

Accepted Manuscript

Mass spectrometry for the analysis of protein lactosylation in milk products

Rosa Anna Siciliano, Maria Fiorella Mazzeo, Simona Arena, Giovanni Renzone, Andrea Scaloni

PII: S0963-9969(12)00463-2
DOI: doi: [10.1016/j.foodres.2012.10.044](https://doi.org/10.1016/j.foodres.2012.10.044)
Reference: FRIN 4330

To appear in: *Food Research International*

Received date: 19 July 2012
Accepted date: 31 October 2012

Please cite this article as: Siciliano, R.A., Mazzeo, M.F., Arena, S., Renzone, G. & Scaloni, A., Mass spectrometry for the analysis of protein lactosylation in milk products, *Food Research International* (2012), doi: [10.1016/j.foodres.2012.10.044](https://doi.org/10.1016/j.foodres.2012.10.044)

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.



MASS SPECTROMETRY FOR THE ANALYSIS OF PROTEIN LACTOSYLATION IN MILK PRODUCTS

Rosa Anna Siciliano ^{a*}, Maria Fiorella Mazzeo ^a,
Simona Arena ^b, Giovanni Renzone ^b and Andrea Scaloni ^{b*}

^a Proteomic and Biomolecular Mass Spectrometry Center, ISA, National Research Council, 83100
Avellino, Italy

^b Proteomics & Mass Spectrometry Laboratory, ISPAAM, National Research Council, 80147
Naples, Italy

Keywords: Milk; Proteomics; Maillard reaction; Thermal treatment; Mass spectrometry

* To whom correspondence should be addressed:

Rosa Anna Siciliano
Proteomic and Biomolecular Mass Spectrometry Center
ISA, National Research Council
via Roma 64, 83100 Avellino, Italy
Email: rsiciliano@isa.cnr.it

Andrea Scaloni
Proteomics & Mass Spectrometry Laboratory
ISPAAM, National Research Council
via Argine 1085, 80147 Naples, Italy
Email: andrea.scaloni@ispaam.cnr.it

ABSTRACT

Milk and dairy products are usually submitted to thermal procedures in order to preserve microbiological safety and to prolong their shelf-life; these actions may eventually induce structural modifications of several components. In particular, milk proteins undergo a non-enzymatic glycation, known as Maillard reaction, in which reducing sugars (such as lactose) mainly react with the ϵ -amino group of Lys residues, leading to the formation of the Amadori product lactulosyl-lysine. Protein lactosylation leads to a decrease of milk nutritional value as it reduces the bioavailability of the essential amino acid lysine and to a potential increase of the specific allergen activity, due to formation of new hapten-like antigens. These aspects are of particular importance in products intended for infant diet, such as milk powders and infant milk formulas; accordingly, a detailed structural characterization of the extent and site-specificity of milk protein lactosylation is mandatory. Analytical methodologies based on mass spectrometry and more recently proteomics have been thoroughly applied to the structural analysis of milk proteins and their modified forms, including glycation products. This review highlights the contribution of mass spectrometry to the extensive investigation of lactosylation events in milk proteins, with a particular focus on the most up-to date technological improvements.

Keywords: Lactosylation, Maillard reaction, Mass spectrometry, Proteomics.

1. INTRODUCTION

In order to preserve microbiological safety and to prolong shelf life, milk and dairy products are usually submitted to thermal procedures (pasteurization and sterilization) prior to retail and consumption. However, these processes induce significant physiochemical changes in milk constituents depending on duration and extent of heating procedure. The most thoroughly studied modification of milk proteins is the Maillard reaction, a non-enzymatic glycation in which the carbonyl group of reducing sugars, such as lactose, primarily reacts with the ϵ -amino group of Lys residues leading to the initial formation of lactosyl-lysine, which structurally rearranges into the more stable Amadori product lactulosyl-lysine (Mauron, 1981) (Fig. 1). These amino acid derivatives are involved in a reaction equilibrium whose direction depends on the environmental pH value. Main lactosylated products are abundant milk soluble proteins, i.e. α -lactoalbumin (α -LA), β -lactoglobulin (β -LG) (whey proteins), and α S1- and β -caseins (CN).

Protein lactosylation results in a decrease of milk nutritional value as it reduces the bioavailability of the essential amino acid lysine. In fact, lactosylated lysines are no longer recognized by gastrointestinal proteases and this also affects milk digestibility. Furthermore, lactosylated proteins may act as hapten-like antigens, increasing the specific allergen activity of individual components (Mauron, 1990; Finot, 1990). These nutritional aspects are of particular importance in products intended for infant diet, such as milk powders and infant milk formulas (a special type of milk product, made from cow milk-derived constituents, i.e. casein or whey proteins, lactose, and butter fat, combined with vegetable oils, starch and other ingredients), used by newborns or infants of varying age (Nasirpour, Scher, & Desobry, 2006). As a consequence of their specific formulation and processing, for instance, infant milk formulas have been demonstrated to present higher levels of protein glycation than other milk products (Birlouez-Aragon et al. 2004, Pischetsrieder, & Henle, 2012), being up to about 15-20% of their protein Lys residues modified by lactosylation (Henle, Walter, & Klostermeyer, 1991). The presence of lactosylated forms can also induce remarkable consequences on technological processes, such as cheesemaking (Singh, & Waungana, 2001), as glycation may potentially influence the ripening process by modifying the protein degradation pattern performed by proteases present in or added to milk, in particular plasmin (Dalsgaard, Nielsen, & Larsen, 2007). On the other hand, controlled glycation carried out under specific conditions, although highly dependent on the saccharide used, has also been proposed as a non toxic technological method to improve functional properties of whey proteins, i.e. solubility, heat stability, emulsifying and foaming properties (Nacka, Chobert, Burova, Léonil, & Haertlé, 1998; Chevalier, Chobert, Mollè, & Haertlé, 2001a; Chevalier, Chobert, Popineau,

Nicolas, & Haertlé, 2001b; Darewicz, & Dziuba, 2001; Thomas, Scher, Desobry-Banon, & Desobry, 2004; Chobert, Gaudin, Dalgalarondo, & Haertlé, 2006; Wang, & Ismail 2012).

The nutritional and technological relevance of milk protein glycation called for an urgent need of detailed structural studies aimed to investigate nature and physical-chemical features of the Maillard reaction products, also defining their quantitative occurrence and the identity of the corresponding modification sites. The introduction of new mass spectrometric techniques suitable for protein and peptide analysis has prompted a boost in these structural studies thus leading to the publication of a significant number of papers. In the last twenty years, analytical methodologies based on mass spectrometry (MS) have been thoroughly applied to characterize different components of milk and dairy products and to assess their quality (Guy, & Fenaille, 2006). In particular, two different soft-ionization techniques, i.e. Electrospray Ionization (ESI) (Fenn, Mann, Meng, Wong, & Whitehouse, 1989) and Matrix Assisted Laser Desorption Ionization (MALDI) (Karas, & Hillenkamp, 1988), usually coupled with quadrupole, Ion Trap (IT) or Time Of Flight (TOF) mass analyzers, have allowed to accurately measure the molecular mass of intact proteins and peptides; these techniques have been applied to the structural analysis of milk proteins and their modified forms, including glycation products (Cunsolo, Muccilli, Saletti, & Foti, 2011; Oliver, 2011). As the formation of lactosyl-lysine and lactulosyl-lysine causes a +324 Da mass shift with respect to the non-modified species, their detection by MS procedures allows to recognize the occurrence of lactosylation events on intact proteins. Furthermore, MS analysis of suitable protein digests (obtained mainly by using trypsin, endoproteinase GluC or AspN) can lead to the assignment of the modification sites. Therefore, these analytical approaches have the ability to provide accurate and detailed information on the extent, nature and location of heat-induced milk protein modifications, thus affording new insight into the glycation process. On the contrary, immunochemical and HPLC- or GC-based methods left unsolved the question of whether specific amino acid residues are preferentially modified.

This review highlights the contribution of mass spectrometric methodologies to the extensive investigation of lactosylation events in milk proteins, with a particular focus on MS-based innovative analytical approaches owing to technological improvements in mass analyzers and fragmentation methods as well as to the development of dedicated proteomic procedures.

2. ESI-MS FOR THE INVESTIGATION OF PROTEIN LACTOSYLATION

ESI-MS coupled on-line or off-line with high performance liquid chromatography (LC) has been widely applied to detect and characterize glycation in milk proteins. Most of the developed methodologies were aimed at investigating the relation between thermal damage induced in milk by

industrial heat treatments and the extent of protein modification. To this purpose, whey proteins (α -LA and β -LG) were regarded as suitable markers due to their physicochemical features, in particular solubility and lack of post-translational modifications (except for disulphide bonds and a partial N-glycosylation of α -LA). On the other hand, the casein fraction is a mixture of isoforms arising from different genetic variants and presents a complex pattern of post-translational modifications (phosphorylation and glycosylation), which can make the interpretation of mass spectra and the assessment of glycation quite challenging. Furthermore, these proteins are mostly present in aggregated structures, casein complex and micelles, which are considered less prone to glycation. Experimental protocols for whey proteins isolation are fast and simple, and include a preliminary centrifugation step to remove milk fat and an acid treatment at pH 4.2-4.6 to precipitate the casein fraction prior to LC analysis.

Pioneering work applying ESI-MS to the detection of multiple glycoforms and to the structural characterization of lactosylated species was carried out on β -LG, used as a model protein submitted to heat treatments *in vitro* in the presence of lactose (as recently thoroughly reviewed by Oliver, 2011). The first direct evidence obtained by ESI-MS on the specific lactosylation of β -LG during the initial stage of the Maillard reaction was reported by Leonil et al. (1997), who analyzed whey proteins separated from mild heat-treated milk samples. Mono- and di-lactosylated derivatives were observed in the ESI mass spectra of intact proteins. By using protease mapping, LC-ESI-MS and tandem mass spectrometric experiments, Lys47 was identified as one of the first lactose-binding sites. At that time, partial lactosylation of β -LG was also observed in fresh milk samples analysed by ESI-MS (Burr, Moore, & Hill, 1996).

Further LC-ESI-MS analysis of the tryptic digest of β -LG submitted to lactosylation reaction under mild heat conditions indicated that lactosylation was widely distributed within the protein structure, involving nine Lys residues out of fifteen residues present in β -LG and the N-terminal group; modification at position 8, 60, 83, 101, 135 and 141 was not detected (Morgan, Léonil, Mollé, & Bouhallab, 1997). However, lactosylation experiments performed under dry state conditions highlighted an increased incorporation of lactose moieties. In fact, all the Lys residues of β -LG, except Lys101, were found to be involved in sugar binding (Morgan et al., 1998), although the detection of the triptic peptide containing Lys101 and its potential modified form could be hampered by specific structural features (Dalgarrondo et al., 1990). In this context, it cannot be ruled out that the lack of detection of lactosylated peptides by MS may also depend on their ionization efficiency and physical-chemical features.

In order to identify the modified residues, LC-ESI tandem mass spectrometry (LC-ESI-MS/MS) operating in neutral loss scanning mode was used for the analysis of a proteolytic digest of β -LG,

which was previously heated in presence of lactose. A more accurate detection of lactosylated peptides was achieved by selectively monitoring the constant mass loss of 216 or 108, arising from singly or doubly charged ions, respectively, which originated from modified peptides as result of the cleavage of the galactose moiety plus the loss of two water molecules (Fig. 2) (Mollé, Morgan, Bouhallab, & Léonil, 1998). Further methodological innovations included nanoESI-MS and nanoESI-MS/MS analyses as well as neutral loss scanning mode and precursor ion scanning mode procedures; these approaches were applied to the structural characterization of lactosylation and galactosylation sites in β -LG modified under solid state condition. It is worth to note that such experimental conditions led to the addition of as many as seventeen lactose and twenty-two galactose moieties. The galactosylation reaction affected all the Lys and Arg residues as well as the N-terminal group of β -LG, while only Arg124 was lactosylated (Fenaille, Morgan, Parisod, Tabet, & Guy, 2004). These results opened the way to the application of ESI-MS based approaches, coupled on-line or off-line to HPLC, to the analysis of a large set of commercial products, such as pasteurized, UHT, sterilized and condensed milks, milk powders or infant milk formulas.

For the first time, Fogliano et al. (1998) performed HPLC separation and off-line ESI-MS analysis of β -LG purified from different commercial milk samples, revealing the presence of mono-lactosylated forms in UHT milk sample (accounting for 3% of the total β -LG) and of mono- and di-lactosylated adducts in samples from sterilized milk. These forms were not detected in pasteurized milk samples thus suggesting that increasing time and temperature of the thermal process favour glycation. LC-MS analysis of the tryptic digest of β -LG from sterilized milk indicated that Lys100 was preferentially lactosylated during industrial milk treatment, together with Lys47. Lys47 and Lys91 were instead the most reactive sites during heat treatment under dry state condition (Morgan et al., 1998; Fenaille, Morgan, Parisod, Tabet, & Guy, 2004).

