DAIRY PRODUCTS AND THE MAILLARD REACTION: A PROMISING FUTURE FOR EXTENSIVE FOOD CHARACTERIZATION BY INTEGRATED PROTEOMICS STUDIES

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Abstract

Heating of milk and dairy products is done using various technological processes with the aim of preserving microbiological safety and extending shelf-life. These treatments result in chemical modifications in milk proteins, mainly generated as a result of the Maillard reaction. Recently, different bottom-up proteomic methods have been applied to characterize the nature of these structural changes and the modified amino acids in model protein systems and/or isolated components from thermally-treated milk samples. On the other hand, different gel-based and shotgun proteomic methods have been utilized to assign glycation, oxidation and glycoxidation protein targets in diverse heated milks. These data are essential to rationalize eventual, different nutritional, amtimicrobial, cell stimulative and antigenic properties of milk products, because humans ingest large quantities of corresponding thermally modified proteins on a daily basis and these molecules also occur in pharmaceuticals and cosmetics. This review provides an updated picture of the procedures developed for the proteomic characterization of variably-heated milk products, highlighting their limits as result of concomitant factors, such as the multiplicity and the different concentration of the compounds to be detected.

Abbreviations

Hex, the Amadori product with D-glucose or D-galactose; G, glyoxal; MG; methylglyoxal; 3-DP, 3-deoxypentosone; 1-DG, 1-deoxyglucosone; 3-DG, 3-deoxyglucosone; 3-DGal, 3-deoxygalactosone; 3-DLact, 3-deoxylactosone; GONE, glucosone; Trios, triosone; -DH, -derived-dihydroxyimidazoline; -H, -derived hydroimidazolone; -He, -derived hemiaminal; GOLD, G-derived Lys dimer; MOLD, MG-derived Lys dimer; DOLD, DG-derived Lys dimer; GODIC, G-derived imidazolium cross-link product; MODIC, MG-derived imidazolium cross-link products DOGDIC, 3-DG-derived imidazolium cross-link products; NL, neutral loss; MRM, multiple reaction monitoring.

Contents

Chemical compounds studied in this article

Lactose (CID 440995)

Glyoxal (CID 7860)

Methylglyoxal (CID 880)

Lactulosyl-L-lysine (CID 3082392)

N^{ε}-carboxymethyl-L-lysine (CID 123800)

Furosine (CID 123889)

Pentosidine (CID 119593)

N^{ε}-carboxyethyl-L-lysine (CID 11241427)

L-methionine sulfoxide (CID 158980)

N-formyl-L-kynurenine (CID 910)

1. Introduction

Cow's milk and other bovine dairy products play a major role in human nutrition. To promote microbiological safety and extend shelf life, most milk is not consumed raw, but undergoes thermal treatments before retail and intake. These actions induce variable physicochemical modifications in milk molecules, depending on the duration/harshness of heating. Pasteurization (15-20 sec at 72-75 °C), and gradually, UHT treatment (2-3 sec at 135-150 °C), sterilization (10–30 min at >110 °C), concentration *in vacuo*, and formation of milk caramel affect the nutritional quality and alter the sensory attributes of the final products. These treatments generate chemicals not present in the raw material, including those due to non-enzymatic processes that occur during heating of combinations of proteins and reducing sugars, in what is known as the Maillard reaction (van Boekel, 1998). This yields a multitude of products depending on: *a*) the different reactions occurring in series and in parallel; *b*) the many proteins present in cow's milk (about 2500 of them); *c*) the reactivity of protein amino acids; and *d*) the most abundant sugars present in the raw material, i.e. lactose, D-glucose and D-galactose, which are in part transformed during heating into lactulose, epilactose and D-tagatose. The effect of thermal treatment plays a central role also in infant formulas, where milk molecules (whey proteins, caseins, lactose, D-glucose, D-galactose and fatty acids) are mixed with additional ingredients, including vegetable oils and other additives, to meet babies' diet requirements (Pischetsrieder & Henle, 2012).

The Maillard reaction was summarized in the Hodge scheme (Hodge, 1953), which was later integrated with the free radical degradation of the initial sugar-adducted intermediates (Figure 1). The initial step (reaction *a*) includes the condensation of the carbonyl moiety from a reducing sugar (*e.g.* lactose) with protein amino groups (N-terminus or lysine side chain), generating a Schiff base (aldimine) product. This unstable derivative rearranges to yield the 1-amino-1-deoxy-2-ketose (ketoamine) adduct, also known as the Amadori product (Figure 1, reaction *b*), which in the case of lactose corresponds to N^{ε}-(1-deoxy-D-lactulos-1-yl)-Lys (also named lactulosyl-lysine). Researchers have demonstrated that some commercial

products, for example skim milk powder, contain up to 50% of the total protein lysines converted into lactulosyl-lysine (Henle, Walter & Klostermeyer, 1991). Lactulosyl-lysine is an early-stage glycation derivative that can be more degraded through various reactions into advanced glycation end-products (AGEs) (Henle, 2005; Arena, Salzano, Renzone, D'Ambrosio & Scaloni, 2014), such as protein unbound furfurals, reductones, pyranones and fragmentation products (carbonyl and hydroxycarbonyl derivatives) (van Boekel, 1998) (Figure 1, reactions *c* and *d*). Researchers have identified the Amadori derivative oxidative degradation product N^{ε}-carboxymethyllysine as the most abundant protein bound AGE occurring in commercial products and heated milk models (Ahmed, Mirshekar-Sayhkal, Kennish, Karachalias, Babaei-Jadidi & Thornalley, 2005; Hegele, Buetler & Delatour, 2008; Assar, Moloney, Lima, Magee & Ames, 2009; Lima, Moloney & Ames, 2010; Nguyen, van der Fels-Klerx & van Boekel, 2016) (Figure 2). N^{ε}-carboxymethyllysine can also be generated as result of other glycation and lipid peroxidation reactions (see below) (Arena *et al.*, 2014). A non-oxidative pathway that involves intramolecular enolization and further elimination of water or galactose can precede above-mentioned reactions, and lead to intermediate glycation protein-monosaccharide products containing a α-dicarbonyl moiety. In particular, N^{ε}-(5,6-dihydroxy-2,3-dioxohexyl)-Lys has been identified as a reactive derivative that can further inter-convert into N^{ε}-(2,3-dihydroxy-5,6-dioxohexyl)-Lys, as a consequence of the mobility of the carbonyl moiety along its sugar group (Figure 2). These reactive intermediates can in turn react with additional amino acids, yielding stable protein cross-linking AGE products. This is the case with glucosepane and crossline, which are cross-linked derivatives generated as a result of the reaction of these carbonylated intermediates with Arg and Lys residues, respectively (Figure 2). Otherwise, these compounds can lose a carbon atom by retro-Claisen ester condensation, thereby generating corresponding pentose adducts; these reactive derivatives can in turn react with additional amino acids to generate other AGEs. An example of five carbon-containing AGE derivative is pentosidine (Figure 2), which Henle and coworkers directly detected in commercial milks (Henle, Schwarzenbolz &

Klostermeyer, 1997) and which is also formed when D-glucose is present in heated protein model mixtures.

On the other hand, carbohydrate adduct fragmentation can happen by retro-aldolization (Figure 1, reaction *d*), yielding stable α-dicarbonyl-containing molecules such as glyoxal (G), methylglyoxal (MG), 3-deoxypentosone (3-DP), glucosone (GONE), 1-deoxyglucosone (1-DG), 3-deoxyglucosone (3-DG), and other derivatives. These reactive compounds can also be formed as result of direct thermal degradation and/or oxidatation of unbound carbohydrates (Figure 1). For instance, lactose heating in milk produces G, MG, 3-DP, 3,4-dideoxypentosone, GONE, galactosone (GAONE), 3-DG and 3-deoxygalactosone (3-DGal). Similarly, D-glucose thermal degradation generates G, MG, triosone (Trios), tetrosone, 3-DP, GONE and 3-DG; in this context, Hellwig and coworkers observed interconversion of 3-DG and 3-DGal. G, MG and glycolaldehyde can also result from oxidative degradation of ascorbic acid or polyunsaturated fatty acids, and (metal-catalyzed) oxidation of protein serines/threonines. The α-dicarbonyl compounds mentioned above can react with protein N-terminus and Lys/Arg side chains yielding additional AGEs, such as aminoketones and Strecker aldehydes of the amino acids (Figure 1, reaction *e*); these derivatives may further condense to form pyrazines, contributing to the smell of heated foods. Linear and cross-linked AGEs formed as deriving from these α-dicarbonyl compounds include: *a*) G-derived N^{ε}-carboxymethyllysine; *b*) MG-derived N^{ε}-carboxyethyllysine, dihydropyrimidine (DHP), tetrahydropyrimidine (THP) and argpyrymidine; *c*) 3-DG-derived pyrraline; *d*) G-, MG-, 3-DG- and 3-DGal-derived-dihydroxyimidazolines (G-DH, MG-DH, 3-DG- and 3-DGal-DH); *e*) G-, MG-, 3-DG- and 3-DGal-derived hydroimidazolones (G-H, MG-H, 3-DG-H and 3-DGal-H); *f*) G-, MG- and 3-DG-derived Lys dimers (GOLD, MOLD, and DOLD); *g*) G-, MG- and 3-DG-derived imidazolium cross-link products (GODIC, MODIC, and DOGDIC); *h*) G-, MG-, 3-DG-and 3-DGal-derived hemiaminals (G-He, MG-He, 3-DG-He and 3-DGal-He) (Henle, 2005; Mittelmaier & Pischetsrieder, 2011; Arena *et al*., 2014) (Figure 2). N^{ε}-carboxymethyllysine (Ahmed *et al*., 2005; Hegele

142 *et al*., 2008; Assar *et al*., 2009; Lima *et al*., 2010; Nguyen *et al*., 2016), N^{ε}-carboxyethyllysine (Ahmed
143 *et al*., 2005), MG-H (Ahmed *et al*., 2005) and pyrraline (Hegele *et al*., 2008) have been directly detected
144 in hydrolysates of heated milk products. These derivatives complement oxalic acid monolysinylamide, a
145 Maillard reaction product deriving from ascorbic acid.
146 The final stages of the Maillard reaction correspond to reactions *f* and *g* in Figure 1, which generate
147 small/large polymeric compounds introducing fluorescence/color to the final material. Step *f* involves
148 the aldol condensation of reductones, furfurals and aldehydes resulting from reactions *c*, *d* and *e*, without
149 the involvement of compounds containing amino groups; step *g* refers to the processes between the same
150 derivatives and amino group-containing molecules, ultimately yielding melanoidins (Hodge, 1953;
151 Hellwig & Henle, 2014). These furan, pyrrole and pyridine ring-containing polymers, having a molecular
152 mass above 100 kDa and a brown/black color, can integrate redox-active compounds and have therefore
153 antioxidant characteristics.
154 The nature and relative concentration of each AGE is governed by the type of reducing sugar and the
155 complex system of parallel processes ongoing together (Figure 1). Thus, thermodynamics and kinetics
156 of: *a*) the generation of the Amadori product; *b*) the concomitant oxidation/degradation of unbound
157 sugars; *c*) the successive formation of different free and protein-bound, reactive α-dicarbonyl derivatives;
158 and *d*) the further reaction of the latter with the polypeptide chain have to be considered with a view to
159 explaining the generation of the ultimate AGE compounds. Thus, these final products can originate from
160 protein non-enzymatic glycation by reducing sugars in pre- and post-Amadori processes, and from
161 alternative reactions where the Amadori adduct is not the starting initiator. AGEs can therefore be
162 produced in both the early and late steps of the Maillard reaction. Accordingly, the term *advanced*
163 *glycation end-products* is inaccurate, but the idea of early, intermediate and advanced glycation products
164 is simple and the corresponding terminology has become widespread.

