10, w/v) before use. For heating experiments, 100 μL of milk or reconstituted milk powder in screw-cap Eppendorf tubes was subjected to heating in a preheated water bath at 90 °C for the indicated length (0, 1, 5, and 20 min) of time prior to extraction.

Samples of milk or reconstituted milk powder were diluted 1 in 10 (v/v) with 150 mM ammonium bicarbonate, pH 6.5, prior to centrifugation for 10 min at 12000 *g*. After the fat layer was removed, the supernatant was retained and used for further analysis.

Purified milk proteins were obtained from Sigma (Poole, United Kingdom): α-La (L-5385), β-Lg (L-3908), and β-casein (C-6905). For SEC-ESI-MS, these were prepared as 0.5 mg/mL solutions in 150 mM ammonium bicarbonate, pH 6.5.

SEC-ESI-MS. Samples of extracted milk or purified milk proteins (see above) (4 μL) were applied to a BioSep S3000 column (300 mm × 7.8 mm) (Phenomenex, Macclesfield, United Kingdom) pre-equilibrated with 150 mM ammonium bicarbonate (pH 6.5) and attached to an Agilent 1100 series binary LC. The flow rate used was 1 mL/min, and the postcolumn flow was split between an Agilent (Cheadle, United Kingdom) 1100 series photodiode array (detection 220−700 nm) and a Bruker (Coventry, United Kingdom) MicroToF (80:20). The HPLC, mass spectrometer, and PDA were operated using Bruker HyStar ver. 3.2. MS settings used were optimized for intact protein detection by infusion of purified β-Lg (Sigma) at 0.1 mg/mL in 150 mM ammonium bicarbonate. The source settings used were as follows: capillary voltage, 4200 V; nebulizer gas, 1 bar; dry gas, 7 L/min; source temperature, 180 °C; and capillary exit, 200 V. MS settings used were as follows: positive ion mode, hexapole RF 740 V, and mass range 150−3000 m/z with data collection in centroided mode. Spectra were analyzed and processed using Bruker Daltonics DataAnalysis software (version 3.4, build 179).

To extract protein masses, electrospray data from the MicroToF mass spectrometer were charge deconvoluted using the MagTran software package, version 1.03 b2.[17] MagTran was run with an isotope envelope width setting of 1−5 Da, signal-to-noise threshold of 5, mass accuracy of 0.1 Da, and a charge state range of 1−30. The charge state was determined by "charge envelope then isotope", with an isotopic peak width setting of 0.25 Da. It was found necessary to divide the anticipated molecular mass range into blocks of typically 5 kDa and commence processing at higher masses to prevent spurious molecular mass features. Several iterations of smoothing were used, with the number of iterations adjusted to give a well-defined molecular mass stable against further smoothing.

Masses were assigned to protein components of milk using the Informall database of allergens (http://foodallergens.ifr.ac.uk) and the

Table 2. Assignment of Masses Observed during SEC-ESI-MS to Milk Components[a]

designation	accession	modification	observed mass	expected mass	mW by SEC (kDa)
caseins					
α-s1 casein	P02662		23614.1	23614.8	102
β-a1 casein	P02666		24021.4	24023.2	
β-a2 casein	P02666		23982.6	23981.6	
β-a1 casein	P02666	+1 lac	24345.5	24347.2	
β-a2 casein	P02666	+1 lac	24304.8	24305.7	
β-lactoglubulin					38.3
β-Lg A	P02754		18361.4	18363.1	
β-Lg B	P02754		18275.1	18277	
β-Lg A	P02754	+ 1 lac	18685.7	18687.2	
α-lactalbumin					16.4
α-lactalbumin	P00711		14174.9/14215.2[b]	14176.9	
α-lactalbumin	P00711	+1 lac	14536.3	14502.9	
purified protein standards					
β-casein	P02662		24022.9	24023.2	112
β-Lg A	P02754		18361.8	18363.1	36.9
α-La	P00711		14174.9/14215.1	14176.9	15.3

[a] Observed masses were obtained by deconvolution of mass spectra obtained by SEC-ESI-MS (Materials and Methods) and averaging across all samples where the mass events were present. Masses were assigned to protein components of milk using the Informall database of allergens (http://foodallergens.ifr.ac.uk) and the Uniprot protein database (www.uniprot.org). Each cysteine was assumed to be cystine in the absence of reduction (−1.008 Da). Additions of 324.1 Da were assumed to be lactosylation events [+342 Da (lactose), −18 Da (H_2O)]. Additional mass events (of 702.2, 1044.5, 1388.5, and 1830.6 Da) were also observed at a retention time 13.9 min and could not be assigned to known milk components but were not present in "lactose free" milks. These mass events are therefore tentatively ascribed to the presence of lactose not covalently attached to protein. In all cases, the average mass is given. The native molecular weight was estimated by SEC retention time relative to those of molecular weight standards. [b] In the case of α-La, the major deconvoluted mass in all milks and in the purified protein standard was approximately 38 Da above the expected value.