In a further study, Siciliano, Rega, Amoresano, & Pucci (2000) detected a small percentage of covalent adducts (accounting for about 3% of total whey proteins) on both α -LA and β -LG purified from pasteurized milk. A relative quantitative analysis was performed by using the ESI-MS transformed mass spectra, on the basis of the relative intensity (ion current) of signals originated by the modified and unmodified forms of each protein, assuming similar ionization efficiencies of the different glycoforms. In whey proteins purified from UHT milk samples, the extent of lactosylation increased (accounting for about 30% of total protein) and di-lactosylated forms were also detected (Fig. 3A-B). Finally, analyses performed on proteins from powdered milk intended for infant nutrition revealed that up to 70% of proteins was modified and showed also the formation of tri-lactosylated forms and adducts modified by the addition of hexose residues (Fig. 3C-D).

Furthermore, thermal denaturation of β -LG was also observed. Interestingly, the lactosylation sites

of α -LA purified from out-of-date UHT milk samples were originally identified by MALDI-TOF-MS analysis of the tryptic protein digest. Among the twelve Lys residues present in α -LA, Lys98 was the main lactosylation site, while Lys114 and/or Lys122 were modified at a less extent.

LC-ESI-MS analysis of particular commercial products, such as different whey protein concentrates, showed that industrial processes have a significant influence on the lactosylation pattern and, in addition to glycation, also cause a significant increment of Met oxidation (Hau, & Bovetto, 2001). On the other hand, LC-ESI-MS data obtained from the analysis of whey proteins submitted to different heat treatments and purified by various dairy products can provide information on protein physico-chemical changes induced by lactosylation and on relative reaction kinetics. The degree of lactosylation was accurately determined from the extracted ion chromatograms of the main multi-charged ion of each form. By using different model systems (single protein, α -LA/ β -LG protein mixture and whole milk), the influence of the other matrix components on protein lactosylation was assessed, demonstrating that α -LA was considerably more lactosylated in whole milk sample than in aqueous protein solution. This behaviour was not observed for β -LG. Variations in retention times, shape of the chromatographic peaks of the extracted ion chromatograms and protein charge state distribution were related to a reduction of the protein hydrophobicity and to conformational changes induced by lactosylation (Czerwenka, Maier, Pittner, & Lindner, 2006). Similarly, Monaci & van Hengel (2007) performed an in depth investigation on structural modifications of β -LG in a model solution or in milk samples submitted to different heat treatments. Authors reported a decrease of protein charge state distribution in lactosylated β -LG, probably due to the ability of the sugar moiety to mask the protonable sites of the protein. A further development of the method reported by Czerwenka, Maier, Pittner, & Lindner (2006) was then introduced by Losito, Carbonara, Monaci, & Palmisano (2007). A new quantification strategy of lactosylation was set up, which was based on peak area integration after multiple ion current extraction and considering all the ions detectable in the multi-charged ESI-MS spectrum for each whey protein form. The higher number of different lactosylated sites in β -LG, compared to α -LA, was related to an enhanced degree of denaturation of this protein, which increased with temperature and time of treatment. In fact, since denaturation induced the partial loss of protein tertiary structure, it also favoured the accessibility of Lys residues to lactose. As for glycation sites of β -LG, Lys69 seemed to be a specific modification site of the B variant (Losito, Stringano, Carulli, & Palmisano, 2010). Mono-, di- and tri-lactosylation of α -LA as well as mono- and di-lactosylation of β -LG were also detected in raw water buffalo milk samples by LC-ESI-MS analyses (as already reported for bovine β -LG (Burr, Moore, & Hill, 1996; Morgan et al., 1999));

Lys47 and Lys69 were indicated as early glycation sites in β -LG (Ferranti, Mamone, Picariello, & Addeo, 2011).

Lactosylation of casein fraction was also studied. A thoroughly investigation was carried out by Scaloni et al. (2002), who integrated immunochemical and MS methodologies for the analysis of raw, pasteurized, UHT and sterilized milk samples. Based on relative intensity of signals in the ESI-MS transformed spectra, mono-lactosylated forms of α S1- and β -CN, accounting for 5% and 6% of total protein content, were detected in pasteurized milk samples. The presence of α S1- and β -CN di-lactosylated adducts in mass spectra obtained from the analysis of UHT and sterilized milk samples demonstrated a more pronounced extent of Maillard reaction, accordingly with the severity of thermal process. Analysis of tryptic and/or endoproteinase AspN digests of modified caseins from UHT and sterilized milk by MALDI-TOF-MS led to define lactosylation sites and to demonstrate that mono- and di-lactosylated α S1- and β -CN existed as a population of different isomers originated from a non-site-specific reaction involving seven out of fourteen and five out of eleven Lys residues present in α S1- and β -CN, respectively. On the contrary, lactosylation occurred specifically at Lys34 of α S1-CN and Lys107 of β -CN in moderately heat-treated milks. More recently, an alternative method was set up that involves native size-exclusion chromatography coupled on-line with ESI-MS; it allowed the analysis of the whole protein milk fraction and the detection of lactosylated forms of whey proteins as well as of A1 and A2 variants of β -CN. This approach also gave information on the protein oligomeric state and aggregation in a single analysis (Johnson, Philo, Watson, & Mills, 2011).

3. PROTEIN LACTOSYLATION INVESTIGATED BY MALDI-TOF-MS

Due to its remarkable tolerance for contaminants and ability to analyse complex protein and peptide mixtures, MALDI-TOF-MS has been thoroughly applied for investigating protein lactosylation in heat treated milk samples. In fact, approaches based on MALDI-TOF-MS did not require HPLC separation of protein mixtures, thus allowing the direct analysis of milk polypeptide species. As for LC-ESI-MS analysis, whey proteins have been mainly regarded as suitable markers of thermal damage; therefore experimental protocols included defatting and casein precipitation before MALDI-TOF-MS analysis. In order to improve mass spectra quality and detection limits, a sample clean up step based on immobilized metal affinity chromatography (IMAC) has been recently introduced aimed at removing interfering compounds that hamper polypeptide ionization. These analyses led to define specific molecular profiles of milk protein fractions that could be related to technological thermal procedures (Meltretter, Birlouez-Aragon, Becker, & Pischetsrieder, 2009).

Pischetsrieder and coworkers particularly contributed to assess MALDI-TOF-MS as a simple and rapid method to detect various glycation and oxidation products as well as to identify modifications sites of whey proteins. First experiments were performed on model solutions of whey proteins simulating milk conditions in the course of thermal treatment (Meltretter, Seeber, Humeny, Becker, & Pischetsrieder, 2007). Analogous experiments were also performed to investigate the influence of the type of carbonyl group (aldose or ketose) of the sugar (galactose or tagatose) on the structural changes of whey proteins during non-enzymatic glycation (Corzo-Martinez, Moreno, Olano, & Villamiel, 2008). On the other hand, MALDI-TOF-MS analysis of commercial dairy products showed that the relative content of the Amadori product in α -LA and β -LG increased with prolonged heating and increased temperature. Although as minor components, mono-lactosylated forms of whey proteins were indeed detected in pasteurized and high temperature milk, while di-lactosylated forms were present in UHT milk samples. Moreover, tri- and quadruply-lactosylated forms of α -LA as well as forms modified by hexose addition were observed in liquid and powdered infant formulas (Meltretter, Birlouez-Aragon, Becker, & Pischetsrieder, 2009). An alternative analytical approach integrating SDS-PAGE, *in-gel* enzymatic digestion of proteins contained in bands of interest and MALDI-TOF-MS analysis of peptide mixtures was also proposed for the identification of lactosylation sites of β -LG (Meltretter, Becker, & Pischetsrieder, 2008).

Interestingly, Fenaille et al. (2006) used MALDI-TOF-MS to demonstrate that different kind of commercial infant formulas (liquids, powders and hypoallergenic powders) exhibited a different degree of α -LA glycation (up to eight lactose moieties); conversely, liquid infant formula showed a lower glycation extent (Fig. 4). The higher number of glycoforms observed in powder samples, compared to liquid samples, could be related to the heating periods involved in the drying step and to the better efficiency of protein glycation at low water activities, as also clearly highlighted by experiments carried out on model systems (French, Harper, Kleinholz, Jones, & Green-Church, 2002). MALDI-TOF-MS has been also recently applied to the investigation of heat induced modifications of whey proteins in model systems mimicking lactose-free milk. In these products, also called high-digestibility milk products developed for lactose-intolerant patients, lactose was almost quantitatively converted into glucose and galactose by an enzymatic degradation process. This study clearly demonstrated a higher reactivity of glucose and galactose compared to lactose. In fact, the analysis of the intact α -LA revealed species with up to seven and fourteen adducts in lactose and sugar mix (hydrolysed lactose) containing solution, respectively, whereas for β -LG the addition of up to three and five sugar moieties was observed in the case of lactose and sugar mix experiments, respectively. However, a lower glycation degree was observed in a commercial UHT lactose-free milk sample (two and three hexose adducts for α -LA and β -LG, respectively), probably

due to the use of a different heating condition with respect to that used in the lactose-free model solution, and in addition milk components may exert a protective effect on whey proteins (Carulli, Calvano, Palmisano, & Pischetsrieder, 2011).

Very recently, MALDI-TOF-MS integrated with chromatography on hydroxyapatite, for the selective enrichment of phosphorylated proteins and peptides, has been applied to investigate lactosylation of whey proteins, caseins and, more interesting, casein phosphopeptides (CPP) in pasteurised and UHT milk, milk powder and milk protein powder (obtained by ultrafiltration and spray drying). Lactosylation was strictly related to the technological treatment, so that the extent of modification of whey proteins and caseins in milk powder was higher than in protein milk powder, probably due to the lower lactose content of the ultrafiltered milk or whey. Moreover, mono-lactosylated forms of CPP were identified in the analysed milk samples (except in raw and pasteurized ones), with α S2–CN(1-24)4P fragment, and β -CN(1-28)4P and (1-27)4P fragments being the main components. Lactosylated β -CN(1-28)4P fragment was tested as suitable signature peptide for the detection of UHT milk additions in raw and pasteurized milk, allowing the detection of 10% addition of UHT milk (Pinto et al., 2012).

4. PROTEOMICS FOR THE ANALYSIS OF PROTEIN LACTOSYLATION

To provide an overall picture of the modifications induced in milk proteins by thermal technological procedures, including their localization in the protein sequence, classical proteomic approaches integrating two-dimensional electrophoresis (2-DE), enzymatic *in gel* protein digestion and analysis of the obtained peptide mixtures by MALDI-TOF-MS or tandem mass spectrometry were applied. A first proteomic study on commercial milk powder led to a reliable identification of single and multiple lactose-conjugates of caseins, α -LA and β -LG (Galvani, Hamdan, & Righetti, 2000). Similarly, 2-DE and nanoESI-MS/MS was applied to resolve the lactosylation pattern of milk proteins purified from infant formula powders. Interestingly, neutral loss MS/MS experiments were carried out to selectively detect lactosylated peptides. Up to ten lactosylation sites were identified in β -LG and α S2-CN, five modification sites in α S1- and β -CN and four in κ -CN. α -LA, with five lactosylated peptides, was regarded as potential marker in milk powder sample to detect chemical modification induced by the processing/storage conditions (Marvin, Parisod, Fay, & Guy, 2002). Very recently, Holland, Gupta, Deeth, & Alewood (2011) carried out a proteomic analysis of the protein fraction purified from UHT milk samples stored at three different temperatures (4°C, 28°C and 40°C) for two months. 2-DE maps of samples stored at higher temperatures were characterized by an increasing number of "stacked spots". MS analysis led to identify lactosylated forms of whey proteins in these spots and revealed modification of nine out of fifteen and eight out

of twelve Lys residues in β -LG and α -LA, respectively, thus highlighting a strict relation between the extent of lactosylation and storage temperature. No information was achieved for other milk protein components. A similar link between extent of lactosylation and storage conditions (temperature, relative humidity and time) was observed in milk protein concentrates by using proteomic techniques. α -LA was selected as suitable marker; this protein was found to be modified by as many as five lactose moieties on eight different Lys residues (Le, Deeth, Bhandari, Alewood, & Holland, 2012).