Meanwhile, heating milk in the presence of oxygen can induce protein oxidation, and the amino acids Met, Cys and Trp have been proved as highly susceptible to corresponding modifications (Bachi, Dalle-Donne & Scaloni, 2013). Although this does not depend directly on the Maillard reaction, lactose may promote oxidation of these amino acids in heated milks (Meltretter, Seeber, Humeny, Becker & Pischetsrieder, 2007). This is ascribable either to: *a*) sugar degradation-derived α-dicarbonyl compounds that can directly cause oxidative transformation of the Lys side chain into aminoadipic semialdehyde (Meltretter *et al*., 2007); or *b*) reactive oxygen species, which are produced as result of the Maillard reaction (Mossine, Linetsky, Glinsky, Ortwerth & Feather, 1999). Methionine sulfoxide has been ascertained as the most abundant oxidized amino acid occurring in heated dairy products (Baxter *et al*., 2007; Meltretter, Becker & Pischetsrieder, 2008; Meltretter, Wust & Pischetsrieder, 2014). Depending on the milk derivative, mutable levels of Cys and Trp oxidized products, *i.e.* sulfinic acid, sulfonic acid, cystine, kynurenine, N-formylkynurenine and hydroxytryptophan, have also been observed (Hoffman & van Mil, 1999; Puscasu & Birlouez-Aragon, 2001; Cho, Singh & Creamer, 2003; Meltretter *et al*., 2007; 2014). In particular, formation of cystine generates thermal-dependent protein aggregates in dairy products (Hoffman *et al*., 1999; Cho *et al*., 2003). Also worth mentioning are the carbonylated adducts deriving from the reaction of amino acids with lipid peroxidation products, such as 4-hydroxy-2-nonenal (Scaloni, *et al*., 2002; Fenaille, Parisod, Tabet & Guy, 2005; Meyer, Baum, Vollmer & Pischetsrieder, 2012), which are also involved in the formation of N^{ε}-carboxymethyllysine (Lima *et al*., 2010).

Other sugar-independent protein derivatives can be observed when milk is heated. These include: *a*) deamidated Asp, whose side chain is converted in the amide counterpart (Meltretter *et al*., 2014); *b*) α-ketoamide at the N-terminal amino acid, as result of the corresponding oxidative deamination (Meltretter *et al*., 2014); *c*) pyrrolidone adduct at the N-terminal Glu (Meltretter *et al*., 2007; 2014); and *d*) lysinoalanine and histidinoalanine cross-linked derivatives that result from the reaction of Lys and His with dehydroalanine, which in turn originates from β-elimination of Cys and Ser residues.

All of these amino acid derivatives (Figures 2) have found a widespread application as markers to assess the nutritional value of milk products (Erbersdobler & Somoza, 2007; Hellwig *et al*., 2014; Wada & Lonnerdal, 2014). Their accretion in thermally treated material generally changes the availability of essential amino acids (Lys, Met and Trp) after digestion, and can also modify the function, digestibility and allergenicity of specific milk proteins (Muscat, Pischetsrieder, Maczurek, Rothemund & Munch, 2009; Arena *et al*., 2014; Hellwig *et al*., 2014; Wada *et al*., 2014; Heilmann *et al*., 2014).

2. Amino acid- and immunoassay-based evaluation of heated milk proteins

Amino acid adducts mentioned above were initially ascertained in heated dairy samples by gas or liquid chromatography (GC or LC) methods coupled with UV/fluorescence-based and/or mass spectrometry (MS)- or tandem mass spectrometry (MS/MS)-based detection procedures, which were used in the analysis of corresponding enzymatic or acid protein hydrolysates. This allowed the detection of furosine (Troise, Fiore, Wiltafsky & Fogliano, 2015), lactulosyl-lysine (Henle *et al*., 1991), N^{ε}-carboxymethyllysine (Ahmed *et al*., 2005; Erbersdobler *et al*., 2007; Hegele *et al*., 2008; Assar *et al*., 2009; Delatour *et al*., 2009; Troise *et al*., 2015), N^{ε}-carboxyethyllysine (Ahmed *et al*., 2005; Troise *et al*., 2015), pyrraline (Hegele *et al*., 2008), MG-H (Ahmed *et al*., 2005), pentosidine (Henle *et al*., 1997), methionine sulfoxide (Baxter *et al*., 2007), lysinoalanine and histidinoalanine in various milk products. As dihydroxyimidazoline and hydroimidazolone derivatives are not stable to extensive acid hydrolysis, they were detected under dedicated experimental conditions. In most cases, the synthesis of isotope-enriched internal standards and the possibility to perform selected ion monitoring (SIM) MS experiments allowed an accurate evaluation of trace quantities of these amino acid adducts. GC-SIM experiments generally involved pre-column derivatization to generate corresponding volatile derivatives.

The recent introduction of LC-ESI-MS/MS with multiple reaction monitoring (MRM) procedures, concurrent fluorescence detection and use of isotopically-labeled internal standards have allowed

concomitant detection of N^{ε}-carboxyethyllysine, N^{ε}-carboxymethyllysine, pyrraline, G-H, MG-H, 3DG-H, argpyrymidine and pentosidine combinations in dairy products as underivatized or derivatized compounds (Ahmed *et al.*, 2005; Hegele *et al.*, 2008; Assar *et al.*, 2009). Comparative quantitative studies on raw, pasteurized, UHT, sterilized, powdered, condensed, and liquid or powdered infant formula milk have demonstrated that heating induces gradual modifications, whose extent depends on how long and at what temperature the raw material is heated.

Identical deductions have been reached using dedicated antibodies developed to measure non-enzymatic glycation, glycoxidation and amino acid oxidation by dot blot, ELISA and western blotting (Bachi *et al.*, 2013; Arena *et al.*, 2014). Under gel-based approaches, for example, immunoblotting has been used to assign the modified proteins on a reference SDS-PAGE profile, so that the relative amount of the modified species could be evaluated in comparative experiments with identical protein sample loading. When applied to dairy products, immunoenzymatic approaches have allowed detection of lactulosyl-lysine (Pallini, Compagnone, Di Stefano, Marini, Coletta & Palleschi, 2001), N^{ε}-carboxymethyllysine (Meyer, Al-Diab, Vollmer & Pischetsrieder, 2011), oxalic acid monolysinylamide, N-formylkynurenine (Ehrenshaft *et al.*, 2009) and carbonylated adducts (Scaloni *et al.*, 2002; Fenaille *et al.*, 2005; Meyer *et al.*, 2012) in heat-modified milk proteins. The greatest challenge in studying milk protein damage has been the sheer diversity of the products to be assayed by dedicated reagents, which has hampered the simultaneous determination of the modified species in commercial products.

3. Single protein-centered characterization heat-dependent modifications

The main disadvantages of the approaches discussed above lie in the absence of information on the identity of the modified proteins, the extent of the modifications, and the assignment of the adducted amino acids. Such information is central for linking a specific protein modification to definite technological or nutritional characteristics of a dairy product. To that end, various MS procedures have

237 been used to identify and structurally elucidate thermally-treated milk proteins as purified from dairy
238 products, taking advantage of the recent improvements in this analytical technique (Siciliano, Mazzeo,
239 Arena, Renzone & Scaloni, 2013; Arena *et al*., 2014). Meanwhile, *in vitro* studies using dedicated
240 reagents (lactose, D-glucose, G, MG or additional sugars and α-dicarbonyl derivatives) able to induce
241 glycation/glycoxidation or oxidation have been realized on isolated proteins to assess the corresponding
242 reactivity in the circumstances of a real technological treatment of milk (Siciliano *et al*., 2013; Arena *et*
243 *al*., 2014). Through direct MS analysis of intact protein components and the the corresponding detection
244 of adducts with a (multiple) mass increase of +324 Da (for lactose) or +162 Da (for D-glucose/D-
245 galactose), compared with the unmodified counterpart, it has been observed that the Amadori derivatives
246 are the most abundant species occurring in mildly-to-moderately heated dairy products (Siciliano *et al*.,
247 2013). Investigations on β-lactoglobulin (Leonil, Molle, Fauquant, Maubois, Pearce & Bouhallab, 1997;
248 Morgan, Bouhallab, Molle, Henry, Maubois & Leonil, 1998; Fogliano *et al*., 1998; Siciliano, Rega,
249 Amoresano & Pucci, 2000; Fenaille, Morgan, Parisod, Tabet & Guy, 2004; Monaci & van Hengel, 2007;
250 Carulli, Calvano, Palmisano & Pischetsrieder, 2011), α-lactalbumin (Siciliano *et al*., 2000; Carulli *et al*.,
251 2011), and αS1- or β-caseins (Scaloni *et al*., 2002; Johnson, Philo, Watson & Mills, 2011) exemplify
252 this observation; they characterized the prevalent processes present in thermally-treated milk models and
253 milk products. This analytical approach was also used to ascertain the thermal history of milk products
254 (Losito, Carbonara, Monaci & Palmisano, 2007; Johnson *et al*., 2011; Sassi, Arena & Scaloni, 2015).
255 Indeed, comparative quantitative studies on a number of dairy products confirmed that thermal treatment
256 promotes protein non-enzymatic glycation, whose magnitude depends on the temperature and time of
257 exposure. Indeed, the corresponding spectrum profiles showed that there is a progressive increase in the
258 number and relative concentration of the Amadori product and oxidized adducts, and the concomitant
259 generation of other uncharacterized compounds. Using dry state heating conditions generally increases
260 the extent of protein modification (Morgan *et al*., 1998; Fenaille *et al*., 2004).