Uniprot protein database (www.uniprot.org). Additions of 324.1 Da were assumed to be lactosylation events. UV data were analyzed and processed using HyStar Post-Processing (version 3.2) software (Bruker, Coventry, United Kingdom).

RESULTS AND DISCUSSION

SEC conditions were selected to maintain milk proteins in oligomeric and conformational states as close to those found in milk as possible, and major milk proteins were identified by comparison of observed deconvoluted masses with theoretical masses derived from sequence accessions. An example of an annotated chromatogram and associated obtained mass spectra for selected SEC peaks for pasteurized milk is shown in Figure 1. It should be noted that due to the constraints of native chromatography, much aggregated material was insoluble and removed by centrifugation prior to analysis. For comparison, a chromatogram and spectra for a milk powder are given in Figure 2. Significant peaks containing UV-absorbing material were observed in pasteurized (Figure 1A) and skimmed milk powder (Figure 2A) at 6.5−7.5 (i), 8.8 (ii), 9.8 (iii), 10.7 (iv), and 13.9 min (v). The protein composition of these peaks was assigned by deconvolution of mass spectra where available as described in Table 2: caseins (ii), β-Lg (iii), and α-La (iv). No identifiable mass events were observed for peak (i), which is eluted at the exclusion limit for the column and comprised UV-absorbing high M_r (>150 kDa) material. Such aggregated protein does not typically ionize under native conditions and is likely to correspond to heavily cross-linked protein formed by heat-induced intermolecular β-sheet structures and disulfide interchange. Denaturation and reduction, possibly followed by proteolysis, would be necessary to further characterize this aggregate fraction. Peak v was present only in none lactose-reduced milks and may be associated with the presence of free lactose in milk products. Both α-La and β-Lg (isoforms A and B) were readily identifiable, but the caseins, being more diverse and including post-translational modifications such as phosphorylation and glycosylation, were more difficult to assign. In addition, caseins are known to be modified with phosphate to varying degrees when analyzed directly in milk.[18] Masses corresponding to unmodified α-s1, β-A1, and β-A2 casein were assigned to within 100 ppm mass accuracy. Some less abundant caseins that have previously been detected by RP-HPLC[19] were unidentifiable, possibly because of relatively poor ionization due to "near-native" conditions or lack of denaturation. However, because of the nature of the separation technique used, all identified caseins elute at identical times (corresponding to a native mW range of 70−170 kDa as estimated by size exclusion elution time) and, thus, were all represented in the same mass spectrum. Caseins are thought to be present as associations of trimers in milk,[20] and our data would indicate either a relatively high stokes-radius trimer or a low radius hexamer. Assignment of modified caseins is less certain than is the case for whey proteins due to increased spectra complexity and lesser intensity of the charge states of each individual casein. In the case of α-La, mass events corresponding to the predicted 14176.9 Da mass of the intact protein were observed in all milks, but in all cases a mass of 14215 Da was consistently present in greater abundance. This was also the case for the purified α-La standard. The reason for the apparent +38 Da addition is unclear but would be consistent with either replacement of a proton with potassium or replacement of two protons with calcium.

Initially, the effect of more extensive thermal treatment was assessed by heating pasteurized milk at 90 °C for 0, 1, 2, 5, and