An analytical strategy based on 2-DE and MALDI-TOF-MS has been very recently developed for the detection of milk adulteration, in particular for monitoring the addition of milk powered derivatives to commercial milk samples (pasteurized and UHT). Proteomic analyses were initially performed in order to define lactosylated peptides specifically found in milk powder that could be regarded as biomarkers of adulteration. Direct MALDI-TOF-MS analysis of the tryptic peptide mixtures of whey protein and casein fractions from intentionally adulterated milk samples allowed to monitor these diagnostic peptides and to detect it down to 1% of adulteration level (Calvano, Monopoli, Loizzo, Faccia, & Zambonin, 2012),

Very recently, integrated shotgun proteomic procedures were developed in our laboratory for global characterization of the various lactosylation protein targets in different commercial milk products, i.e. raw, pasteurized, UHT and powdered for infant nutrition (Arena et al., 2010; Arena, Renzone, Novi, & Scaloni, 2011). To this purpose, soluble milk proteins, soluble milk proteins enriched for less abundant components by the ProteoMiner™ combinatorial hexapeptide ligand library technology (D'Amato et al., 2009), or protein components from purified milk fat globule (MFG) particles were resolved by SDS-PAGE. In the second case, the ProteoMiner™ technology was used depending on its effective equalization outcome when applied on protein mixtures in which analytes present at very different concentrations occur together. Slices from whole SDS-PAGE lanes were then trypsinolyzed and/or digested with endoprotease LysC, and extracted protein digests were enriched for lactosylated peptides by affinity chromatography on *m*-aminophenylboronic acid covalently attached to insoluble agarose material (Fig. 5A). This support was previously used to perform proteomic profiling studies on non-enzymatically glycosylated proteins from human serum and erythrocyte samples (Lee, Kim, Ha, Lee, & Choo, 2005; Gontarev, Shmanai, Frey, Kvach, & Schweigert, 2007; Frolov, & Hoffman, 2008; Zhang et al., 2007a; Zhang et al., 2008a; Zhang et al., 2008b); its application in enriching glycosylated peptides directly from whole protein digests allowed identifying modified proteins present even at low-abundance levels. Comparative experiments with different proteases demonstrated that trypsin can be efficiently used in high-throughput shotgun studies, although endoprotease LysC also worked properly to this

purpose (Zhang et al., 2008b). Thus, we used the boronic acid-functionalized resin to directly bind to *cis*-diol groups on lactosylated peptides under alkaline conditions to form a reversible five-member ring complex (Fig. 5B), whereas most of the non-modified species were washed free (Arena et al., 2010; Arena, Renzone, Novi, & Scaloni, 2011). In the case of lactosylated species, diol functionalities were present both on glucose and galactose cyclic structures. Bound lactosylated peptides were then eluted by washing with volatile acids to reduce salt residues within samples. As an example, Figure 6 shows the MALDI-TOF mass spectrum profiles of peptide mixtures obtained from gel slice protein digests deriving from fat globules of raw, pasteurized, UHT and powdered milk, respectively, after phenylboronic acid affinity trapping (Arena, Renzone, Novi, & Scaloni, 2011). An increasing signal complexity due to enriched lactosylated peptides was observed in the recorded mass spectra; their measured signal intensity well paralleled with milk thermal treatment. Signal number/intensity of the modified species (with respect to that of the unmodified ones) proved the efficacy of using *m*-aminophenylboronic acid-agarose to enrich lactosylated peptides directly from whole protein digests.

In order to directly characterize lactosylated peptides, simultaneously providing information on the nature of the protein modified and the tagged amino acid(s), labelled peptide mixtures eluted from phenylboronate chromatography were analyzed by performing nanoLiquid Chromatography-Electrospray Ionization-Linear Ion Trap-Tandem Mass Spectrometry (nLC-ESI-LIT-MS/MS) experiments with Electron Transfer Dissociation (ETD) fragmentation (Arena et al., 2010; Arena, Renzone, Novi, & Scaloni, 2011). This experimental set up was also successfully used to identify modification sites in digests of non-enzymatically glycosylated proteins from human serum/erythrocytes (Zhang et al., 2007a, Zhang et al., 2007b, Zhang et al., 2008a, Zhang et al., 2008b). The use of supplemental collisional activation after electron transfer allowed increasing the number of modified peptides identified in shotgun experiments. We and others verified that collision induced dissociation (CID) fragmentation of glycosylated peptides generates mass spectra characterized by non sequence-informative ion species associated with the cleavage of the glycosidic bond (Mollé, Morgan, Bouhallab, & Léonil, 1998; Zhang et al., 2007a, Zhang et al., 2007b; Arena et al., 2010), bearing neutral loss of various H₂O molecules (in particular, the furylium ion), and rare b- and y-ions. Accordingly, lactosylated peptides from soluble milk proteins were poorly identified with confidence in the course of preliminary CID experiments (Arena et al., 2010). For example, Figure 7 shows the MS/MS spectra of the $[M+4H]^{4+}$ ion corresponding to the di-lactosylated peptide (78-100) from β -LGB obtained under CID and ETD conditions. Under CID fragmentation (Fig. 7A), sequence-informative b- and y-ions resulting from peptide backbone cleavage were rare in the mass spectra and poorly identified with confidence. The most abundant ions were associated with the

cleavage of the glycosidic bond, with retention of the glycosidic O atom by the species that carries the reducing end, plus neutral losses of various molecules of H₂O. Losses of one to four molecules of H₂O from glycosylated peptides during MS/MS have been well documented in the literature (Zhang et al., 2007b). Worth mentioning was the relative abundance of the furylium ion under CID conditions. This ion, associated with the loss of a galactose unit plus two H₂O molecules from the parent ion (Fig. 2), has been previously described in MS/MS studies on lactose and glucose-modified peptides (Mollé, Morgan, Bouhallab, & Léonil, 1998; Zhang et al., 2007a, Zhang et al., 2007b). Neutral loss pattern of variable number of water molecules and formation of the furylium ion were generally charge-independent, as already reported. When the [M+4H]⁴⁺ ion from the same peptide was selected as precursor for analysis by ETD, a series of abundant c- and z-type ions were detected (Fig. 7B). Contrary to what was observed under CID conditions, neither furylium ions nor ions corresponding to neutral losses of H₂O from the lactose modification site were observed, clearly demonstrating that the Amadori moiety is stable under ETD conditions and bond cleavage is less dependent on peptide composition and side-chain modification(s). The abundance and the almost completeness of the c- and z-type ion series observed, regardless of whether the lactosylation site was located in the middle of the sequence or close to the N-terminus, greatly facilitated peptide sequencing and modification site assignment. In order to increase the number of lactosylated peptides characterized, each sample was subjected to a first nLC-ESI-LIT-MS/MS analysis, where data-dependent product ion scanning procedure over the 5 most abundant ions and subsequent ETD fragmentation of peptide precursor ions were performed; then, a second analysis was executed on the same sample with the same acquisition method, but excluding fragmentation of the peptides confidently identified in the first run. As already reported for proteins subjected to glucose addition, most of the sequence-informative ETD spectra derived from fragmentation of triply and quadruply charged ions. These results were in good agreement with previous data from proteomic characterization of glucosylated proteins in human serum/erythrocyte samples (Zhang et al., 2007a, Zhang et al., 2008a, Zhang et al., 2008b) and definitively confirmed ETD as the most efficient fragmentation mode for the analysis of non-enzymatically glycosylated proteins (Zhang et al., 2007b, Zhang et al., 2008b).

When applied to soluble milk fraction or soluble fraction enriched for less abundant components by ProteoMiner™ technology, this shotgun strategy allowed the identification of 271 non-redundant lactosylation sites in 33 proteins (Arena et al., 2010). Most of these protein species escaped to detection by Western blotting following 2-DE analysis of skimmed milk or whey samples (Marvin, Parisod, Fay, & Guy, 2002). Similarly, 157 novel non-redundant modification sites and 35 MFG proteins never reported so far as being lactosylated were identified in protein components from

purified fat globules (Arena, Renzone, Novi, & Scaloni, 2011), in addition to the 153 ones present in other 21 proteins detected as adsorbed on this fatty fraction, which were already characterized in the soluble milk portion (Arena et al., 2010). Shotgun analysis allowed the characterization of a variable number of modification sites within each protein; their number increased with harder milk processing conditions. In fact, a significant protein lactosylation was observed in UHT and powdered for infant nutrition milk preparations, which well paralleled with the harshness of thermal treatment. In addition, our analysis showed a good reproducibility of the lactosylation data, since modified sites ascertained in pasteurized milk were also present in UHT and powder milk samples; similarly, lactosylation sites observed in the UHT milk were generally conserved in powdered milk. Exceptions were due to protein compositional changes in milk samples depending on the technological processes used for their productions. As expected, most of the lactosylation sites were identified in abundant milk proteins, such as β -LG, α -LA, α S1-, α S2-, β - and κ -CN, glycosylation-dependent cell adhesion molecule 1, lactotransferrin, xanthine dehydrogenase, peripilin 2, lactadherin, fatty acid binding protein and lactoperoxidase, which account for more than 95% of the total protein content in milk. A good qualitative agreement with previous studies on lactosylation sites in β -LG, α -LA, α S1- and β -CN (Morgan, Léonil, Mollé, & Bouhallab, 1997; Leonil et al., 1997; Fogliano et al., 1998; Siciliano, Rega, Amoresano, & Pucci, 2000; Scaloni et al., 2002; Fenaille, Morgan, Parisod, Tabet, & Guy, 2004; Meltretter, Becker, & Pischetsrieder, 2008) was observed. The widespread modification observed for proteins from UHT and powder milk samples perfectly matched with previous quantitative amino acid determination on whole protein hydrolysates, which demonstrated that about 15-20% of the Lys residues is blocked in the Amadori product (Henle, Walter, & Klostermeyer, 1991), and with MS analysis of intact β -LG, α -LA, α S1- and β -CN, which unveiled a population of un-, mono-, di- and tri-lactosylated species (Leoni et al., 1997; Fogliano et al., 1998; Siciliano, Rega, Amoresano, & Pucci, 2000; Scaloni et al., 2002; Czerwenka, Maier, Pittner, & Lindner, 2006). These researches emphasized a progressive decreased nutritional value of milk, as result of a diminished bioavailability of the essential amino acid lysine, which well paralleled the harshness of product heating (Rérat, Calmes, Vaissade, & Finot, 2002; Henle, 2007; Sebekova, & Somoza, 2007).

A global picture of the modification sites ascertained in lactosylated proteins from different milk preparations is reported in Table 1, which also shows the number of modified amino acids detected in each polypeptide chain with respect to the total number of Lys residues. Variable values were observed for the different milk proteins, with almost a third of the polypeptide species showing a widespread modification (a ratio value of more than 0.5). The observed difference between milk proteins may be ascribable to: i) a different reactivity of their Lys residues toward lactose, as result

of eventual ion pairing with acid or adjacent amino acids in the polypeptide three-dimensional arrangement (Fogliano et al., 1998); ii) a selective masking effect of other macromolecules within supramolecular structures (in micelle or fat globule assembling); iii) a diverse response of their structure to unfolding processes caused by thermal treatment (Boye, & Alli, 2000; de la Fuente, & Singh, 2002), which should result in a variable exposition of internal amino acids to lactosylation; iv) a dissimilar distribution of their concentration in the samples subjected to lactosylation analysis. In the latter case, the aminophenylboronic acid resin should have allowed preferential enrichment (and final detection) of lactosylated peptides from more abundant proteins, notwithstanding its modification degree and our attempt to compensate their wide dynamic concentration range by protein equalization or by limiting abundant proteins into specific gel portions to be further characterized for modification sites by independent experiments. Nevertheless, these studies provided the most detailed and wide inventory of lactosylated proteins present in raw, pasteurized, UHT and powdered milk for infant nutrition samples so far, also assigning modification sites (Arena et al., 2010, Arena, Renzone, Novi, & Scaloni, 2011). Their impact on technical and sensory properties of dairy products has to be further deeply investigated. On the other hand, this analysis did not provide quantitative results, thus missing information on preferential/more reactive amino acids in each protein, among the whole list of lactosylation sites assigned.