These findings have been confirmed by qualitative studies on the nature of the modifications detected and the number of assigned, modified amino acids, as ascertained by comparative peptide mapping experiments on isolated milk proteins; the latter were performed with nanoLC-ESI or MALDI-TOF MS procedures (Leonil *et al*., 1997; Morgan *et al*., 1998; Fogliano *et al*., 1998; Siciliano *et al*., 2000; Scaloni *et al*., 2002; Meltretter *et al*., 2007; 2008; Lima *et al*., 2009; Carulli *et al*., 2011; Dyer *et al*., 2016). For lactulosyl-lysine derivatives, preventive reduction with $NaBH_4$ stabilized protein adducts before enzymatic digestion (Siciliano *et al*., 2000; Scaloni *et al*., 2002). These studies demonstrated a relevant time- and temperature-dependent formation of modified peptides containing lactulosyl-lysine, hexose-associated Amadori products, and N^{ε}-carboxymethyllysine (showing a Δm = +58 Da) at specific Lys sites. For poorly glycated intact proteins, lysines more exposed on the molecular surface or having a structure-predictable higher reactivity showed a high propensity to modification (Fogliano *et al*., 1998). Conversely, when moderately/highly modified intact proteins were considered, widespread modification at all lysines was observed. These investigations also demonstrated production of Met/Trp/Cys oxidation products, as well as lysine aldehyde and pyrrolidone adducts in samples subjected to heating for variable times or at moderate temperatures; corresponding spectrum profiles showed signals at +16, -1 and -18 Da, respectively, with respect to non-modified counterparts (Meltretter *et al*., 2007; 2008; Carulli *et al*., 2011; Dyer *et al*., 2016). In the case of milk models treated with D-glucose or MG for prolonged times, at high temperatures, MG-H and MD-DH adducts (showing a Δm = +54 and +72 Da, respectively) were also observed at specific Arg residues (Lima *et al*., 2009). In most of these cases, MS/MS experiments were performed to correct for the uncertainty caused by the concomitant presence of multiple modifiable amino acids in the adducted peptides (Morgan, Leonil, Molle & Bouhallab, 1997; Molle, Morgan, Bouhallab & Leonil, 1998; Lima *et al*., 2009; Dyer *et al*., 2016).

Recently, Meltretter and coworkers used ultrahigh-performance liquid chromatography-electrospray ionization tandem mass spectrometry (UPLC-ESI-MS/MS) for the systematic characterization and site-

specific assignment of heat-induced modifications in β-lactoglobulin from milk products subjected to thermal treatment (Meltretter, Wust & Pischetsrieder, 2013). To this purpose, β-lactoglobulin was heated *in vitro* with lactose under selected conditions to maximize the formation of non-enzymatic modifications. Based on known protein modifications occurring following oxidation and the Maillard reaction, the protein digest underwent full scan and enhanced resolution scan experiments, together with enhanced product ion scans. Next, these authors identified the main glycation/glycoxidation/oxidation, and deamidation products at Lys, Arg, Met, Cys, Trp, Asn, and the N-terminus. By using MS data, they developed a highly sensitive scheduled MRM method suitable for the analysis of milk products, detecting 19 different structures and 26 modified sites in β-lactoglobulin from various commercial dairy products. As expected, the number of modified species correlated with the extremity (temperature and time) of the heat treatment. The same researchers then used this approach to study site-specific relative modifications of β-lactoglobulin in thermally-treated milks and different dairy products, with the aim of ascertaining their thermal or non-thermal origin and assigning marker candidates for milk processing (Meltretter *et al.*, 2014). They found that: *a*) site-specific assessment of lactulosyl-lysine is a more sensitive marker for mild thermal treatment than the overall content of this adduct in the protein; *b*) N^{ε}-carboxymethyllysine and N-terminal ketoamide are of thermal origin and may be considered as good markers for fairly harsh thermal treatments, whereas N^{ε}-carboxyethyllysine reflects thermal/non-thermal processes; *c*) MG-derived modifications at Arg residues are far less relevant than other ones; *d*) oxidation at Met and Cys is a fairly weak indicator of heat impact; and *e*) Trp oxidation adducts kynurenine and N-formylkynurenine are of non-thermal origin and become further degraded during milk processing.

Because reactive oxygen species can be generated as result of the Maillard reaction, autoxidation of sugars and lipid peroxidation, Wüst and Pischetsrieder have used an analogous MRM approach to assess the influence of milk fat on Met oxidation (Wust & Pischetsrieder, 2016). By performing a quantitative

analysis of β-lactoglobulin, α-lactalbumin and αS1-casein oxidation at different Met sites in protein samples from raw, UHT and evaporated milk samples with different fat contents, they found that oxidation at most protein Met sites was not affected by milk fat. They therefore conclude that lipid oxidation products are not the major cause of Met oxidation in milk.

Through tandem mass spectrometry experiments have been widely used to identify adducted residues in modified peptides, collision induced dissociation (CID) analysis of Amadori product-containing components has shown the almost absence of sequence-informative *b*- and *y*-type ions in the corresponding spectra, thus hampering their sequence-dependent molecular assignment (Molle *et al.*, 1998; Arena *et al.*, 2010). For lactosylated peptides, the most intense fragment ions result from the breakdown of the glycosidic bond, with the maintenance of the glycosidic oxygen by the moiety having the reducing end, together with neutral loss (NL) of two/three water molecules, yielding the corresponding $C_6H_{12}O_6$ + 2 H_2O species, also named furylium ion (-216 Da). NL of one and two water molecules (-18 and -36 Da, respectively), and $C_6H_{12}O_6$ + 2 H_2O + HCHO (-246 Da) was also detected. NL pattern of various water molecules and the generation of furylium ion generally did not depend on the charge of the peptide under investigation. The above is summarized in Fig. 3, showing the CID spectrum of the $[M+4H]^{4+}$ ion from the lactosylated peptide (74-99) present in modified lactotransferrin. Although *b*- and *y*-type ions are rare in CID spectra, Le and coworkers have used the above findings to develop an MRM-based procedure for the quantitative analysis of lactosylated peptides in β-lactoglobulin and α-lactalbumin, as resulting from milk powder samples subjected to storage under diverse environmental conditions (Le, Deeth, Bhandari, Alewood & Holland, 2013). Authors specifically selected NL of 162 and 216 Da, *i.e.* the cleavage of galactose and the formation of the furylium ion, respectively, as operative MRM transitions, and based the quantification of modified species on the corresponding peak areas. The method appears to be feasible, given the good correlation between MRM and furosine content results, with respect to the storage time of the samples.

In order to overcome the sequencing limitations associated with CID fragmentation of Amadori product-containing species, researchers developed a dedicated method based on electron transfer dissociation (ETD) analysis (Arena *et al.*, 2010). Under these experimental conditions, neither furylium ions nor ions corresponding to NL of water molecules from the modified amino acid were detected, thus highlighting that the adducted residue is stable during ETD fragmentation. The cleavage of above-mentioned chemical bonds was independent on side-chain modification and peptide composition. The intensity and near-completeness of the different *c*- and *z*-type ions present in the spectrum, independently from the location of the modified amino acid in the polypeptide chain, makes easy peptide sequencing and adducted residue assignment. During analysis, the number of modified peptides identified was increased using supplemental collisional activation after electron transfer (Arena, Renzone, Novi & Scaloni, 2011). This condition is exemplified in Fig. 3B, showing peptide fragmentation with supplemental collisional activation under ETD conditions of the $[M+4H]^{4+}$ ion from the lactosylated peptide (74-99) present in modified lactotransferrin. Unlike what was found in the case of CID fragmentation (Fig. 3A), abundant sequence-informative *c*- and *z*-type ions were observed, greatly facilitating peptide sequencing and assignment of the adducted amino acid. These studies were demonstrative of the essential role played by ETD-based methods in shotgun proteomic analysis of heat-dependent milk modifications.

4. Proteomic characterization of heat-dependent protein modifications

Classical proteomic approaches based on the integration of two-dimensional electrophoresis (2-DE), *in-gel* enzymatic protein digestion and MALDI-TOF MS or nanoLC-ESI-MS/MS analysis of the corresponding peptide mixtures have been used to provide an overall picture of the modified proteins present in milk after it is heated, including modified amino acids assignment. The ability of this electrophoretic technique to display and quantify thousands of polypeptide species in a single experiment has made it possible to assess small variations of protein representation in milk samples after different

356 heat treatments, also purifying components for their further MS-based description. In the first proteomic
357 study on commercial milk powder samples, Galvani and coworkers were able to reliably identify single
358 and multiple lactose-conjugates of β-lactoglobulin, α-lactalbumin and caseins (Galvani, Hamdan &
359 Righetti, 2000). Marvin and coworkers later used 2-DE, trypsinolysis and nanoLC-ESI-MS/MS to
360 evaluate the lactosylation profile of milk proteins in powder formulas for infant nutrition (Marvin,
361 Parisod, Fay & Guy, 2002). Indeed, NL fragmentation experiments *ad hoc* performed to detect
362 lactosylated species revealed up to 10 lactosylated lysines in β-lactoglobulin and αS2-casein, 5 in αS1-
363 and β-casein, and 4 in κ-casein. α-Lactalbumin was selected as a potential indicator to reveal
364 processing/storage-dependent chemical modifications in milk samples. Later on, Holland and colleagues
365 applied gel-based proteomic methods to investigate protein modifications in UHT milk samples as
366 dedendent on the timimg and temperature of storage (Holland, Gupta, Deeth & Alewood, 2011). 2-DE
367 proteomic maps of these samples showed the occurrence of: *a*) components that were associated with
368 non-disulfide cross-linked αS1-, αS2- and β-casein species, which migrated diffusely above the position
369 of the corresponding monomers; *b*) non-conventional acid protein isoforms (mainly for αS1-casein), due
370 to Asp/Glu deamidation; and *c*) vertical stacked whey protein spots that were associated with
371 corresponding lactosylated products. The higher the storage temperature, the more extensive were these
372 proteomic variations. Mass spectrometric analysis of corresponding protein digests demonstrated that
373 the cross-linked species mainly contained αS1-casein adducts, but the authors also observed a
374 heterogeneous population of cross-linked forms containing αS2- and β-caseins. Tandem mass
375 spectrometry allowed assignment of deamidated residues in αS1-casein. Similarly, it determined that the
376 stacked spots of β-lactoglobulin and α-lactalbumin were due to modified protein adducts containing 9
377 and 8 lactosylated lysines, respectively. A close correlation between the extent of lactosylation and
378 storage temperature was ascertained. No additional information was obtained for the remaining milk
379 proteins. Later on, the same authors confirmed an analogous link between the extent of lactosylation and

storage conditions (time, temperature and relative humidity) in milk concentrates again using a gel-based proteomic approach (Le, Deeth, Bhandari, Alewood & Holland, 2012). α-Lactalbumin was chosen as proper marker of sample storage and was shown to contain as many as 5 lactose moieties on 8 different lysines.