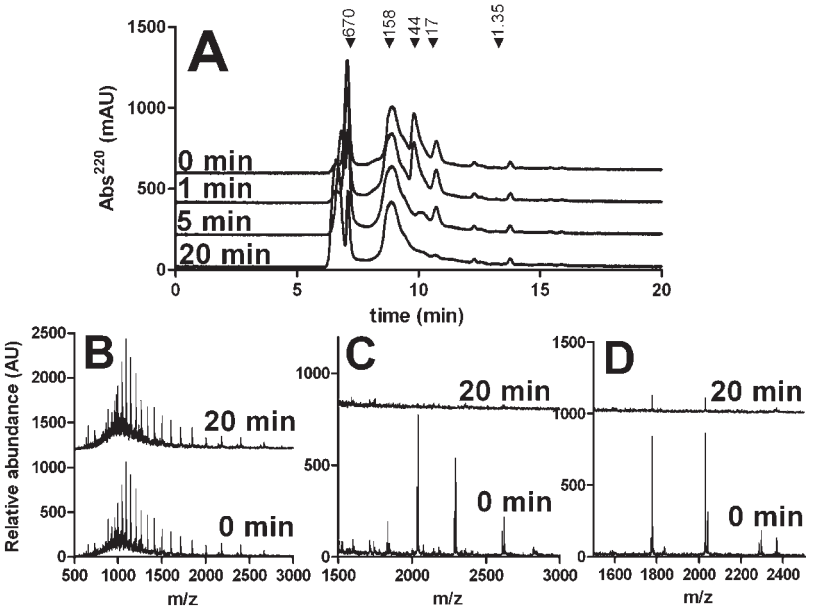

Figure 3. Effect of heating on SEC behavior of pasteurized milk (milk 5). Panel A shows the elution of milk components from the column as detected by UV absorbance at 220 nm [molecular mass (kDa) and elution of marker proteins are shown across the top]. The spectra of the three major protein groups recovered from 0 and 20 min heated samples are shown as follows: (B) caseins, (C) β-Lg, and (D) α-La.

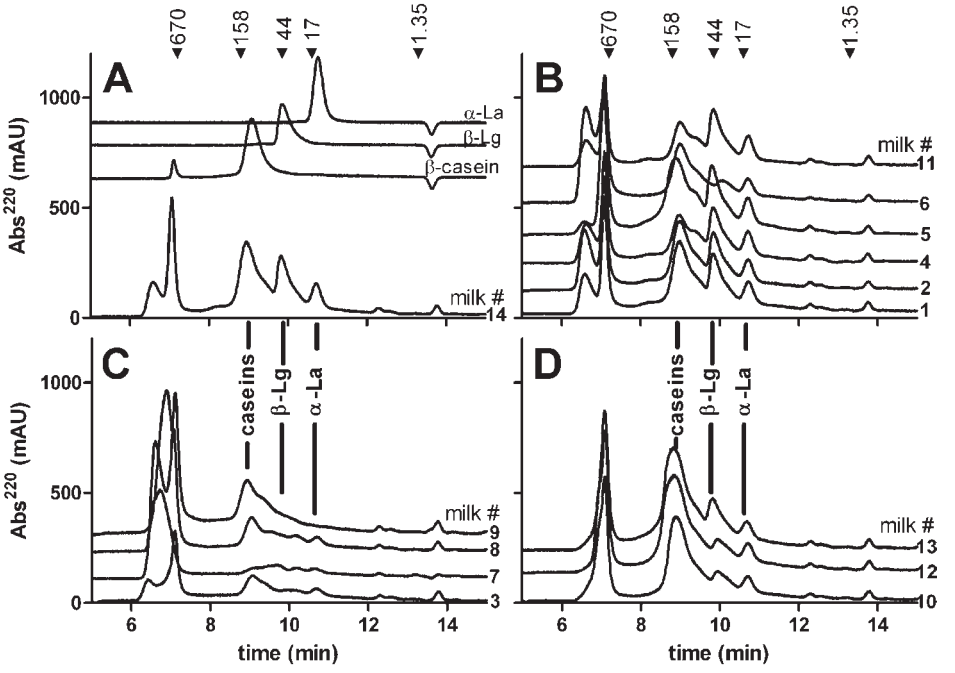

Figure 4. SEC profiles of protein standards (A) and milks grouped by processing category. (A) Purified protein standards (unpasteurized milk is shown for comparison), (B) pasteurized milks, (C) shelf-stable liquid milk, and (D) skimmed milk powders. Peaks are labeled by protein having been identified by mass spectra (see Table 2). Milk numbers corresponding to those in Table 1 are indicated. Molecular mass (kDa) and elution of marker proteins are shown across the top.