A visual inspection of the lactosylation sites within the available protein spectroscopic and crystallographic structures revealed that most of the modified amino acids occurred on the macromolecule surface, although some internal or substrate-binding regions seemed also affected (Arena et al., 2010). On this basis, the Maillard reaction should highly influence the three-dimensional structure of various milk proteins, as already investigated in model systems (Boye, & Alli, 2000; de la Fuente, & Singh, 2002), with significant consequences on their functional activity. Since it has been demonstrated that various milk components are resistant to proteolysis in the gastrointestinal tract (Sorva, Mäkinen-Kiljunen, & Juntunen-Backman, 1994; Castell, Friedrich, Kuhn, & Poppe, 1997; Lönnerdal, 2003; Moreno, Rubio, Olano, & Clemente, 2006), and directly should contribute to a number of biological activities within infant intestine or other organs (Lönnerdal, 2003; Gauthier, Pouliot, & Maubois, 2006; Jouan, Pouliot, Gauthier, & Laforest, 2006; Wheeler, Hodgkinson, Prosser, & Davis, 2007; Casado, Affolter, & Kussmann, 2009; D'Alessandro, Scaloni, & Zolla, 2010; D'Alessandro, Zolla, & Scaloni, 2011), their widespread lactosylation may have important consequences on the nutritional and health-beneficial characteristics of this aliment. In fact, we have demonstrated that milk proteins involved in the delivery of nutrients (lactotransferrin, apolipoprotein A-I, A-IV and E, α S1-, α S2-, β - and κ -CN, serum albumin, α -LA, β -LG, vitamin D-binding protein, secretoglobulin 1D2, perilipin, xanthine

dehydrogenase, butyrophilin, NPC2, and fatty acid-binding protein), the defence response against pathogens and related inflammatory processes (lactotransferrin, lactadherin, α -LA, κ -CN, polymeric Ig receptor, Ig light chain, Ig K, nucleobindin 1 and 2, clusterin, endopin 1b, serpin A1, A3-1, A3-4 and A3-7, lactoperoxidase, xanthine dehydrogenase, CD9, CD14, CD36, CD46, CRISP3, B2M, S100A9, S100A12, MGC137014 and serum amyloid A protein) and the regulation of cellular proliferative/differentiation events (osteopontin, fibroblast growth factor-binding protein 1, angiogenin, CDC42, CREG1, GTPase KRas, peptidyl-prolyl *cis-trans* isomerase A, glycosylation-dependent cell adhesion molecule 1 and Zn- α 2-glycoprotein) are modified as a consequence of a drastic heat treatment. Thus, thermal processing may eventually determine a reduced function of the proteins mentioned above. This issue could have important consequences when milk is the unique source of nutrients, as in infant diet (R  rat, Calmes, Vaissade, & Finot, 2002; Henle, 2007; Sebekova K, & Somoza, 2007).

Since lactosylated lysines are no longer recognized by proteolytic enzymes, the Maillard reaction may also seriously affect gastrointestinal digestion of milk proteins (Dalsgaard, Nielsen, & Larsen, 2007). This issue was addressed by comparing the digestibility of the MFG proteins present in different commercial samples, under partially denaturing conditions and treatment with trypsin (Arena, Renzone, Novi, & Scaloni, 2011). Tricine-SDS-PAGE and SDS-PAGE analyses demonstrated an increasing resistance to proteolysis for pasteurized, UHT and powdered milk proteins, respectively, as proved by the progressive appearance/maintenance of high-molecular mass polypeptide bands in the corresponding digests. This reduced rate/efficiency of protein digestion well parallels with the increasing harshness of milk thermal processing and then to the corresponding polypeptide lactosylation extent. These results were in good agreement with previous studies on isolated β -LG samples preventively treated with galactose, tagatose or galacto-oligosaccharides, which were subjected to an *in vitro* simulated gastrointestinal digestion consisting of a treatment with pepsin in acid environment, followed by incubation with trypsin/chymotrypsin at a neutral pH value (Sanz, Corzo-Martinez, Rastall, Olano, & Moreno, 2007; Moreno, Quintanilla-Lopez, Lebron-Aguillar, Olano, & Sanz, 2008; Corzo-Martinez, Lebron-Aguillar, Villamiel, Quintanilla-Lopez, & Moreno, 2009; Corzo-Martinez, Soria, Belloque, Villamiel, & Moreno, 2010). Not considering possible physiological implications on assumption of poorly-digestible foods, this point has a first important significance for some soluble milk proteins that, after an impaired proteolysis, may present a reduced release of sequence-encrypted peptides with antimicrobial, opioid, antihypertensive and antithrombotic properties (Clare, & Swaisgood, 2000; Meisel, 2005; Haque, & Chand, 2008). Examples in this context are: i) β -LG-derived antimicrobial (15-20), (78-83) and (92-100) peptides, ACE inhibitor β -lactorphin (142-148) and opioid (102-105)

peptides; ii) α -LA-derived antimicrobial (1-5) and (17-31)S-S(109-114) peptides; iii) α S2-CN-derived antimicrobial casocidin I (150-188) peptide and ACE peptide inhibitors (189-192) and (198-202); iv) κ -CN-derived antithrombotic (112-116) and opioid casoxin C (25-34) peptides; v) β -CN-derived ACE inhibitor β -casokin 7 (177-183); vi) α S1-CN-derived ACE peptide inhibitors (23-34), (104-109) and (194-199). Also in this case, a reduced biological activity of these components may also be the consequential effect of protein lactosylation. Since some of these proteins were found as highly adsorbed on fat globules following milk homogenization/heating procedures, the beneficial effects of their occurrence in cream products should be hampered by the harshness of food thermal treatment (Michalski, & Januel, 2006), which may limit their digestion as result of a widespread lactosylation. On the other hand, a reduced digestibility of lactosylated milk proteins may also determine an increased allergenic response to specific dairy products; in fact, it has been reported that proteins resistant to digestion may behave as major food allergens (Astwood, Leach, & Fuchs, 1996; Wal, 2001). Extensive lactosylation of surface-exposed lysines in specific milk proteins should also *per se* affect the allergenic impact of these nutrients; in fact, lactose moieties introduced on protein structure after drastic heating have been described acting as hapten-like antigens (Karamanova et al., 2004; Gruber, Vieths, Wangorsch, Nerkamp, & Hofmann, 2004). In this perspective, preliminary evidences have recently been reported for β -LG treated with galactose or tagatose and the corresponding hydrolysates, which were both assayed for their IgG- and IgE-reactivities (Corzo-Martinez, Soria, Belloque, Villamiel, & Moreno, 2010),

5. CONCLUSIONS

Advanced mass spectrometric and proteomic technologies significantly contributed to draw a thorough and detailed picture of lactosylation of milk proteins, further confirming that Maillard reaction can modify a large number of Lys residues in several proteins. Based on assumption that a limited lactosylation has little or no effect on the ionization potential of proteins and that the distribution profile of molecular ion peaks of different glycosylated forms in a mass spectrum reflect their solution composition in a mixture, different ESI- and MALDI-based studies provided information on the occurrence of lactosylated proteins in milk products and on the semiquantitative estimation of their modification degree (Siciliano, Rega, Amoresano, & Pucci, 2000; Yeboah, & Yaylayan, 2001; Losito, Carbonara, Monaci, & Palmisano, 2007; Meltretter, Birlouez-Aragon, Becker, & Pischetsrieder, 2009; Carulli, Calvano, Palmisano, & Pischetsrieder, 2011). Accordingly, various MS-based analytical strategies have been often proposed as rapid and reliable tools for routinely monitoring milk adulterations or illegal practices but, to the best of our knowledge, only few recent studies truly addressed this issue (Calvano, Monopoli, Loizzo, Faccia, & Zambonin,

2012; Pinto et al., 2012). The application of these methods to the analysis of a wider range of dairy products is mandatory for their validation as official methods to control milk quality. However, since all these investigations utilized MS-based procedures for the detection of specific lactosylated derivatives in various untreated or thermally-treated milk samples and their mixtures, without using any labelled internal standard compound having identical ionization properties, we believe that the problem of the absolute quantification of the Amadori products in dairy products has not been sufficiently solved in all its features. In this context, the development of dedicated LC-ESI-MS-based approaches also based on the use of labelled proteotypic peptides have to be strongly encouraged; in a next future, these approaches will definitively prevail over the methods mentioned above or the outdated procedures measuring lactosyl-lysine and lactulosyl-lysine in protein hydrolysates (Henle, Walter, & Klostermeyer, 1991; Pischetsrieder, & Henle, 2012). In addition, no data have been reported so far on the quantitative evaluation of glycation in minor protein components from milk samples submitted to different technological treatments; therefore, information on more reactive amino acids in these proteins are still missing. Future studies applying MS procedures on isolated protein components treated *in vitro* with unlabelled/labelled lactose mixtures or quantitative shotgun proteomic approaches on differently treated milk samples should be advisable to gain information on the Lys residues more prone to react with sugars, also in these less abundant proteins. The themes described above and those associated with further oxidative decomposition of lactosylated proteins, or other carbonylation reactions observed during industrial milk treatment, which determine the addition of advanced lipo-oxidation end-products on proteins or the generation of additional advanced glyco-oxidation products and oxidized amino acids (Henle, Schwarzenbolz, & Klostermeyer, 1997; Drusch, Faist, & Erbersdobler, 1999; Pischetsrieder, Gross, & Schoetter, 1999; Birlouez-Aragon et al., 2004; Fenaille, Parisod, Tabet, & Guy, 2005; Michalski, Calzada, Makino, Michaud, & Guichardant, 2008; Meyer, Al-Diab, Vollmer, & Pischetrieder, 2011; Pischetsrieder, & Henle, 2012), have to be evaluated in a next future for a global estimation of the nutritional and toxicological properties of milk products deriving from severe heat treatments.

ACKNOWLEDGMENTS

This work was supported by funds from the Italian Ministry of Economy and Finance to CNR and ENEA for the project "Innovazione e sviluppo del Mezzogiorno - Conoscenze Integrate per Sostenibilità ed Innovazione del Made in Italy Agroalimentare" - Legge n.191/2009, and from Regione Campania for Rete di Spettrometria di Massa della Campania (RESMAC).

REFERENCES

- Arena, S., Renzone, G., Novi, G., Paffetti, A., Bernardini, G., Santucci, A., & Scaloni, A. (2010). Modern proteomic methodologies for the characterization of lactosylation protein targets in milk. *Proteomics*, 10, 3414-3434.
- Arena, S., Renzone, G., Novi, G., & Scaloni, A. (2011). Redox proteomics of fat globules unveils broad protein lactosylation and compositional changes in milk samples subjected to various technological procedures. *Journal of Proteomics*, 74, 2453-2475.
- Astwood, J. D., Leach, J. N., & Fuchs, R. L. (1996). Stability of food allergens to digestion in vitro. *Nature Biotechnology*, 14, 1269-1273.
- Birlouez-Aragon, I., Pischetsrieder, M., Leclerc, J., Morales, F. J., Hasenkopf, K., Kientsch-Engel, R., Ducauze, C. J., & Rutledge, D. (2004). Assessment of protein glycation markers in infant formulas. *Food Chemistry*, 87, 253-259.
- Boye, J. I., & Alli, I. (2000). Thermal denaturation of mixtures of β -lactalbumin and α -lactoglobulin: a differential scanning calorimetric study. *Food Research International*, 33, 673-682.
- Burr, R., Moore, C. H., & Hill, J. P. (1996). Evidence of multiple glycosylation of bovine β -lactoglobulin by electrospray ionisation mass spectrometry. *Milchwissenschaft*, 51, 488-492.
- Calvano, C. D., Monopoli, A., Loizzo, P., Faccia, M., & Zambonin, C. (2012). Proteomic approach based on MALDI-TOF MS to detect powdered milk in fresh cow's milk. *Journal of Agricultural and Food Chemistry*, in press. dx.doi.org/10.1021/jf302999s.
- Carulli, S., Calvano, C. D., Palmisano, F., & Pischetsrieder, M. (2011). MALDI-TOF-MS characterization of glycation products of whey proteins in a glucose/galactose model system and lactose-free milk. *Journal of Agricultural and Food Chemistry*, 59, 1793-1803.
- Casado, B., Affolter, M., & Kussmann, M. (2009). OMICS-rooted studies of milk proteins, oligosaccharides and lipids. *Journal of Proteomics*, 73, 196-208.
- Castell, J. V., Friedrich, G., Kuhn, C. S., & Poppe, G. E. (1997). Intestinal absorption of undegraded proteins in men: presence of bromelain in plasma after oral intake. *American Journal of Physiology*, 273, G139-146.
- Chevalier, F., Chobert, J. M., Mollè, D., & Haertlé, T. (2001)a. Maillard glycation of β lactoglobulin with several sugars: Comparative study of the properties of the obtained polymers and of the substituted sites. *Lait*, 81, 651-666.
- Chevalier, F., Chobert, J. M., Popineau, Y., Nicolas, M. G., & Haertlé, T. (2001)b. Improvement of functional properties of β -lactoglobulin glycosylated through the Maillard reaction is related to the nature of the sugar. *International Dairy Journal*, 11, 145-152.