Based on above-mentioned integration of 2-DE, *in-gel* trypsinolysis and MALDI-TOF MS analyses, Calvano and coworkers developed a dedicated procedure to detect milk adulteration and monitor the addition of powdered milk derivatives into pasteurized and UHT counterparts (Calvano, Monopoli, Loizzo, Faccia & Zambonin, 2013). To begin, they performed proteomic analyses to define glycated, oxidized (Cys/Trp/Trp), lactosylated, deamidated, and N^{ε}-carboxymethyllysine- or aminoadipic semialdheyde-containing peptides specifically detected in milk powder; these adducted species could have been considered as adulteration markers. Modified residues then were assigned in β-lactoglobulin, α-lactalbumin, αS1-, αS2-, β- and κ-caseins. MALDI-TOF MS experiments on tryptic digests of whey and casein fractions from *ad hoc* adulterated milk samples made it possible to evaluate these diagnostic components and reveal them down to a 1% level of adulteration.

On the other hand, Chevalier and Kelly (Chevalier & Kelly, 2010) specifically evaluated disulfide bond formation in milk proteins from thermally-treated milk samples by using 2-DE experiments under reducing and non-reducing conditions, which were associated with MS measurements on selected protein *in-gel* digests. Proteomic analysis of raw milk demonstrated that almost 18, 25 and 46% of αS2-casein, β-lactoglobulin, and k-casein molecules, respectively, are involved in disulfide-linked hetero- and homo-polymers, whereas αS1- and β-casein mainly are present in the reduced form. After heating, amounts of reduced κ-casein, β-lactoglobulin and serum albumin, decreased by 75, 75 and 85%, respectively, with the formation of disulfide-containing aggregates. MS identified homo- and heteropolymers of κ- and αS2-casein in heated samples; molecular aggregates involving only κ-casein or only αS2-casein

403 accounted for 12 and 43% of the total polymers, respectively, suggesting their prominent function in the
404 generation of intermolecular S-S bridging between proteins. These observations confirmed previous data
405 on thermally-treated and pressurized milk samples, in which copolymers ranging from 440 to 2000 kDa
406 or more than 2000 kDa were detected (Hoffman *et al*., 1999; Cho *et al*., 2003; Nabhan, Girardet,
407 Campagna, Gaillard & Le Roux, 2004). For dimers and trimers of κ-casein, Holland and coworkers used
408 dedicated MS procedures to ascertain the nature of the S-S linked peptides and the specific cysteines
409 involved in disulfides (Holland, Deeth & Alewood, 2008). Similar research by Livney and Dalgleish
410 using a milk model fully characterized homo- and heteromeric dimers and trimers that contained κ-
411 casein and β-lactoglobulin (Livney & Dalgleish 2004). The interaction and the association of milk
412 proteins *via* S-S bridges appear to have a protecting role for milk micelle maintenance and against the
413 generation of fibril aggregates.
414
415 **5. Shotgun proteomics of heat-dependent protein modifications**
416 Recent advances in MS and chromatographic technologies have streamlined the examination of whole
417 proteomes according the so-called shotgun proteomic approach. In this case, total protein extracts are
418 digested *in-solution* and resulting peptide mixtures are resolved through two-dimensional
419 chromatography combined with MS/MS analysis, making the experiment amenable in a gel-free set up.
420 When focusing on protein modifications we must consider that only a part of the proteome bears protein
421 modifications. Limitations in the selective analysis of this part can be overcome by using specific
422 procedures to selectively trap the modified peptides and/or reveal them among all those present in the
423 digest (Bachi *et al*., 2013). Because of the broad diversity of the chemical moieties introduced on the
424 amino acid side chains following non-enzymatic glycation, glycoxidation and oxidation processes
425 associated with thermal treatment of milk (Figure 2), no specific precursor ion scanning or NL

426 experiments can be realized in a unique LC-MS/MS run; thus, all possible modified peptides must be
427 considered in the course of subsequent database searching.
428 Combined shotgun proteomic procedures have therefore been developed for the global identification and
429 structural analysis of the different glycation and glycoxidation protein targets in raw, pasteurized, UHT
430 and powdered infant formula milk samples (Arena *et al*., 2010; 2011; Renzone, Arena & Scaloni, 2015).
431 For this purpose, soluble milk proteins and counterparts enriched for less abundant species by the
432 combinatorial peptide ligand library technology (D'Amato *et al*., 2009) as well as milk fat globule (MFG)
433 components have been resolved by mono-dimensional electrophoresis (Figure 4). The first technology
434 was selected for its effectiveness in equalizing concentration of proteins in samples where analytes
435 present very different quantities. Then, portions from entire electrophoretic lanes were digested with
436 trypsin (Arena *et al*., 2010), and a part of the corresponding digests was enriched for non-enzymatically
437 glycated peptides by affinity chromatography on agarose functionalized with *m*-aminophenylboronic
438 acid. Its use in trapping peptides bearing a *cis*-diol moiety directly from total protein digests made it
439 possible to identify modified proteins even at low-concentration levels. Authors used it to bind glycated
440 or glycoxidized peptides from milk under alkaline conditions to form corresponding reversible five-
441 member ring complexes, while most of the non-modified species were washed away (Arena *et al*., 2010;
442 2011; Renzone *et al*., 2015). Bound glycated/glycoxidized peptides were then eluted by washing with
443 volatile organic acids. MALDI-TOF MS analysis of eluted peptide digests from diverse dairy products
444 showed corresponding signal intensities and complexities that closely paralleled the harshness of milk
445 heating, proving the efficacy of this approach in enriching glycated/glycoxidized peptides from milk.
446 In order to identify glycated/glycoxidized peptides, while elucidating the identity of the modified protein,
447 the nature of the adducted chemical moiety and the modified residue(s), Scaloni and coworkers analyzed
448 either peptide digests directly deriving from gel and their counterparts subjected to phenylboronate
449 chromatography using nLC-ESI-LIT-MS/MS with ETD fragmentation and supplemental collisional

activation (Arena *et al*., 2010; 2011; Renzone *et al*., 2015) (Figure 4). This fragmentation technique was chosen because: *a*) CID of lactosylated peptides is associated with mass spectra having a poor intensity of sequence-informative ions (Arena *et al*., 2010) (see above); and *b*) parallel experiments using ETD and CID on peptides modified as Amadori products with D-glucose/D-galactose and corresponding dehydrated adducts, G-DH, Triose-DH, N^{ε}-carboxymethyllysine and pyrraline derivatives showed in the first four cases the best fragmentation spectra with ETD (Suppl. Figure S1), while the occurrence of no evident differences in spectral characteristics were present for the remaining molecules (Figure 5) (Renzone *et al*., 2015). To increase the amount of modified peptides identified, authors put each sample through two consecutive nLC-ESI-LIT-MS/MS runs, performing data-dependent product ion scanning over the five most intense precursor ions, which were automatically subjected to ETD fragmentation; during the second run, they excluded automatic fragmentation of peptides already identified in the first analysis. To identify lactosylated and AGE-containing peptides, authors then searched nLC-ESI-LIT-MS/MS raw data files against a non-redundant sequence database of *B. taurus*, chosing carbamidomethylation at Cys as fixed modification, and variable oxidations at Trp and Met, and the number of glycation/glycoxidation reactions at Lys and Arg (Figure 2) as non-fixed modifications.

When used for the analysis of soluble milk fraction or counterpart enriched for minoritary components by the combinatorial peptide ligand library technology, this shotgun approach permitted identifying 271 non-redundant sites of lactosylation in 33 proteins (Arena *et al*., 2010). Most of these proteins escape detection when combined 2-DE/immunoblotting experiments were used for the analysis of skimmed milk or whey (Marvin *et al*., 2002). Subsequently, 157 novel non-redundant lactosylation sites in 35 MFG proteins never before reported as being lactosylated were found in the fat globule portion (Arena *et al*., 2011), in addition to the 153 present in another 21 proteins detected as adsorbed on the MFG membrane, which were already identified in the soluble milk fraction (Arena *et al*., 2010). Overall, Scaloni and coworkers identified a total of 428 non-redundant sites of lactosylation in 68 proteins; most of that have

474 already been reported as involved in the delivery of nutrients, the defensive reaction against pathogenic
475 organisms, molecular processes associated with inflammation, and cell differentiative/proliferative
476 mechanisms.
477 This approach enabled the identification of a mutable number of modified amino acids within each
478 protein; as expected, their quantity rose with more extreme milk processing conditions. It also presented
479 a good reproducibility of the data on modification, because lactosylated residues identified in pasteurized
480 milk also occurred in UHT and powdered milk samples. Analogously, lactosylated amino acids observed
481 in UHT milk samples were mostly conserved in powdered counterparts. As predictable, most of the
482 modified residues were detected in main milk proteins, namely β-lactoglobulin, α-lactalbumin, all
483 caseins, lactotransferrin, lactoperoxidase, glycosylation-dependent cell adhesion molecule 1, lactadherin,
484 fatty acid binding protein, xanthine dehydrogenase and peripilin 2, which account for about 95% of the
485 whole polypeptide content of milk. The analyses also showed good qualitative coherence with earlier
486 investigations on lactosylated amino acids in main milk proteins (Leonil *et al*., 1997; Morgan *et al*., 1997;
487 Fogliano *et al*., 1998; Siciliano *et al*., 2000; Scaloni *et al*., 2002; Meltretter *et al*., 2008). The widespread
488 modification detected for components from UHT and powdered milk samples was in very good
489 agreement with quantitative amino acid analysis data on equivalent hydrolysates (Henle et al., 1991), as
490 well as MS measurements on intact β-lactoglobulin, α-lactalbumin, αS1- and β-casein, which already
491 ascertained protein populations containing mono-, di- and tri-lactosylated proteins, together with the non-
492 modified counterparts (Leonil *et al*., 1997; Fogliano *et al*., 1998; Siciliano *et al*., 2000; Scaloni *et al*.,
493 2002).
494 This shotgun approach was also used to identify AGE-modified proteins in soluble and MFG milk
495 portions; it identified 31 proteins bearing 308 intermediate (IG) and advanced glycoxidation (AGE)
496 derivatives, including cross-linked ones (Renzone *et al*., 2015). Besides confirming all lactosylated
497 amino acids ascertained in our previous investigations (Arena *et al*., 2010; 2011), this study also