20 min (Figure 3). Loss of dimeric β-Lg was observed first, followed by monomeric α-La, but at no stage during the heating was there evidence that caseins became aggregated. This is consistent with previous findings that whey proteins are more susceptible to heat-induced denaturation and aggregation than the caseins.[21] Heat-induced cross-linking of caseins to β-Lg has previously been described as a mechanism for the aggregation of milk proteins during thermal processing.[22] However, our observation that β-Lg can undergo aggregation without apparent cross-linking to casein would suggest that widespread occurrence of β-Lg cross-linking to caseins is not pivotal to all protein aggregation events in heated milk products.

The protein composition of a range of commercially available milk products processed to different degrees was assessed enabling their classification into three broad groups as follows: (1) milks with no or minimal heat treatment including raw and pasteurized liquid milks, (2) moderately modified thermally processed liquid milks, and (3) extensively modified milks comprising milk powders. UV spectra of SEC-ESI runs of all milks used are shown in Figure 4. The SEC profiles of raw and pasteurized milks were very similar and in all cases were characterized by relatively high abundance (UV and ion count) of both whey and caseins in an unaggregated "nativelike" state. A relatively minor impact of protein oligomeric state is consistent with pasteurization, which typically involves heating to 71.7 °C for 15−20 s—a relatively mild heat treatment. Shelf-stable liquid milk products, which have undergone more extensive thermal treatment including ultra heat treatment (typically a brief heating to 135 °C for 1−2 s) and an evaporated milk (typically 115−118 °C for 15 min) had reduced levels of monomeric α-La and dimeric β-Lg as compared to the raw/pasteurized milk group. In all of the milks, apart from an extensively thermally modified infant formula milk (recommended for infants under 1 year old), the casein remained in its native oligomeric state. The third group of products was the milk powders, where proteins were resolved into discrete peaks corresponding to α-La and β-Lg, which, while less abundant than in other milk samples, were still easily observed in the chromatograms and mass spectra.

In addition to the identification of major milk proteins, the SEC-ESI-MS method also allowed characterization of protein modification—specifically lactosylation—in milk products. The method presented here provides rapid analysis of lactosylation state of the major milk proteins without extensive sample pretreatment. Shifts in m/z corresponding to +324 Da additions were observable for all major milk proteins. Data for lactosylation of β-Lg are shown as this protein exhibited the greatest degree of lactosylation. Lactosylation was observed primarily in the powdered milks (Figure 5) with some occurrence in the thermally processed milk group. Lactosylation of β-Lg was also detected in various highly processed liquid milk products, but the extent of modification was less than that in milk powders. NIST SRM 1549 milk powder possessed a greater degree of lactosylation on all of the major milk proteins than commercial milk powders. NIST SRM 1549 is γ-irradiated for storage stability,[23] and it is possible that this irradiation results in lactosylation in addition to those produced by the spray-drying process. However, conditions during spray drying could also account for observed differences in lactosylation, and further study is required to examine the effect, if any, of irradiation on protein modifications.

Analysis of charge state distribution can be used to give an indication of protein structure,[24] with increasing protein unfolding leading to higher average charge and greater spread of charge states. The charge states obtained in this work were lower and less diverse than those previously obtained using denaturing separation conditions, for example, for β-Lg using reverse phase HPLC[16] or SDS-PAGE for casein.[25] This is particularly noticeable for casein spectra, which did not display typical charge distribution but demonstrated a bimodal character with an apparent lower charge form (Figures 1C and 2C) not present with denaturing separations, possibly indicating two differently folded forms of casein. Within our experiments, the charge-state distribution of all of the observed proteins remained surprisingly constant in both commercially processed and laboratory-heated