- Chobert, J. M., Gaudin, J. C., Dalgarrondo, M., & Haertlé, T. (2006). Impact of Maillard type glycation on properties of beta-lactoglobulin. *Biotechnology Advances* 24, 629-632.
- Clare, D. A., & Swaisgood, H. E. (2000). Bioactive milk peptides: a prospectus. *Journal of Dairy Science*, 83, 1187-1195.
- Corzo-Martínez, M., Moreno, F. J., Olano, A., & Villamiel, M. (2008). Structural characterization of bovine beta-lactoglobulin-galactose/tagatose Maillard complexes by electrophoretic, chromatographic, and spectroscopic methods. *Journal of Agricultural and Food Chemistry*, 56, 4244-4252.
- Corzo-Martínez, M., Lebrón-Aguilar, R., Villamiel, M., Quintanilla-López, J. E., & Moreno, F. J. (2009). Application of liquid chromatography-tandem mass spectrometry for the characterization of galactosylated and tagatosylated beta-lactoglobulin peptides derived from in vitro gastrointestinal digestion. *Journal of Chromatography A*, 1216, 7205-7212.
- Corzo-Martínez, M., Soria, A.C., Belloque, J., Villamiel, M., & Moreno, F. J. (2010). Effect of glycation on the gastrointestinal digestibility and immunoreactivity of bovine beta-lactoglobulin. *International Dairy Journal*, 20, 742-752.
- Cunsolo, V., Muccilli, V., Saletti, R., & Foti, S. (2011). Applications of mass spectrometry techniques in the investigation of milk proteome. *European Journal of Mass Spectrometry (Chichester, Eng)*, 17, 305-320.
- Czerwenka, C., Maier, I., Pittner, F., & Lindner, W. (2006). Investigation of the lactosylation of whey proteins by liquid chromatography-mass spectrometry. *Journal of Agricultural and Food Chemistry*, 54, 8874-8882.
- Dalgarrondo, M., Chobert, J. M., Dufour, E., Bertrand-Harb, C., Dumont, J. P., & Haertlé, T. (1990). Influence of pH on the structural changes of β -lactoglobulin studied by tryptic hydrolysis. *Biochimica et Biophysica Acta*, 1077, 31-34.
- D'Alessandro, A., Scaloni, A., & Zolla, L. (2010). Human milk proteins: an interactomics and updated functional overview. *Journal of Proteome Research*, 9, 3339-3373.
- D'Alessandro, A., Zolla, L., & Scaloni, A. (2011). The bovine milk proteome: cherishing, nourishing and fostering molecular complexity. An interactomics and functional overview. *Molecular BioSystems*, 7, 579-597.
- Dalsgaard, T. K., Nielsen, J. H., & Larsen, L. B. (2007). Proteolysis of milk proteins lactosylated in model systems. *Molecular Nutrition & Food Research*, 51, 404-414.
- D'Amato, A., Bachi, A., Fasoli, E., Boschetti, E., Peltre, G., S  n  chal, H., & Righetti, P. G. (2009). In-depth exploration of cow's whey proteome via combinatorial peptide ligand libraries. *Journal of Proteome Research*, 8, 3925-3936.

- Darewicz, M., & Dziuba, J. (2001). The effect of glycosylation on emulsifying and structural properties of bovine β -casein. *Nahrung*, 45, 15-20.
- de la Fuente, M. A., & Singh, H. H. Y. (2002). Recent advances in the characterisation of heat induced aggregates and intermediates of whey proteins. *Trends in Food Science & Technology*, 13, 262-274.
- Drusch, S., Faist, V., & Erbersdobler, H. F. (1999). Determination of N(epsilon)-carboxymethyllysine in milk products by a modified reversed-phase HPLC method. *Food Chemistry*, 65, 547-553.
- Fenaille, F., Morgan, F., Parisod, V., Tabet, J. C., & Guy, P. A. (2004). Solid-state glycation of beta-lactoglobulin by lactose and galactose: localization of the modified amino acids using mass spectrometric techniques. *Journal of Mass Spectrometry*, 39, 16-28.
- Fenaille, F., Parisod, V., Tabet, J. C., & Guy, P. A. (2005). Carbonylation of milk powder proteins as a consequence of processing conditions. *Proteomics*, 5, 3097-3104.
- Fenaille, F., Parisod, V., Visani, P., Populaire, S., Tabet, J. C., & Guy, P. A. (2006). Modifications of milk constituents during processing, a preliminary benchmarking study. *International Dairy Journal*, 16, 728-739.
- Fenn, J. B., Mann, M., Meng, C. K., Wong, S. F., & Whitehouse C. M. (1989). Electrospray ionization for mass spectrometry of large biomolecules. *Science*, 246, 64-71.
- Ferranti, P., Mamone, G., Picariello, G., & Addeo, F. (2011). The "dark side" of β -lactoglobulin: unedited structural features suggest unexpected functions. *Journal of Chromatography A*, 1218, 3423-3431.
- Finot, P. A. (1990). Metabolism and physiological effects of Maillard reaction products. In P. A. Finot, H. Aeselbacher, R. F. Hurrell, R. Liardon (Eds.), *The Maillard Reaction in Food Processing, Human Nutrition and Physiology* (pp 259-27) Birkhaeuser, Boston, MA.
- Fogliano, V., Monti, S. M., Visconti, A., Randazzo, G., Facchiano, A. M., Colonna, G., Ritieni, A. (1998). Identification of a β -lactoglobulin lactosylation site. *Biochimica et Biophysica Acta*, 1388, 295-304.
- French, S. J., Harper, W. J., Kleinholz, N. M., Jones, R. B., & Green-Church, K. B. (2002). Maillard reaction induced lactose attachment to bovine beta-lactoglobulin, electrospray ionization and matrix-assisted laser desorption/ionization examination. *Journal of Agricultural and Food Chemistry*, 50, 820-823.
- Frolov, A., & Hoffmann, R. (2008). Analysis of amadori peptides enriched by boronic acid affinity chromatography. *Annals of the New York Academy of Sciences*, 1126, 253-256.

- Galvani, M., Hamdan, M., & Righetti, P.G. (2000). Two-dimensional gel electrophoresis/matrix-assisted laser desorption/ionisation mass spectrometry of a milk powder. *Rapid Communications in Mass Spectrometry*, 14, 1889-1897.
- Gauthier, S. F., Pouliot, Y., & Maubois, J. L. (2006). Growth factors from bovine milk and colostrum: composition, extraction and biological activities. *Lait*, 86, 99-125.
- Gontarev, S., Shmanai, V., Frey, S. K., Kvach, M., & Schweigert, F. J. (2007). Application of phenylboronic acid modified hydrogel affinity chips for high-throughput mass spectrometric analysis of glycosylated proteins. *Rapid Communications in Mass Spectrometry*, 21, 1-6.
- Gruber, P., Vieths, S., Wangorsch, A., Nerkamp, J., & Hofmann T. (2004). Maillard reaction and enzymatic browning affect the allergenicity of Pru av 1, the major allergen from cherry (*Prunus avium*). *Journal of Agricultural and Food Chemistry*, 52, 4002-4007.
- Guy, P. A., & Fenaille, F. (2006). Contribution of mass spectrometry to assess quality of milk-based products. *Mass Spectrometry Reviews*, 25, 290-326.
- Haque, E., & Chand, R. (2008). Antihypertensive and antimicrobial bioactive peptides from milk proteins. *European Food Research and Technology*, 227, 7-15.
- Hau, J., & Bovetto, L. (2001). Characterisation of modified whey protein in milk ingredients by liquid chromatography coupled to electrospray ionisation mass spectrometry. *Journal of Chromatography A*, 926, 105-112.
- Henle, T., Walter, H., & Klostermeyer, H. (1991). Evaluation of the extent of the early Maillard-reaction in milk products by direct measurement of the Amadori-product lactulosyllysine. *Zeitschrift für Lebensmittel-Untersuchung und -Forschung A*, 193, 119-122.
- Henle, T., Schwarzenbolz, U., & Klostermeyer, H. (1997). Detection and quantification of pentosidine in foods. *Zeitschrift für Lebensmittel-Untersuchung und -Forschung A*, 204, 95-98.
- Henle, T. (2007). Dietary advanced glycation end products-a risk to human health? A call for an interdisciplinary debate. *Molecular Nutrition & Food Research*, 51, 1075-1078.
- Holland, J. W., Gupta, R., Deeth, H. C., & Alewood, P. F. (2011). Proteomic analysis of temperature-dependent changes in stored UHT milk. *Journal of Agricultural and Food Chemistry*, 59, 1837-1846.
- Johnson, P., Philo, M., Watson, A., & Mills, E. N. (2011). Rapid fingerprinting of milk thermal processing history by intact protein mass spectrometry with nondenaturing chromatography. *Journal of Agricultural and Food Chemistry*, 59, 12420-12427.
- Jouan, P. N., Pouliot, Y., Gauthier, S. F., & Laforest, J. P. (2006). Hormones in bovine milk and milk products: A survey. *International Dairy Journal*, 16, 1408-1414.

- Karamanova, L., Fukal, L., Kodicek, M., Rauch, P., Mills, E., & Morgan, M. (2003). Immunoprobosc for thermally-induced alteration in whey protein structure and their application to the analysis of thermally-treated milks. *Food and Agricultural Immunology*, 15, 77-91.
- Karas, M., & Hillenkamp, F. (1988). Laser desorption ionization of proteins with molecular masses exceeding 10,000 daltons. *Analytical Chemistry*, 60, 2299-2301.
- Le, T. T., Deeth, H. C., Bhandari, B., Alewood, P. F., & Holland, J. W. (2012). A proteomic approach to detect lactosylation and other chemical changes in stored milk protein concentrate. *Food Chemistry*, 132, 655-662.
- Lee, J. H., Kim, Y., Ha, M. Y., Lee, E. K., & Choo, J. (2005). Immobilization of aminophenylboronic acid on magnetic beads for the direct determination of glycoproteins by matrix assisted laser desorption ionization mass spectrometry. *Journal of the American Society for Mass Spectrometry*, 16, 1456-1460.
- Leonil, J., Molle, D., Fauquant, J., Maubois, J. L., Pearce, R. J., & Bouhallab, S. (1997). Characterization by ionization mass spectrometry of lactosyl β -lactoglobulin conjugates formed during heat treatment of milk and whey and identification of one lactose-binding site. *Journal of Dairy Science*, 80, 2270-2281.
- Lönnerdal, B. (2003). Nutritional and physiologic significance of human milk proteins. *American Journal of Clinical Nutrition*, 77, 1537S-1543S.
- Losito, I., Carbonara, T., Monaci, L., & Palmisano, F. (2007). Evaluation of the thermal history of bovine milk from the lactosylation of whey proteins, an investigation by liquid chromatography-electrospray ionization mass spectrometry. *Analytical and Bioanalytical Chemistry*, 389, 2065-2074.
- Losito, I., Stringano, E., Carulli, S., & Palmisano, F. (2010). Correlation between lactosylation and denaturation of major whey proteins, an investigation by liquid chromatography-electrospray ionization mass spectrometry. *Analytical and Bioanalytical Chemistry*, 396, 2293-2306.
- Marvin, L. F., Parisod, V., Fay, L. B., & Guy, P. A. (2002). Characterization of lactosylated proteins of infant formula powders using two-dimensional gel electrophoresis and nanoelectrospray mass spectrometry. *Electrophoresis*, 23, 2505-2512.
- Mauron, J. (1981). The Maillard reaction in food, a critical review from the nutritional standpoint. *Progress in Food and Nutrition Science*, 5, 5-35.
- Mauron, J. (1990). Influence of processing on protein quality. *Journal of Nutritional Science and Vitaminology*, 36, S57-69.
- Meisel, H. (2005). Biochemical properties of peptides encrypted in bovine milk proteins. *Current Medicinal Chemistry*, 12, 1915-1919.