498 characterized 40 additional lactulosyl-lysine derivatives, yielding the widest record of proteins bearing
499 non-enzymatic glycation in commercial milk samples. The results have been confirmed by recent
500 investigations in this field (Milkovska-Stamenova & Hoffmann, 2016*a*; 2016*b*). Focusing on IG and
501 AGE derivatives, Scaloni and coworkers recognized 289 modified peptides in the milk samples, bearing:
502 a) lactulosyl-lysine-H_2O (+306 Da), lactulosyl-lysine-$2H_2O$ (+288 Da), the Amadori product with D-
503 glucose or D-galactose (Hex)/3-DG-He/3-DGal-He (+162 Da), Hex-H_2O (+144 Da), Hex-$2H_2O$ (+126
504 Da), N^{ε}-carboxymethyllysine/G-He (+58 Da), N^{ε}-carboxyethyllysine/MG-He (+72 Da), pyrraline (+108
505 Da), 3-DPen-He (+132 Da) and lactosone-derived hemiaminal (+340 Da) adducts at Lys residues; and
506 b) argpyrymidine (+80 Da), G-H (+40 Da), MG-H (+54 Da), G-DH/G-He (+58 Da), MG-DH/MG-He
507 (+72 Da), 3-DG-DH/3-DGal-DH/3-DG-He/3-DGal-He (+162 Da), 3-DLact-DH/3-DLact-He (+324 Da),
508 3-DLact-H (+306 Da) and Trios-DH/Trios-He (+88 Da) adducts at Arg. Sometimes, IG and AGE
509 assignment was not conclusive, because identical mass difference values are assignable to different
510 adducts on the same residue. However, recent studies on hemiaminals have found these compounds not
511 to be stable for extended lengths of time (Mittelmaier *et al*., 2011; Meltretter *et al*., 2013); thus, they may
512 be omitted from the list reported above.
513 Overall, 3 IG/AGE-containing peptides were detected in raw milk, 30 in pasteurized milk, 112 in UHT
514 milk, and 281 in infant formula samples. They were related to 169 non-redundant IG/AGE-product-
515 modified amino acids in milk protein components; the latter have already been detected as lactosylated
516 in this food, thus strengthening the reliability of data. The most frequent IG/AGE adducts were the
517 Amadori product with D-glucose or D-galactose/3-DG-He/3-DGal-He, the dehydrated Amadori product
518 with lactose, N^{ε}-carboxymethyllysine/G-He, G-DH/G-He, 3-DG-DH/3-DGal-DH/3-DG-He/3-DGal-He
519 and 3-DLact-DH/3-DLact-He (about 52, 10, 8, 5, 4 and 3% of all modified, non-lactosylated components,
520 respectively). We also identified 16 peptide adducts presenting a mass increment of +40 or +54 Da
521 localized at specific lysines, which corresponded to G and MG-dependent Schiff bases.

522 Finally, we observed 3 linear cross-linked peptides with an intra-molecular bond at Lys and Lys/Arg
523 residues; molecular mass of parent ion and corresponding sequencing data assigned it to DOLD,
524 DOGDIC and pentosidine adducts (Figure 6). Concomitant identification of fragment ions related both
525 linked residues ensured no uncertainty in the assignment of these cross-linked peptides. Some of these
526 structural species have alredy been reported in previous investigations on model protein-sugar mixtures
527 subjected to heating under controlled conditions. The nature of these cross-linked peptides and of the
528 other linear IG/AGE products ascertained in dairy samples suggested G, 3-DG and 3-DGal as the most
529 reactive α-dicarbonylated species present therein, in agreement with lactose oxidative degradation
530 products identified in heated milk.
531 Indeed, identification of IG/AGE-containing peptides in various milk fractions strongly paralleled the
532 detection of the corresponding proteins and the corresponding lactosylated counterparts in the same
533 samples (Arena *et al*., 2010; 2011) and, in the latter case, even in milk types subjected to milder
534 processing conditions. These results confirm that, depending on the reactivity of each protein Lys residue
535 and the severity of milk heating, adductable lysines first react with lactose to yield the corresponding
536 Amadori products. These early adduction derivatives are further oxidized/modified to yield the
537 corresponding IG and AGE counterparts. Conversely, non-modified arginines and lysines in these
538 species can react with D-glucose, D-galactose, or lactose degradation/oxidation products (G, MG, 3-
539 DLact, 3-DG, 3-DGal, 3-DPen, lactosone and triosone), ultimately determining the production of AGE
540 counterparts. The progressively heightened detection of IG/AGE products in milk samples subjected to
541 increasingly harsh processing conditions is consistent with corresponding GC-MS- or LC-MS-derived
542 quantitative data on amino acid derivatives in total hydrolysates (Ahmed *et al*., 2005; Erbersdobler *et al*.,
543 2007; Pischetsrieder *et al*., 2012).
544 The results reported in these studies for whey proteins and caseins concurs with recently published data
545 on specific components from raw milk, commercial dairy counterparts and other protein models heated

546 in the presence of sugars, in terms of the identity of IG/AGE products ascertained and the modified
547 lysines/arginines detected therein (Meltretter *et al*., 2007; 2008; 2013; Carulli *et al*., 2011; Holland *et al*.,
548 2011; Calvano *et al*., 2013). Any discrepancies among these investigations can be attributed to the
549 chromatography on phenylboronate-derivatized support, which specifically enriched diol-containing
550 IG/AGE-modified peptides in all the analyzed fractions.
551 Visual analysis of the crystallographic/spectroscopic structural models of the labeled milk proteins
552 reveals that modified amino acids generally occurred on the external molecular face, although a few inner
553 residues were also labelled. Thus, the Maillard reaction may affect the conformation of these proteins
554 with large effects on their function. Because some milk proteins may resist to gastrointestinal digestion
555 (Castell, Friedrich, Kuhn & Poppe, 1997; Lonnerdal, 2003) and extrinsecate their function directly in the
556 human gut or additional organs (Lonnerdal, 2003; D'Alessandro, Zolla & Scaloni, 2011), their
557 modification as a consequence of heating can have important reflections for the nutritional/nutraceutical
558 characteristics of food. The shotgun investigations discussed above demonstrate that different milk
559 proteins whose function is related to the delivery of nutrients, the defensive reaction against pathogenic
560 organisms, molecular processes associated with inflammation, and cell differentiative/proliferative
561 mechanisms (Arena *et al*., 2010; 2011; Renzone *et al*., 2015) undergo glycation/glycoxidation after
562 heating, thus meaning that severe thermal treatment may affect the corresponding activity (Sebekova &
563 Somoza, 2007; Henle, 2007). This may have significant consequences when milk is the only origin of
564 nutrient/defensive compounds, as in the infant nutrition.
565 Since proteases do not recognize modified amino acids, the Maillard reaction can also determine
566 alterations in the gastrointestinal processing of milk proteins (Dalsgaard, Nielsen & Larsen, 2007).
567 Recent studies have investigated these possible changes by comparatively evaluating the digestion of
568 whey proteins and caseins from various dairy products (Arena *et al*., 2011; Wada *et al*., 2014). For major
569 soluble components, more *in vivo*/*in vitro* digestion recalcitrance of the intact proteins was measured by

570 SDS-PAGE in untreated and pasteurized milks than in UHT and sterilized counterparts. However, protein
571 sensitivity to digestion (as assayed by the Kjedahl method) displayed a slightly contrary trend, as
572 previously reported for infant-directed dairy products (Rudloff & Lonnerdal, 1992; Rutherfurd &
573 Moughan, 2005). This condition was rationalized to mean that industrial thermal processing can increase
574 milk protein digestibility by generating conformational changes that make molecular species more prone
575 to proteolysis, but this augmentation is counterweighted by temperature-dependent Maillard reaction
576 adductions that reduce molecular digestibility (Wada *et al*., 2014). When fatty globular proteins were
577 considered, Scaloni and coworkers observed an augmented component recalcitrance to digestion with
578 trypsin when moving from pasteurized to UHT to powdered milk (Arena *et al*., 2011). On the whole, the
579 data reported above confirmed other investigations on various model protein-saccharide systems (Corzo-
580 Martinez, Soria, Belloque, Villamiel & Moreno, 2010). Aside from the potential physiological
581 implications of assuming poorly-digestible material as food, the finding is of definite importance for
582 various soluble milk components that, following compromised proteolytic digestion, may generate
583 reduced amounts of well-known sequence-encrypted polypeptides with ACE inhibitor, antothrombotic,
584 opioid and antimicrobial properties in strongly heated milk. A limited bioactivity of these molecules can
585 perhaps also be associated with their undesired glycation/glycoxidation. Meanwhile, the poorer
586 digestibility of milk proteins undergoing the Maillard reaction may affect their allergenicity in harshly-
587 treated commercial milk products; in this context, Astwood and coworkers and Wal (Astwood, Leach &
588 Fuchs, 1996; Wal, 2001) have reported that digestion-recalcitrant food proteins may act as major
589 allergens. Related to this issue is also the fact that extensive glycation/glycoxidation of surface-accessible
590 amino acids in various milk proteins can also affect corresponding allergenic potential (Karamanova,
591 Fukal, Kodicek, Rauch, Mills & Morgan, 2003; Gruber, Vieths, Wangorsch, Nerkamp & Hofman, 2004;
592 Heilmann *et al*., 2014).
593

6. Conclusions

Although important progress has been made in studying the Maillard reaction in dairy products, important issues of a chemical, biological and immunological nature remain unsolved. After a preliminary stage focused on model systems and/or purified milk proteins, in which researchers characterized the main Maillard reaction products, new efforts currently are underway identifying all modified molecules in dairy products and elucidating the chemical structure of the corresponding derivatives. In this context, proteomic methods nowaday play an essential role, as underlined in the final sections of this manuscript. This information is essential for understanding the nutritional, biological and toxicological characteristics of milk, because humans ingest large amounts of heated milk proteins on a daily basis, and for formula-fed infants they are the unique source of polypeptides. The available data on the consequence of heating this food on the biological activity and allergenic potential of milk proteins remains conflicting (Astwood *et al*., 1996; Wal, 2001; Karamanova *et al*., 2003; Lonnerdal, 2003; Gruber *et al*., 2004; Sebekova *et al*., 2007; Henle, 2007; Muscat *et al*., 2009; Corzo-Martinez *et al*., 2010; D'Alessandro *et al*., 2011; Shandilya, Kapila, Haq, Kapila & Kansal, 2013; Hellwig *et al*., 2014; Heilmann *et al*., 2014, Verhoeckx et al., 2015 and references therein), potentially due to the variable behavior of the different milk molecules toward heat-dependent chemical modification and unfolding. Information on heat-induced adductions in milk proteins is also essential for understanding the technical properties of novel milk-origin products, now common in foods, pharmaceuticals and cosmetics. Protein modification data will contribute to the generation of complete milk-focused data catalogue, which may eventually allow food producers to heat-treat their products as carefully as possible to achieve controlled quantities of AGEs. Further studies using quantitative MS methods are therefore needed to gain information on the residues most prone to generating adducts in different milk proteins (Bachi *et al*., 2013; Siciliano *et al*., 2014; Arena *et al*., 2014). In this context, recent preliminary proteomic investigations on quantitative basis (Le *et al*., 2013; Meltretter *et al*., 2014; Wust *et al*., 2016; Milkovska-