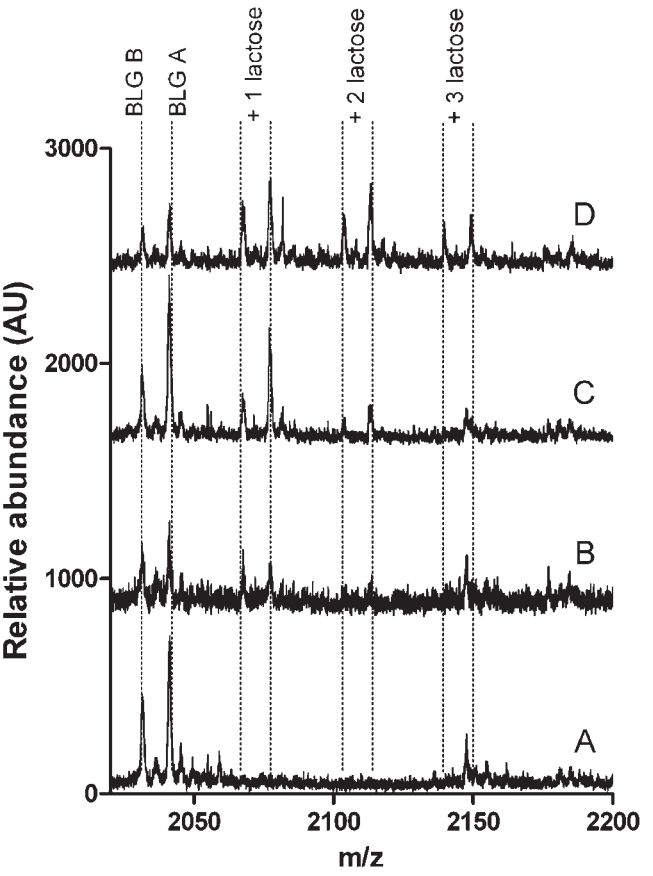

Figure 5. Lactosylation of β-Lg in (A) pasteurized milk 5, (B) heavily thermally treated milk 8, (C) skimmed milk powder 10, and (D) skimmed milk powder 12 as assessed by a single charge state (9+) of β-Lg after SEC-ESI MS. The 9+ charge state of β-Lg A and B are indicated, as are the m/z shifts corresponding to additions of 1, 2, and 3 lactose units to both isoforms.

milk samples after SEC (data not shown). This contrasts with previous reverse-phase HPLC-ESI analysis, which indicated that heating of β-Lg led to higher average charge state.[16] One potential explanation for this behavior is that heat-induced structural changes, which may result in different charge-state distribution with denaturing separations, result in aggregation in our system, rendering the protein nondetectable in the mass spectrometer.

While the application of LC-ESI-MS to milk protein analysis is not of itself novel, milk products have not previously been analyzed under conditions designed to maintain native structure prior to ionization. Native separation of proteins prior to MS analysis has distinct advantages and disadvantages. Typically, such techniques are lower resolution and can also present challenges with respect to efficient ionization of intact proteins (strong acidic conditions are not possible). However, as information on the oligomeric status of the proteins is maintained, such methods are sensitive to processing-induced changes in protein state. The oligomeric state, particularly of the whey proteins α-La and β-Lg, appears to be significantly linked to thermal processing of liquid milk products, both in off-the-shelf commercial products and in laboratory heating experiments on pasteurized milks.

Lactosylation, a common milk protein modification, was readily observed using SEC-ESI-MS. Observation of extensive lactosylation appears to be an indicator of spray drying in the

production of milk powders. Interestingly, the NIST SRM 1549 milk proteins were all far more heavily modified than commercial milk powder counterparts, suggesting that SEC-ESI MS may provide a rapid and simple means of detecting nonstandard processing of milks. The NIST SRM 1549 is a commonly used standard for evaluation and calibration of commercial enzyme-linked immunosorbent assay (ELISA) allergen test kits.[26] From our analyses, however, it would seem that SRM 1549 is significantly different from commercial milk powders with respect to protein modification. Although the impact of these modifications on ELISA detection is currently unknown, we would suggest caution in the use of calibrant material, which has not been thoroughly characterized with respect to protein composition and modification.

The method described herein enables categorization of milk products by thermal treatment history on the basis of protein oligermeric state and modification. Other methods, such as analysis of lactulose content, can also be used to indicate the degree of thermal treatment.[27] However, lactulose can degrade in solution by β-elimination[28] and may be unsuitable as a marker of thermal history in some long-storage liquid milk products. In addition, certain processes in which information on protein state is crucial (e.g., production of milk hydrosylates for allergic consumers, production of reference materials for ELISA of protein content) necessitate protein-based methods of assessment. We suggest that native separation methods and MS provide a rapid, reproducible method for the characterization of major milk proteins and consequently processing history.

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Funding Sources

This work was partly funded by MoniQA (Monitoring and Quality Assurance in the Food Supply Chain), a European funded initiative within the 6th framework programme (N0-FOOD-CT-2006-036337), and the UK Biotechnological and Biological Research Council through an Institute Strategic Programme Grant to IFR.

ACKNOWLEDGMENT

We thank Neil Rigby (IFR) for his practical assistance and Norton Dairy (Frettenham, Norfolk) for the kind gift of unpasteurized milk.

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