- Meltretter, J., Seeber, S., Humeny, A., Becker, C. M., & Pischetsrieder, M. (2007). Site-specific formation of Maillard, oxidation, and condensation products from whey proteins during reaction with lactose. *Journal of Agricultural and Food Chemistry*, 55, 6096-6103.
- Meltretter, J., Becker, C. M., & Pischetsrieder, M. (2008). Identification and site-specific relative quantification of beta-lactoglobulin modifications in heated milk and dairy products. *Journal of Agricultural and Food Chemistry*, 56, 5165-5171.
- Meltretter, J., Birlouez-Aragon, I., Becker, C. M., & Pischetsrieder, M. (2009). Assessment of heat treatment of dairy products by MALDI-TOF-MS. *Molecular Nutrition & Food Research*, 53, 1487-1495.
- Meyer, B., Al-Diab, D., Vollmer, G., Pischetsrieder, M. (2011). Mapping the glycooxidation product N^ε-carboxymethyllysine in the milk proteome. *Proteomics*, 11, 420-428.
- Michalski, M. C., & Januel, C. (2006). Does homogenization affect the human health properties of cow's milk? *Trends in Food Science & Technology*, 17, 423-437.
- Michalski, M. C., Calzada, C., Makino, A., Michaud, S., & Guichardant, M. (2008). Oxidation products of polyunsaturated fatty acids in infant formulas compared to human milk-a preliminary study. *Molecular Nutrition & Food Research*, 52, 1478-1485.
- Mollé, D., Morgan, F., Bouhallab, S., & Léonil J. (1998). Selective detection of lactolated peptides in hydrolysates by liquid chromatography/electrospray tandem mass spectrometry. *Analytical Biochemistry*, 259, 152-161.
- Monaci, L., & van Hengel, A. J. (2007). Effect of heat treatment on the detection of intact bovine beta-lactoglobulins by LC mass spectrometry. *Journal of Agricultural and Food Chemistry*, 55, 2985-2992.
- Moreno, F. J., Rubio, L. A., Olano, A., & Clemente, A. (2006). Uptake of 2S albumin allergens, Ber e 1 and Ses i 1, across human intestinal epithelial Caco-2 cell monolayers. *Journal of Agricultural and Food Chemistry*, 54, 8631-8639.
- Moreno, F. J., Quintanilla-López, J. E., Lebrón-Aguilar, R., Olano, A., & Sanz, M. L. (2008). Mass spectrometric characterization of glycated beta-lactoglobulin peptides derived from galacto-oligosaccharides surviving the in vitro gastrointestinal digestion. *Journal of the American Society for Mass Spectrometry*, 19, 927-937.
- Morgan, F., Léonil, J., Mollé, D., & Bouhallab, S. (1997). Nonenzymatic lactosylation of bovine beta-lactoglobulin under mild heat treatment leads to structural heterogeneity of the glycoforms. *Biochemical and Biophysical Research Communications*, 236, 413-417.

- Morgan, F., Bouhallab, S., Mollé, D., Henry, G., Maubois, J. L., & Leôn timer, J. (1998). Lactolation of β -lactoglobulin monitored by electrospray ionisation mass spectrometry. *International Dairy Journal*, 8, 95-98.
- Morgan, F., Mollé, D., Henry, G., Vénier, A., Léon timer, J., Peltre, G., Levieux, D., Maubois, J. L., & Bouhallab, S. (1999). Glycation of bovine β -lactoglobulin: Effect on the protein structure. *International Journal of Food Science and Technology*, 34, 429-435.
- Nacka, F., Chobert, J. M., Burova, T., Léon timer, J., & Haertlé, T. (1998). Induction of new physicochemical and functional properties by the glycosylation of whey proteins. *Journal of Protein Chemistry*, 17, 495-503.
- Nasirpour, A., Scher, J., & Desobry, S. (2006). Baby foods, formulations and interactions. *Critical Reviews in Food Science and Nutrition*, 46, 665-681.
- Oliver, C.M. (2011). Insight into the glycation of milk proteins: an ESI- and MALDI-MS perspective. *Critical Reviews in Food Science and Nutrition*, 51, 410-431.
- Pinto, G., Caira, S., Cuollo, M., Fierro, O., Nicolai, M. A., Chianese, L., & Addeo, F. (2012). Lactosylated casein phosphopeptides as specific indicators of heated milks. *Analytical and Bioanalytical Chemistry*, 402, 1961-1972.
- Pischetsrieder, M., Gross, U., & Schoetter, C. (1999). Detection of Maillard products of lactose in heated or processed milk by HPLC/DAD. *Zeitschrift für Lebensmittel-Untersuchung und -Forschung A*, 208, 172-177.
- Pischetsrieder, M., & Henle T. (2012). Glycation products in infant formulas: chemical, analytical and physiological aspects. *Amino Acids*, 42, 1111-1118.
- Rérat, A., Calmes, R., Vaissade, P., & Finot, P. A. (2002). Nutritional and metabolic consequences of the early Maillard reaction of heat treated milk in the pig. Significance for man. *European Journal of Nutrition*, 41, 1-11.
- Sanz, M. L., Corzo-Martínez, M., Rastall, R. A., Olano, A., & Moreno, F. J. (2007). Characterization and in vitro digestibility of bovine beta-lactoglobulin glycated with galactooligosaccharides. *Journal of Agricultural and Food Chemistry*, 55, 7916-7925.
- Scaloni, A., Perillo, V., Franco, P., Fedele, E., Froio, R., Ferrara, L., & Bergamo, P. (2002). Characterization of heat-induced lactosylation products in caseins by immunoenzymatic and mass spectrometric methodologies. *Biochimica et Biophysica Acta*, 1598, 30-39.
- Sebekova, K., & Somoza, V. (2007). Dietary advanced glycation endproducts (AGEs) and their health effects. *Molecular Nutrition & Food Research*, 51, 1079-1084.
- Siciliano, R., Rega, B., Amoresano, A., & Pucci P. (2000). Modern mass spectrometric methodologies in monitoring milk quality. *Analytical Chemistry*, 72, 408-415.

- Singh, H., & Waungana, A. (2001). Influence of heat treatment of milk on cheesemaking properties. *International Dairy Journal*, 11, 543-551.
- Sorva, R., Mäkinen-Kiljunen, S., & Juntunen-Backman, K. (1994). Beta-lactoglobulin secretion in human milk varies widely after cow's milk ingestion in mothers of infants with cow's milk allergy. *Journal of Allergy and Clinical Immunology*, 93, 787-792.
- Thomas, M. E. C., Scher, J., Desobry-Banon, S., & Desobry, S. (2004). Milk powders ageing: Effect on physical and functional properties. *Critical Reviews in Food Science and Nutrition*, 44, 297-322.
- Wal, J. M. (2001). Structure and function of milk allergens. *Allergy*, 56 Suppl 67, 35-38.
- Wang, Q., & Ismail B. (2012). Effect of Maillard-induced glycosylation on the nutritional quality, solubility, thermal stability and molecular configuration of whey protein. *International Dairy Journal*, 25, 112-122.
- Wheeler, T. T., Hodgkinson, A. J., Prosser, C. G., & Davis, S. R. (2007). Immune components of colostrum and milk-a historical perspective. *Journal of Mammary Gland Biology and Neoplasia*, 12, 237-247.
- Yeboah, F. K., & Yaylayan, V. A. (2001) Analysis of glycated proteins by mass spectrometric techniques: qualitative and quantitative aspects. *Nahrung*, 45, 164-171.
- Zhang, Q., Tang, N., Brock, J. W., Mottaz, H. M., Ames, J. M., Baynes, J. W., Smith, R. D., & Metz, T. O. (2007)a. Enrichment and analysis of nonenzymatically glycated peptides: boronate affinity chromatography coupled with electron-transfer dissociation mass spectrometry. *Journal of Proteome Research*, 6, 2323-2330.
- Zhang, Q., Frolov, A., Tang, N., Hoffmann, R., van de Goor, T., Metz, T. O., & Smith, R. D. (2007)b. Application of electron transfer dissociation mass spectrometry in analyses of non-enzymatically glycated peptides. *Rapid Communications in Mass Spectrometry*, 21, 661-666.
- Zhang, Q., Tang, N., Schepmoes, A. A., Phillips, L. S., Smith, R. D., & Metz, T. O. (2008)a. Proteomic profiling of nonenzymatically glycated proteins in human plasma and erythrocyte membranes. *Journal of Proteome Research*, 7, 2025-2032.
- Zhang, Q., Schepmoes, A. A., Brock, J. W., Wu, S., Moore, R. J., Purvine, S. O., Baynes, J. W., Smith, R. D., & Metz, T. O. (2008)b. Improved methods for the enrichment and analysis of glycated peptides. *Analytical Chemistry*, 80, 9822-9829.

Table 1. Lactosylation sites in soluble and fat globule proteins as isolated from different commercial milk samples and identified after phenyl boronic acid trapping and MS analysis of modified peptides. This table describes the nature and the number of the observed lactosylated Lys residues with respect to the total number of lysines in each polypeptide chain (Arena et al., 2010, 2011). No information on preferential modification sites is reported.

PROTEIN LACTOSYLATION SITES	LACTOSYLATED LYS/TOTAL LYS
P02754/ B5B0D4 - Beta-lactoglobulin K8, K14, K47, K60, K69, K70, K75, K77, K83, K91, K100, K101, K135, K138, K141	15/15
P00711 - Alpha-lactalbumin K5, K13, K16, K58, K62, K79, K93, K94, K98, K108, K114, K122	12/12
P02769/ B0JYQ0 - Serum albumin K4, K12, K41, K51, K64, K76, K93, K106, K116, K127, K131, K132, K136, K159, K180, K187, K204, K211, K221, K224, K232, K239, K242, K261, K273, K275, K280, K285, K294, K312, K316, K322, K350, K362, K375, K377, K388, K413, K465, K471, K474, K499, K504, K520, K523, K524, K533, K535, K537, K544, K556, K563, K573	53/59
P80195 - Glycosylation-dependent cell adhesion molecule 1 K35, K47, K64, K71, K73, K106, K116, K120, K123, K134	10/12
P02666 - Beta-casein K32, K48, K97, K99, K105, K107, K113, K169, K176	9/11
P02662 - Alpha-S1-casein K3, K7, K34, K36, K83, K102, K105, K124, K132, K193	11/14
P15497 - Apolipoprotein A-I K11, K22, K39, K58, K76, K87, K105, K106, K132, K139, K181, K193, K199, K204, K206, K224, K236	17/22
P02663 - Alpha-S2-casein K24, K32, K41, K76, K80, K91, K113, K150, K152, K158, K165, K166, K173, K181, K188, K191, K197, K199	18/24
P10790 - Fatty acid-binding protein K14, K21, K58, K65, K79, K81, K90, K96, K130	9/13
Q95114 - Lactadherin K145, K156, K177, K202, K214, K266, K275, K356, K364, K376, K377	11/16

P24627 - Lactotransferrin	36/54
K4, K28, K53, K73, K85, K99, K100, K151, K197, K221, K243, K263, K269, K273, K277, K282, K301, K313, K329, K339, K356, K386, K404, K419, K440, K441, K452, K454, K522, K579, K608, K628, K633, K637, K650, K673	
P02668 - Kappa-casein	6/9
K21, K24, K46, K63, K86, K111	
Q32PJ2 - Apolipoprotein A-IV	17/27
K23, K24, K45, K58, K59, K77, K79, K103, K226, K227, K235, K239, K296, K308, K325, K330, K339	
Q9TUM6 - Perilipin-2	16/32
K44, K52, K55, K116, K151, K153, K195, K200, K204, K213, K226, K245, K273, K280, K364, K375	
P34955 - Serpin A1	16/32
K67, K133, K152, K154, K161, K166, K191, K194, K199, K257, K264, K272, K277, K321, K333, K378	
Q1RMN8 - Immunoglobulin light chain	5/10
K152, K169, K189, K191, K207	
P35541 - Serum amyloid A protein	3/7
K29, K55, K111	
Q0IIG8 - Ras-related protein Rab-18	6/15
K21, K48, K58, K138, K142, K183	
Q0IIH5 - Nucleobindin 2	18/50
K35, K53, K60, K66, K78, K95, K125, K136, K147, K160, K169, K182, K187, K202, K296, K336, K348, K362	
P34955 - Alpha-1-antiproteinase	11/32
K67, K161, K191, K194, K215, K264, K272, K321, K329, K333, K378	
A0JNP2 - Secretoglobin, family 1D, member 2	2/6
K27, K38	
P18892 - Butyrophilin subfamily 1 member A1	7/23
K89, K114, K268, K297, K423, K441, K467	
P80457 - Xanthine dehydrogenase/oxidase	23/89
K39, K94, K224, K242, K248, K322, K432, K449, K615, K617, K702, K712, K770, K800, K846, K851, K889, K901, K947, K985, K988, K1227, K1290	
P10152 - Angiogenin-1	2/9
K18, K51	

Q0IIA4 - Glycoprotein 2 K61, K62, K381	3/14
P80025 - Lactoperoxidase K173, K299, K308, K428, K479, K556, K563	7/33
P81265 - Polymeric immunoglobulin receptor K21, K91, K270, K271, K357, K446, K474, K557, K589	9/48
Q2HJH2 - Ras-related protein Rab-1B K58, K116, K187	3/16
P26201 - Platelet glycoprotein 4 K39, K51, K55, K217, K222, K331	6/34
Q9TTE1 - Serpin A3-1 K161, K252, K259, K284	4/23
P17697 - Clusterin K33, K54, K378, K417	4/28
Q03247 - Apolipoprotein E K94, K155	2/13
Q27960 - Sodium-dependent phosphate transport protein K20, K33, K254, K272, K668, K676	6/41
Q4GZT4 - ATP-binding cassette sub-family G member 2 K156, K176, K323, K343, K346, K351	6/41
Q148D9 - Cellular repressor of E1A-stimulated genes 1 K149	1/7
Q3ZEJ6 - Endopin 1b K37, K161, K188	3/25
A2I7N0 - Serpin A3-4 K161, K167, K284	3/25
P01116 - GTPase KRas K87, K127	2/16
A1L528 - RAB1A, member RAS oncogene family K61, K187	2/17
A2I7N3 - Serpin A3-7 K120, K186, K254	3/26
P01888 - Beta-2-microglobulin K74	1/9