618 Stamenova *et al.*, 2016*a*; 2016*b*) have confirmed that the degree of site-specific modification well
619 parallels with the harshness of heating, but have also highlighted that it changes significantly among the
620 brands of UHT products and powdered infant formulas (Milkovska-Stamenova *et al.*, 2016*a*). This
621 indicates that a company's specific production processes need to be considered apart from the generic
622 classification of milk as pasteurized, UHT or powdered. By making the right adjustments to their
623 technical processes, companies should be able to reduce the level of modification in both UHT and
624 powdered milk products. Furthermore, recent research has found that lactose-free milks also present a
625 degree of glycation that varies highly among various brands, with lactose-free UHT milks and infant
626 formulas showing the highest levels (Milkovska-Stamenova *et al.*, 2016*b*). All of this information is
627 essential for connecting the extent of the Maillard reaction occurring in various commercial products to
628 potential nutritional consequences. The issues described above and those relating to other modifications
629 from the industrial treatment of milk (Pischetsrieder *et al*., 2012; Bachi *et al*., 2013) should be fully
630 evaluated for a comprehensive understanding of the nutraceutical and toxicological properties of dairy
631 products.
632
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641

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Legend to Figures

Figure 1. The Hodge Diagram. The initial reaction between a reducing sugar and a protein –NH_2 group forms the unstable Schiff base (reaction *a*), which then slowly rearranges to form the corresponding Amadori product (reaction *b*). Degradation of the Amadori product (reaction *c*). Formation of reactive carbonyl and α-dicarbonyl compounds (reaction *d*). Formation of Strecker aldehydes of amino acids and aminoketones (reaction *e*). Aldol condensation of furfurals, reductones, and aldehydes produced in reaction *c*, *d* and *e* without the intervention of NH_2-containing compounds (reaction *f*). Reaction of furfurals, reductones, and aldehydes produced in reaction *c*, *d* and *e* with NH_2-containing derivatives to form melanoidins (reaction *g*). Free radical-mediated formation of carbonyl fission products from the reducing sugar (Namiki pathway) (reaction *h*).

Figure 2. Chemical structure of linear and cross-linked early, intermediate and advanced glycosylation end-product (AGE) derivatives putatively formed in milk products. (**A**) Linear and cross-linked derivatives occurring exclusively at Lys. LL, the Amadori compound with lactose; LL-H_2O, the mono-dehydrated derivative of the Amadori compound with lactose; LL-$2H_2O$, the bis-dehydrated derivative of the Amadori compound with lactose; N^{ε}-(5,6-dihydroxy-2,3-dioxohexyl)-Lys; N^{ε}-(2,3-dihydroxy-5,6-dioxohexyl)-Lys; Hex, the Amadori compound with D-glucose/D-galactose; Hex-H_2O, the mono-dehydrated derivative of the Amadori compound with D-glucose/D-galactose; Hex-$2H_2O$, the bis-dehydrated derivative of the Amadori compound with D-glucose/D-galactose; PYR, pyrraline; CML, N^{ε}-carboxymethyl-lysine; CEL, N^{ε}-carboxyethyl-lysine; AFGP, 1-alkyl-2-formyl-3,4-glycosyl-pyrrole; GOLD, glyoxal-derived lysine dimer; MOLD, methylglyoxal-derived lysine dimer; POLD, 3-deoxypentosone-derived lysine dimer; DOLD, 3-deoxyglucosone/3-deoxygalactosone-derived lysine dimer; DLactOLD, 3-deoxylactosone-derived lysine dimer; GLUCOLD, glucosone-derived lysine dimer; LACTOLD, lactosone-derived lysine dimer; Hex-derived fluorolink, D-glucose/D-galactose-

892 associated fluorolink; Lact-derived fluorolink, lactose-associated fluorolink; Hex-derived crossline, D-
893 glucose/D-galactose-associated crossline; Lact-derived crossline, lactose-associated crossline. (**B**)
894 Linear and cross-linked derivatives occurring either at Lys and Arg. G-He, glyoxal-derived hemiaminal;
895 MG-He, methylglyoxal-derived hemiaminal; Trios-He, triosone-derived hemiaminal; 3-DPen-He, 3-
896 deoxypentosone-derived hemiaminal; 3-DG-He, 3-deoxyglucosone-derived hemiaminal; 3-DGal-He, 3-
897 deoxygalactosone-derived hemiaminal; 3-DLact-He, 3-deoxylactosone-derived hemiaminal; Gluc-He,
898 glucosone-derived hemiaminal; Gal-He, galactosone-derived hemiaminal; Lact-He, lactosone-derived
899 hemiaminal; PENT, pentosidine; glucosepane, 6-[2-{[(4*S*)-4-ammonio-5-oxido-5-oxopentyl]amino}-
900 6,7-dihydroxy-6,7,8,8*a*-tetrahydroimidazo[4,5-*b*]-azepin-4(5*H*)-yl]-L-norleucinate; GODIC, glyoxal-
901 derived imidazolium cross-link; MODIC, methylglyoxal-derived imidazolium cross-link; DPenDIC, 3-
902 deoxypentosone-derived imidazolium cross-link; DOGDIC, 3-deoxyglucosone/3-deoxygalactosone-
903 derived imidazolium cross-link; DLactDIC, 3-deoxylactosone-derived imidazolium cross-link. (**C**)
904 Linear and cross-linked derivatives occurring exclusively at Arg. G-DH, glyoxal-derived
905 dihydroxyimidazoline; MG-DH, methylglyoxal-derived dihydroxyimidazoline; Trios-DH, triosone-
906 derived dihydroxyimidazoline; 3-DPen-DH, 3-deoxypentosone-derived dihydroxyimidazoline; 3-DG-
907 DH, 3-deoxyglucosone-derived dihydroxyimidazoline; 3-DGal-DH, 3-deoxygalactosone-derived
908 dihydroxyimidazoline; 3-DLact-DH, 3-deoxylactosone-derived dihydroxyimidazoline; G-H, glyoxal-
909 derived hydroimidazolone; MG-H, methylglyoxal-derived hydroimidazolone; Trios-H, triosone-derived
910 hydroimidazolone; 3-DPen-H, 3-deoxypentosone-derived hydroimidazolone; 3-DG-H, 3-
911 deoxyglucosone-derived hydroimidazolone; 3-DGal-H, 3-deoxygalactosone-derived hydroimidazolone;
912 3-DLact-H, 3-deoxylactosone-derived hydroimidazolone; RPYR, argpyrimidine; DHP, N^{δ}-(4-carboxy-
913 4,6-dimethyl-5-hydroxy-1,4-di-hydropyrimidine-2-yl)ornithine; THP, N^{δ}-(4-carboxy-4,6-dimethyl-5,6-
914 di-hydroxy-1,4,5,6-tetra-hydropyrimidine-2-yl)ornithine.
915

Figure 3. Comparison of the spectra obtained for a lactosylated peptide under CID and ETD fragmentation conditions. CID (**A**) and ETD (**B**) tandem mass spectra of the $[M+4H]^{4+}$ ion at *m/z* 804.41, which was associated with the tryptic lactosylated peptide (74–99) from lactotransferrin; k represents lactose adduct to lysine. The spectra were acquired with alternating CID and ETD scanning. Identified fragment ions are labeled above and below the peptide sequence in both panels.

Figure 4. Schematic diagram illustrating the experimental approach used for shotgun proteomic analysis of heat-dependent milk protein modifications. PB chrom, phenylboronate chromatography. Whey proteins added with amounts of counterparts from corresponding skimmed milk was used to prepare soluble components that were further subjected to peptide ligand library-based enrichment; this mixed material is named in the figure as equalized whey.

Figure 5. Comparison of the spectra obtained for a N^{ε}-carboxymethyllysine-containing peptide under CID and ETD fragmentation conditions. CID (panel A) and ETD (panel B) MS/MS spectra of the $[M+3H]^{3+}$ ion at *m/z* 565.5 associated with the N^{ε}-carboxymethyllysine-containing peptide (125-138) from β-lactoglobulin, where k represents the carboxymethyl adduct to Lys135. The spectra were acquired with alternating CID and ETD scanning.

Figure 6. Fragmentation spectrum of a cross-linked peptide bearing an intramolecular DOLD moiety. ETD MS/MS spectrum of the $[M+3H]^{3+}$ ion at *m/z* 447.0 associated with the DOLD-containing peptide (113-123) from serotransferrin, where the DOLD cross-linking between Lys114 and Lys115 is indicated.

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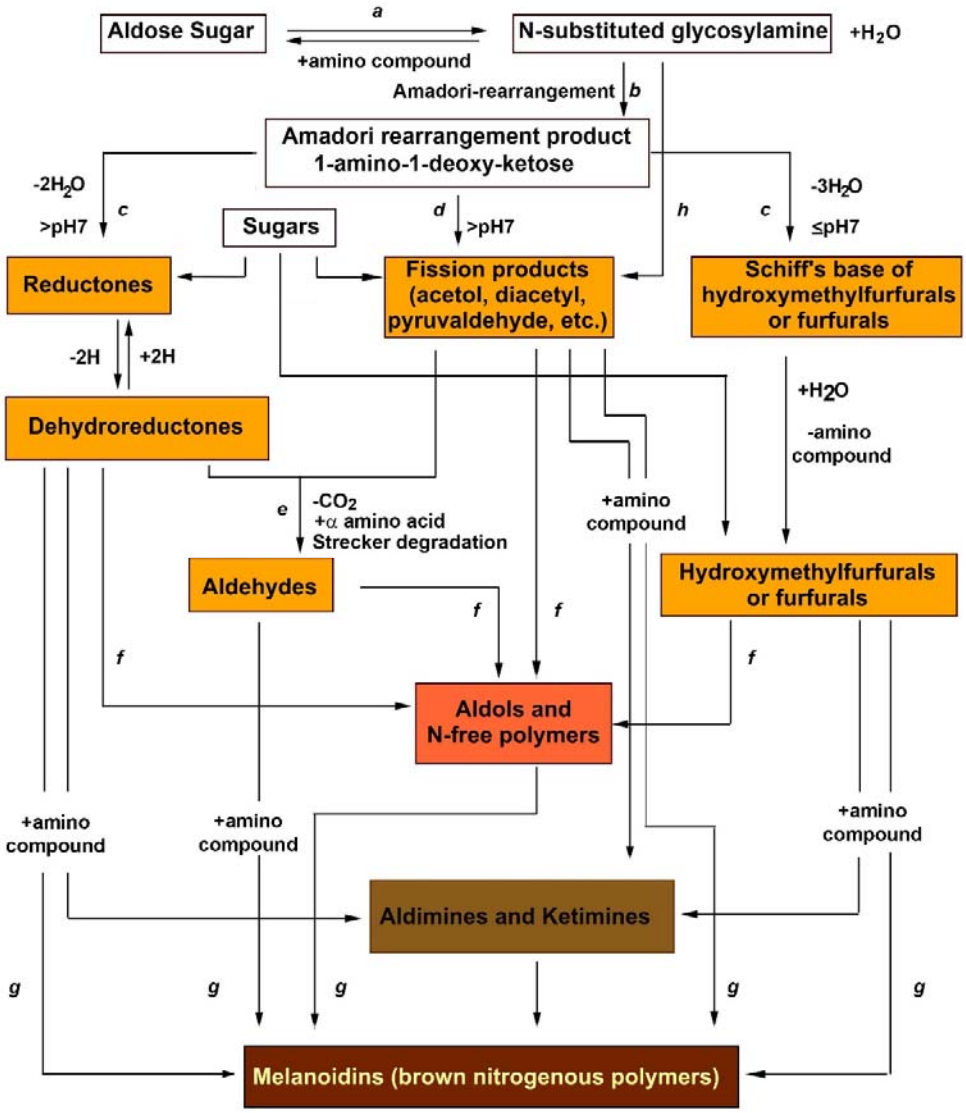

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942 **Arena et al., Figure 1**

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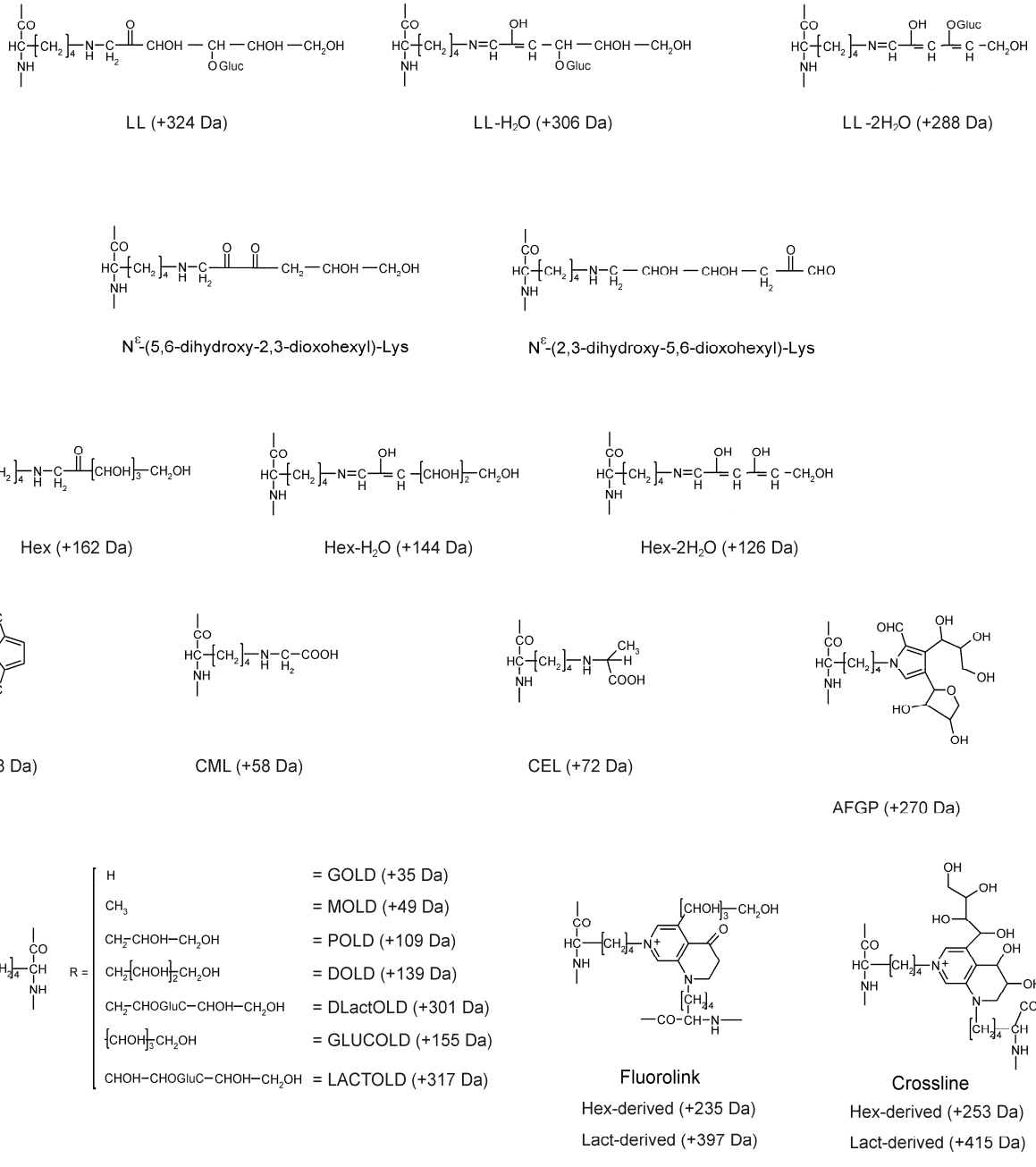

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947 **Arena et al., Figure 2A**

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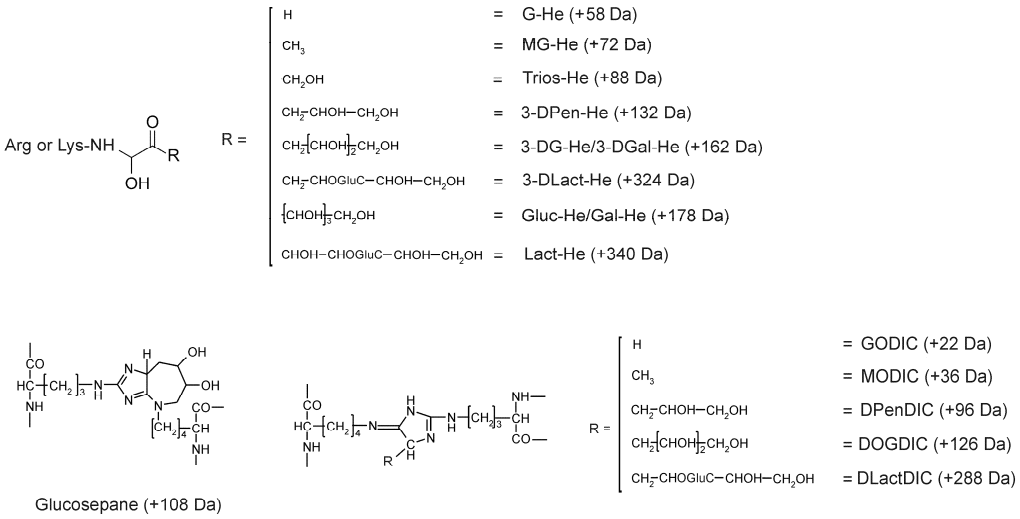
B



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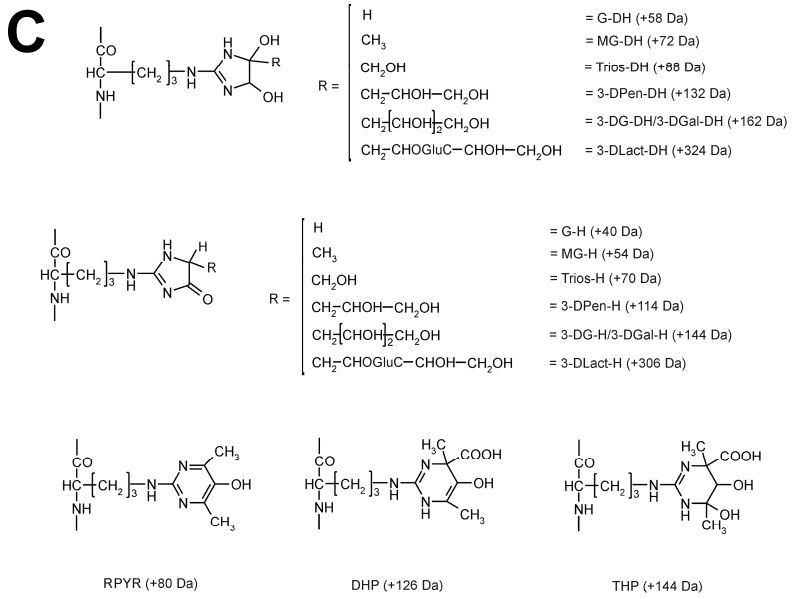
C



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955 **Arena et al., Figure 2B and 2C**

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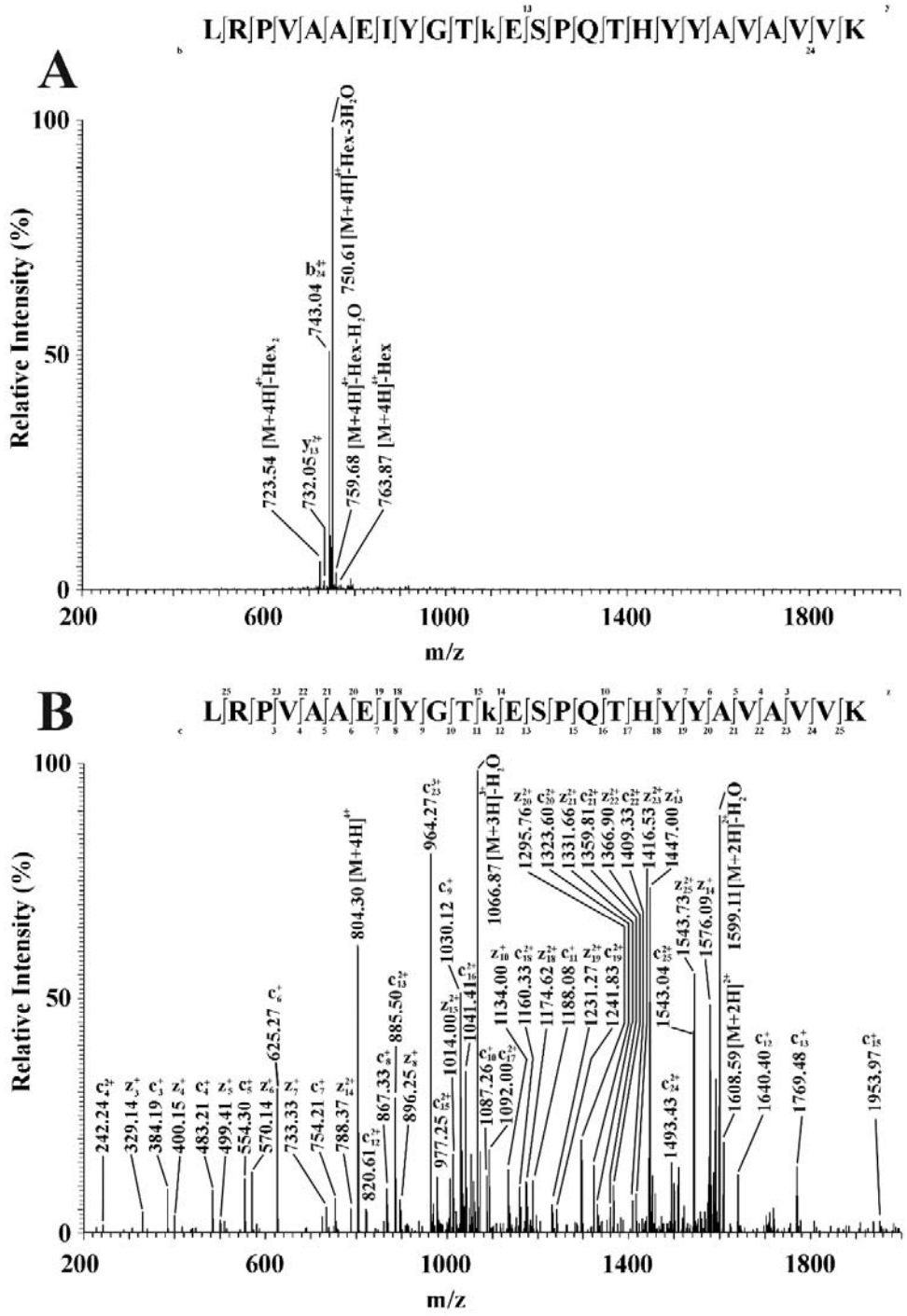