P28783 - Protein S100-A9 K35	1/10
P79105 - Protein S100-A12 K82	1/10
Q3ZCH5 - Zn-α2-glycoprotein K147	1/10
Q29443 - Serotransferrin K18, K651	2/64
Q3MHN5 - Vitamin D-binding protein K222, K385	2/38
A6QLS9 - RAB10 protein K59	1/21
A6QNW7 - CD5L protein K245	1/31
B6VAP7 - CDC42 protein K153	1/16
P01966 - Hemoglobin subunit alpha K61	1/11
P0CH28 - Polyubiquitin-C K48	1/63
P30932 - CD9 antigen K120	1/14
P61585 - Transforming protein RhoA K118	1/17
P62935 - Peptidyl-prolyl cis-trans isomerase A K48	1/14
P79345 - Epididymal secretory protein E1 K86	1/12
Q13748 - Tubulin alpha-3C/D chain K304	1/19
Q2KIT0 - Hibernation-associated plasma protein HP-20-like K155	1/11
Q3ZCL0 - Cysteine-rich secretory protein 2 K22	1/15

Q6VE48 - Membrane cofactor protein K216	1/17
Q71SP7 - Fatty acid synthase K1853	1/90
A1L555 - Prosaposin K127, K172, K399, K463, K472	5/35
B0JYP6 - Immunoglobulin K K213	1/16
P31096 - Osteopontin K19, K29, K210	3/23
Q3MHX6 - Protein OS-9 K348, K482	2/47
P01045 - Kininogen 2 Isoform LMW K213, K219, K374, K384	4/31
Q9MZ06 - Fibroblast growth factor-binding protein 1 K104	1/23
Q0P569 - Nucleobindin 1 K390	1/29
Q95122 - Monocyte differentiation antigen CD14 K59	1/11

LEGEND TO FIGURES

Figure 1. Reaction of a protein Lys residue with lactose to form the Amadori product lactulosyl-lysine.

Figure 2. Collision-induced dissociation pathway for a lactosylated peptide (Amadori product) in furylium ion form.

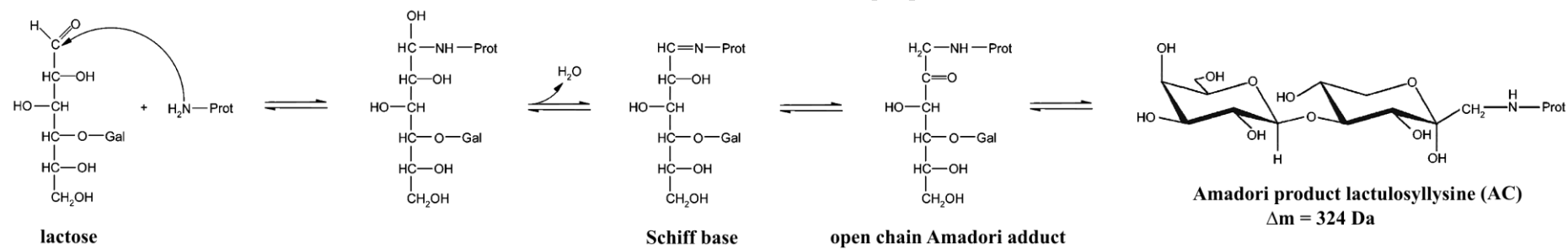
Figure 3. ESI-MS analysis of whey proteins from processed milk samples. Transformed ESI-MS spectra of glycosylated α -LA and β -LG from a UHT milk sample (panel A and B). Transformed ESI-MS spectra of glycosylated α -LA and β -LG from a powder milk sample (panel C and D). Individual molecular species are indicated by capital letters and the corresponding measured mass values and the nature of the various components are reported. Reproduced from Siciliano et al. (2000) with permission from American Chemical Society, copyright 2012.

Figure 4. MALDI-TOF mass spectra (acquired in linear mode), centered on α -LA, of different powder infant formulas (identified as P_i, i = 1–8). P3*, P4* and P7*: samples containing maltodextrins; P8**: a lactose-free sample; ○: hexose adduct (+ 162 Da); ▼: lactose adduct (+ 324 Da). Reproduced from Fenaille et al. (2001) with permission from Elsevier Science, copyright 2012.

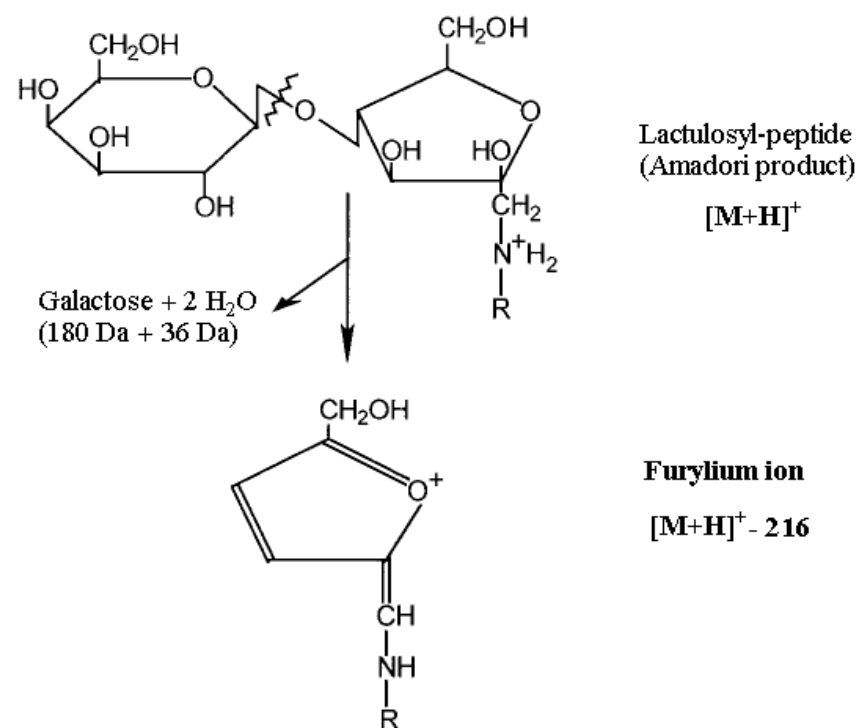
Figure 5. Structure of *m*-aminophenylboronic affinity gel, which is covalently bound to agarose as the solid matrix (panel A). The equilibria between boronic acids and *cis*-diol-containing compounds is shown (panel B). Affinity attachment, elution, and regeneration of boronic acid are also shown.

Figure 6. Comparative MALDI-TOF MS analysis of enriched tryptic digests of MFG proteins from raw (panel A), pasteurized (panel B), UHT (panel C) and powdered milk for infant nutrition (panel D) following phenyl boronic acid affinity trapping. Asterisks indicate peptides further identified by nLC-ESI-LIT-MS/MS; circles indicate peptides further characterized as lactosylated by tandem mass spectrometry experiments. Average mass values associated to linear spectrum traces are reported. Reproduced from Arena et al. (2011) with permission from Elsevier Science, copyright 2012.

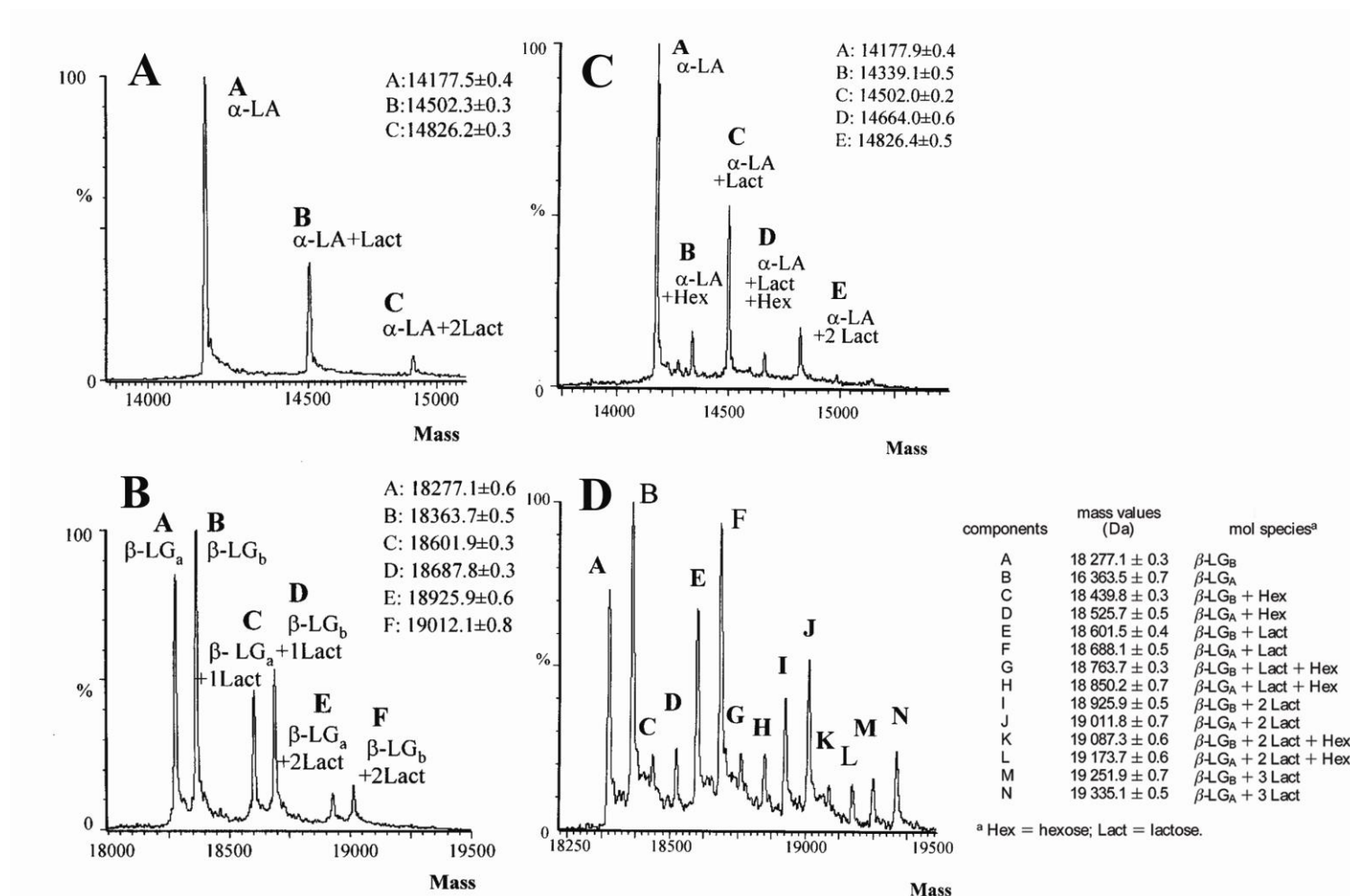
Figure 7. CID (panel A) and ETD (panel B) tandem mass spectrometry analysis of the $[M+4H]^{4+}$ ion at m/z 817.8 associated with the di-lactosylated peptide (78-100) from β -LG, where k represents lactose adduct to lysine. The mass spectra were acquired with alternating ETD and CID scanning. Reproduced from Arena et al. (2010) with permission from WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.



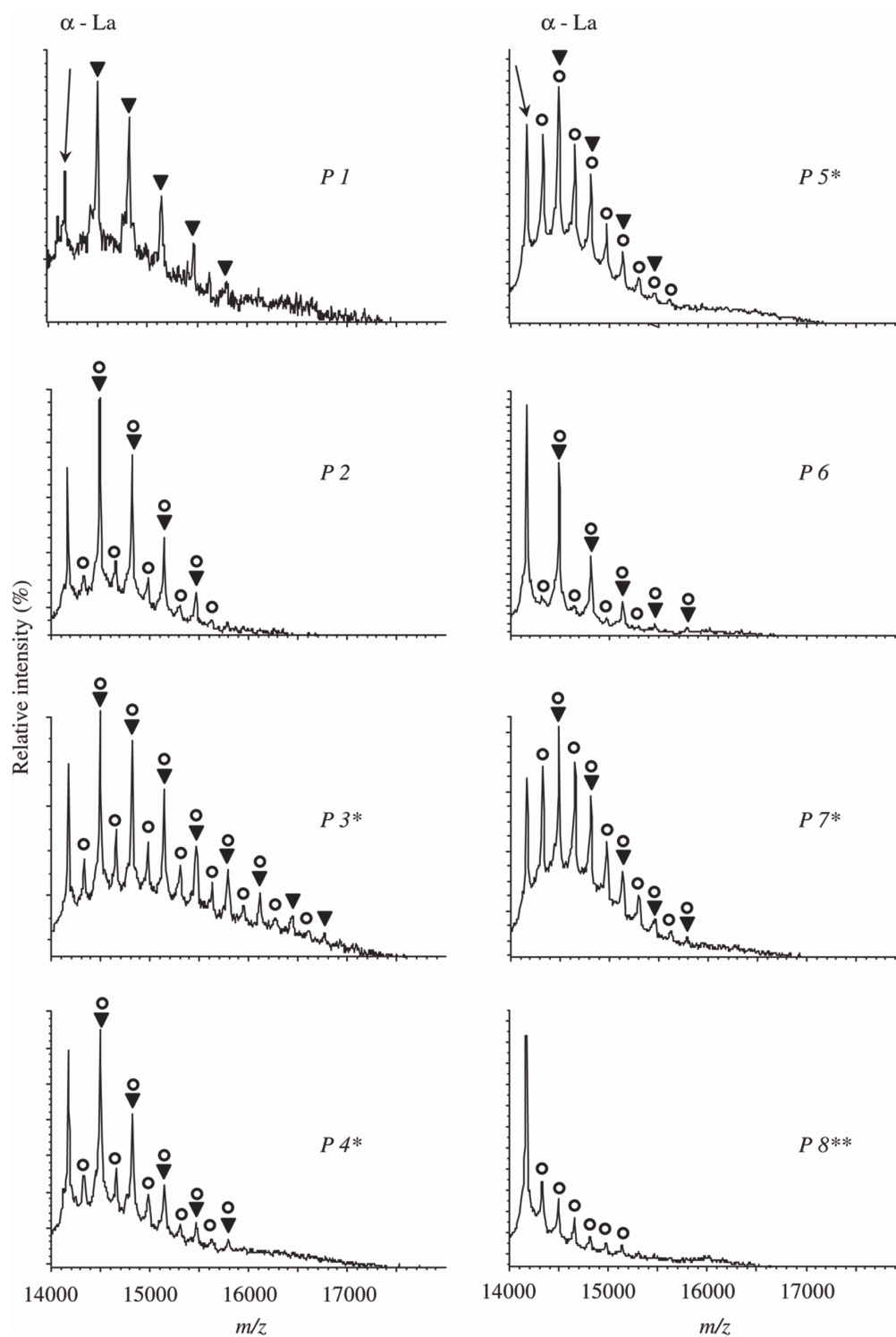
Siciliano et al., Figure 1



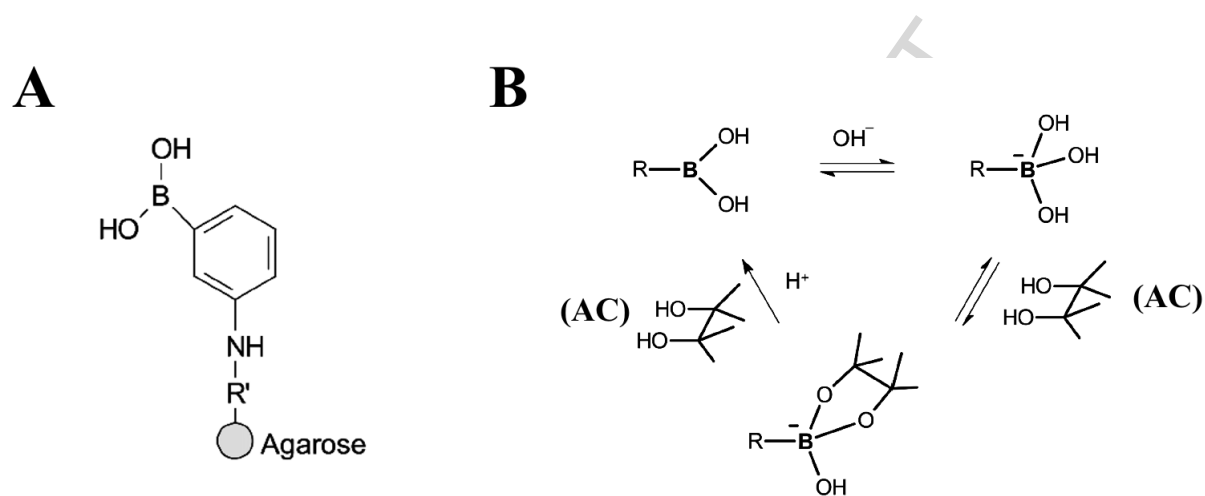
Siciliano et al., Figure 2



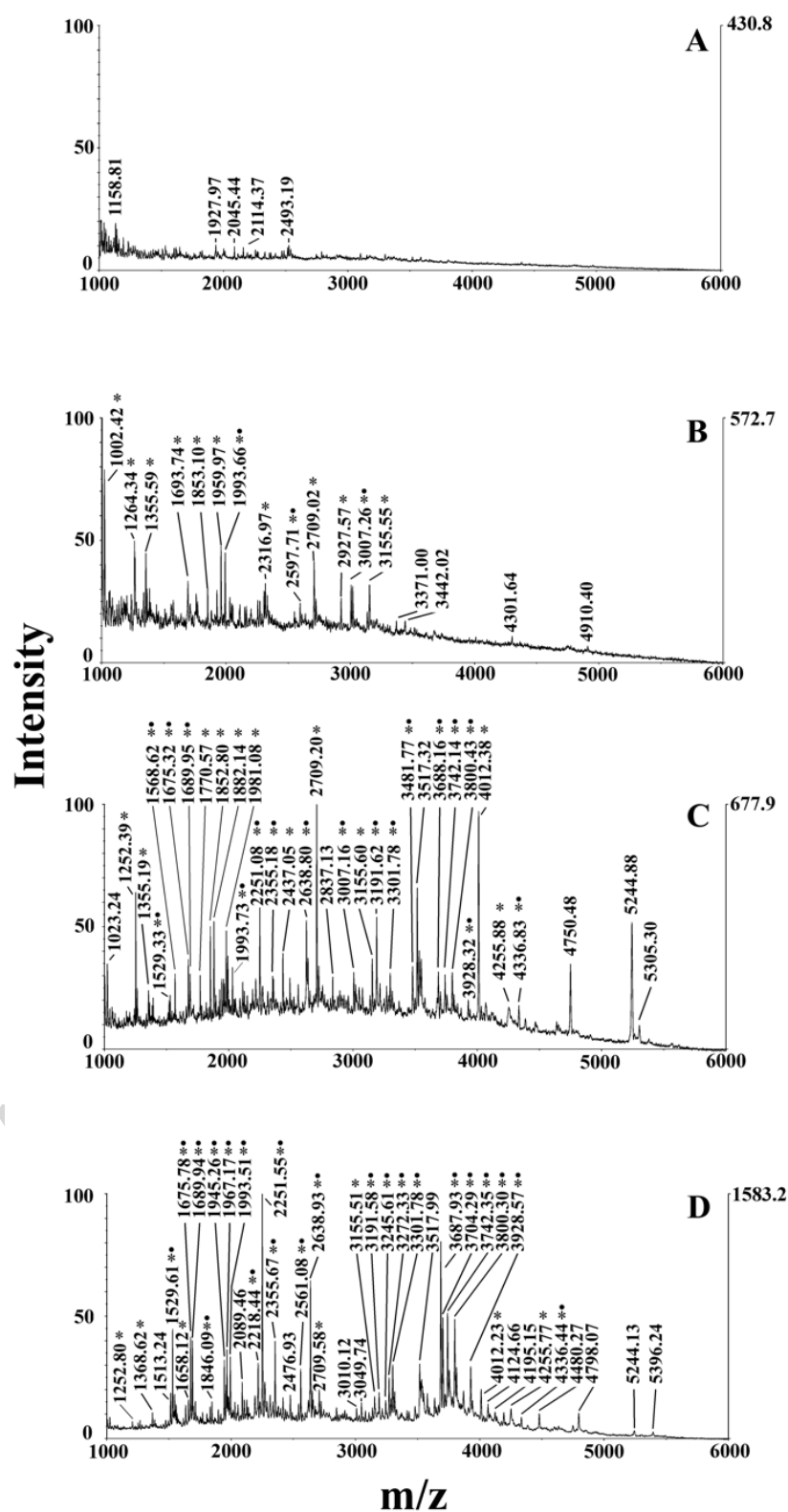
Siciliano et al., Figure 3



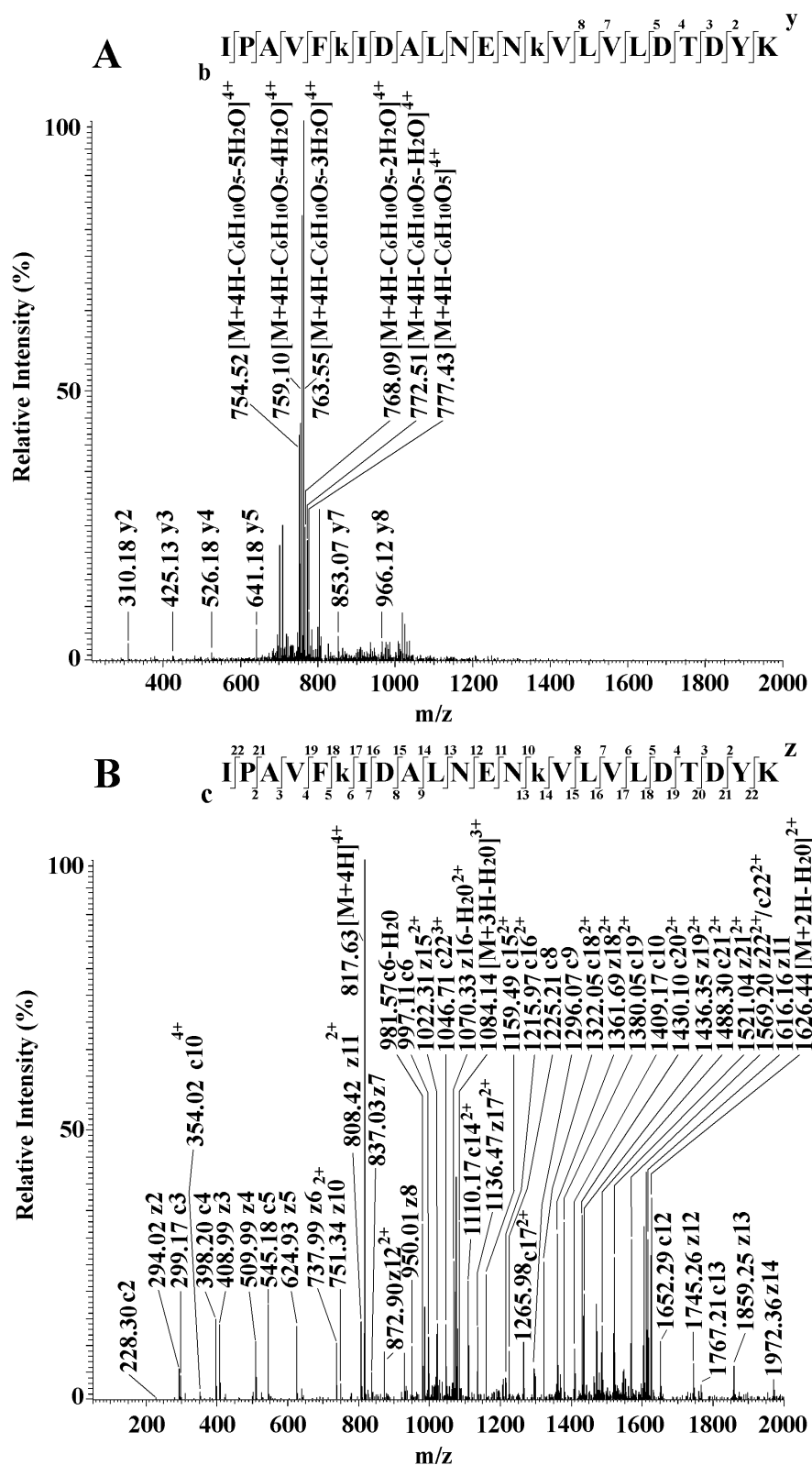
Siciliano et al., Figure 4



Siciliano et al., Figure 5



Siciliano et al., Figure 6



Siciliano et al., Figure 7