Arena et al., Figure 3

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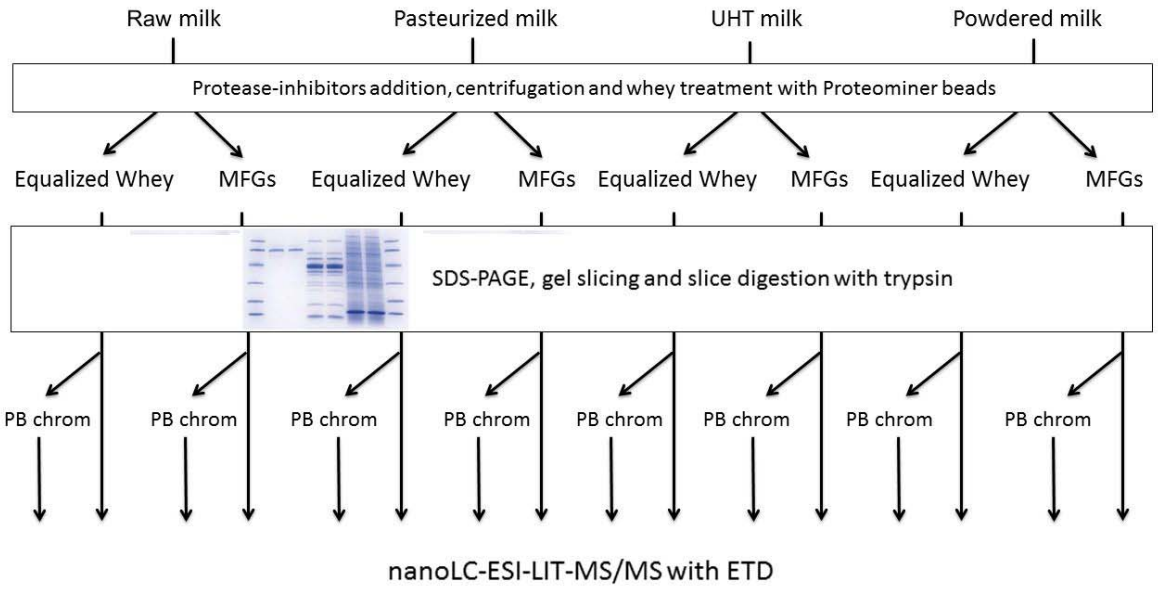

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968 **Arena et al., Figure 4**

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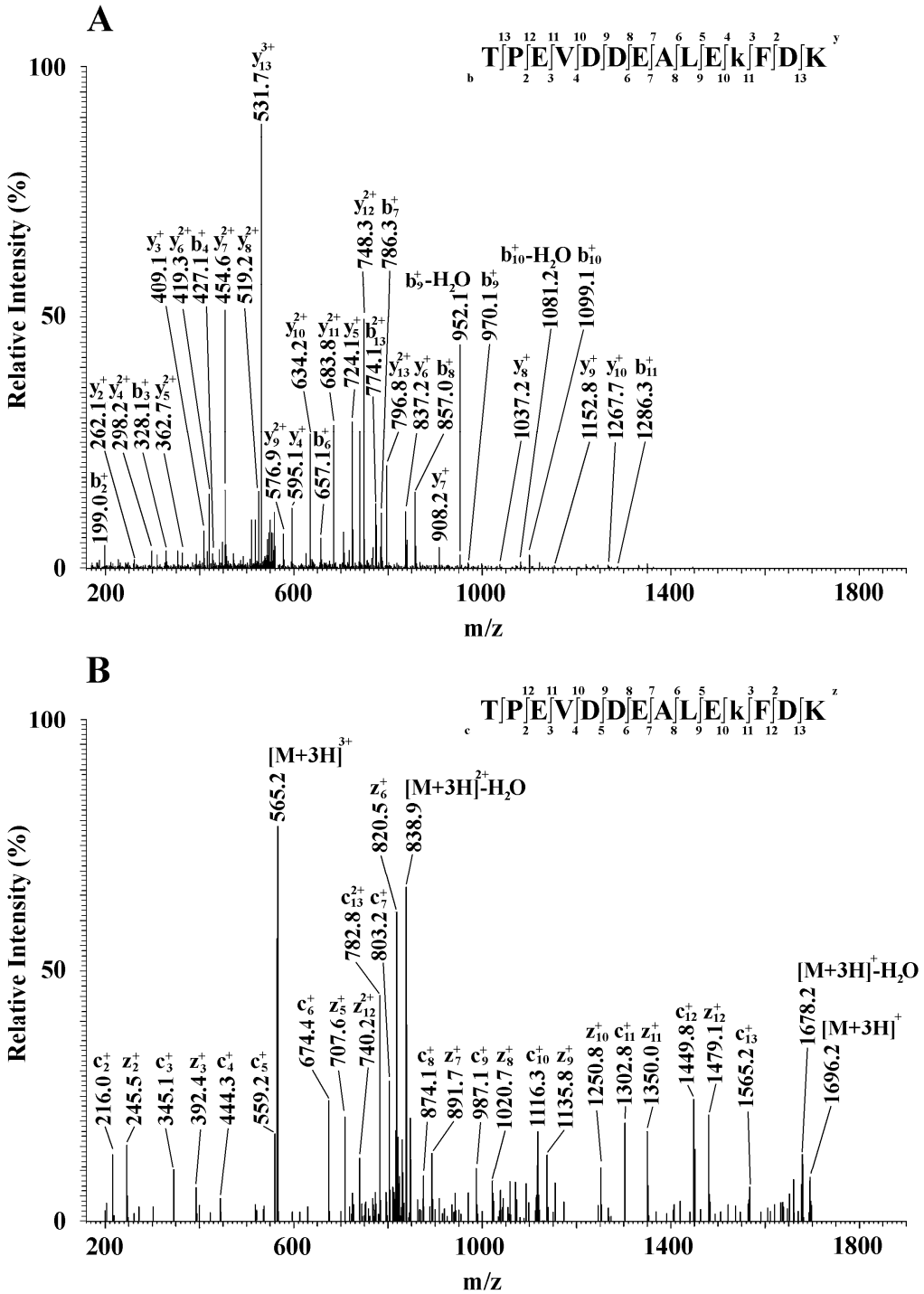
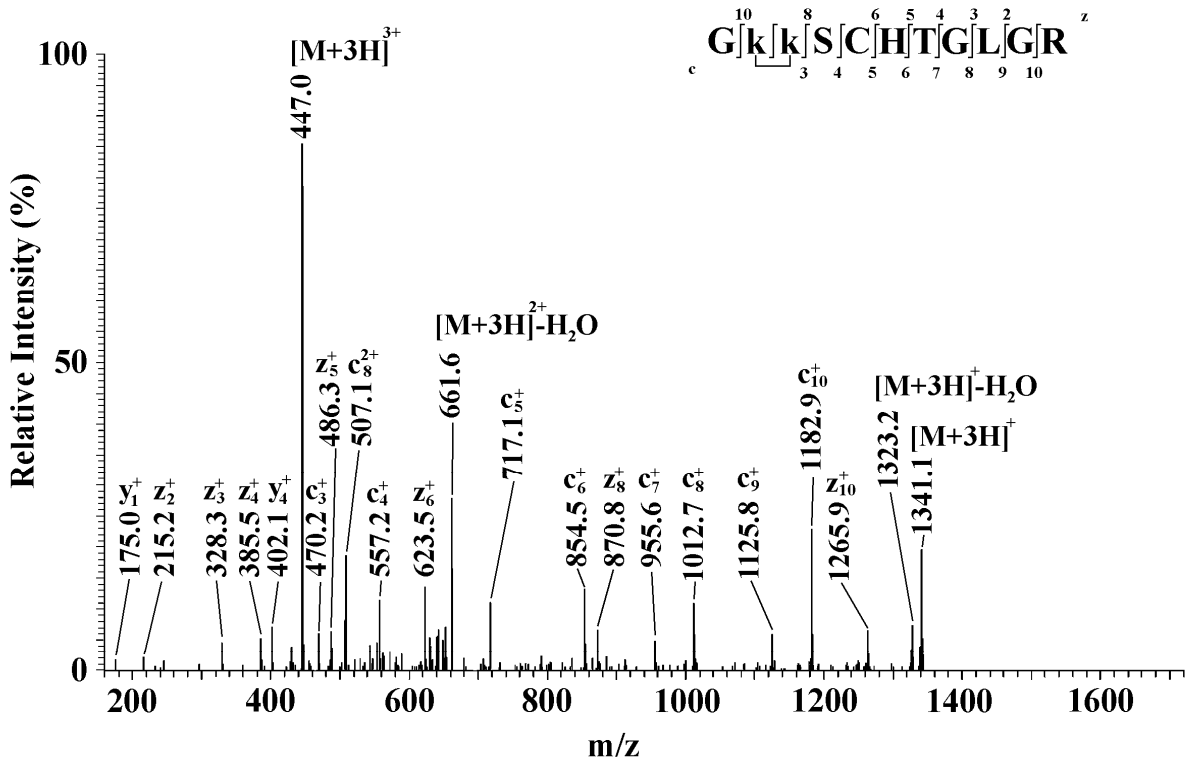


Arena et al., Figure 5



Arena et al., Figure 6