JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Proteomic Approach Based on MALDI-TOF MS To Detect Powdered Milk in Fresh Cow's Milk

Cosima Damiana Calvano,*[,†] Antonio Monopoli,[†] Pasqua Loizzo,[‡] Michele Faccia,[‡] and Carlo Zambonin[†,#]

[†]Dipartimento di Chimica, [‡]Dipartimento di Scienza del Suolo, della Pianta e degli Alimenti, and [#]Centro Interdipartimentale SMART, Università degli Studi di Bari Aldo Moro, via Orabona 4, 70126 Bari, Italy

ABSTRACT: Milk and cheese are expensive foodstuffs, and their consumption is spread among the population because of their high nutritional value; for this reason they are often subjected to adulterations. Among the common illegal practices, the addition of powdered derivatives seems very difficult to detect because the adulterant materials have almost the same chemical composition of liquid milk. However, the high temperatures (180−200 °C) used for milk powder production could imply the occurrence of some protein modifications (e.g., glycation, lactosylation, oxidation, deamidation, dehydration). The modified proteins or peptides could then be used as markers for the presence of powdered milk. In this work, matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) was employed to analyze tryptic digests relevant to samples of raw liquid (without heat treatment), commercial liquid, and powdered cow's milk. Samples were subjected to two-dimensional gel electrophoresis (2-DE); differences among liquid and powder milk were detected at this stage and eventually confirmed by MALDI analysis of the in gel digested proteins. Some diagnostic peptides of powdered milk, attributed to modified whey proteins and/or caseins, were identified. Then, a faster procedure was optimized, consisting of the separation of caseins from milk whey and the subsequent in-solution digestion of the two fractions, with the advantage of obtaining almost the same information in a limited amount of time. Finally, analyses were carried out with the fast procedure on liquid milk samples adulterated with powdered milk at different percentages, and diagnostic peptides were detected down to 1% of adulteration level.

KEYWORDS: *adulteration, cow, powdered milk, MALDI, peptide, 2D gel*

INTRODUCTION

Milk and cheese are expensive foodstuffs, and their consumption is spread among the population because of their high nutritional value; for this reason some dishonest producers, who are tempted by the possibility to enlarge their profits, commit different frauds.[1] To protect public health, continuous efforts are made by authorities to adopt strict measures to prevent adulteration that promotes fraud and/or that might be negative to the health of consumers. These efforts are still inadequate because adulterations are more and more sophisticated and, in some cases, due to the lack of adequate detection techniques.

According to the legislation that regulates the production and marketing of milk and dairy products in different countries, the most common illegal practices are the selling of skimmed milk and semiskimmed milk instead of whole milk,[2] milk dilution by water,[3] the addition of milk anhydrous products to liquid milk,[4] and the mixing of high-quality milk such as buffalo's, sheep's and goat's with cow's milk.[5]

The adulteration of fresh milk by reconstituted milk and the selling of reconstituted milk as fresh product can be economically advantageous when either a surplus of milk powder exists or the importation of dried milk powder is subsidized. The European Union (EU) regulation does not allow the use of powdered milk or milk proteins in the production of Protected Designation of Origin cheeses (UE Regulation no. 510/2006 of 20.03.2006), and in some countries, such as Italy, it is not allowed in any type of cheesemaking process (art. 1 no. 138 of 11.04.1974). When legislation prohibits this practice, it becomes necessary to develop new analytical methods able to detect reconstituted milk sold as (or mixed with) fresh milk by discriminating some particular features of dried milk, which has almost the same chemical composition as fresh milk.

However, to obtain milk powder, fresh milk is submitted to heat and pressure treatments during processing, causing chemical changes that affect different components, mainly proteins and carbohydrates.[6−9] Usually they result in interactions between the amino acid lateral groups, degradation reactions of amino acid lateral chains, interactions with lipids, unfolding of proteins, and whey protein association with casein micelles.[10] In addition, deamidation, protein cross-linking, and interactions between carbohydrates and proteins can also be observed.[11] In fact, the relatively high concentration of reducing carbohydrates (lactose) and lysine-rich proteins makes thermally induced nonenzymatic reactions (Maillard reaction) favorable. During heating, advanced Maillard reaction products, such as glyoxal and methylglyoxal, may react with lysine (and, to a lesser extent, cysteine, methionine, and tryptophan[12]) to form cross-linked proteins.[13] Under severe conditions an active compound, dehydroalanine, is formed, which reacts with lysine via its double bond producing lysinoalanine (LAL). As a consequence, lower bioavailability of important amino acids (e.g., lysine[14]) and a general deterioration of functional

Special Issue: IX Italian Congress of Food Chemistry

Received: July 11, 2012
Revised: August 28, 2012
Accepted: August 29, 2012
Published: August 29, 2012

ACS Publications © 2012 American Chemical Society

properties, with remarkable effects also on technological processes (e.g., cheesemaking) involving whey proteins, are observed. These results may not be simply applied to every manufacturer because different heating procedures during pasteurization or different mixing steps during homogenization may be used, but it is reasonable to conclude that eventually modified-protein milk can be related to the presence of milk powder.

Methods to individuate milk powder adulteration include the detection of furosine or lysinoalanine by liquid[15] or gas chromatography,[16] the determination of empirical parameters by using ultraviolet and visible spectra of milk,[17] and the determination of hydroxymethylfurfural (HMF).[18] Moreover, measurements of fluorescence at different wavelengths or the fluorescence of advanced Maillard products and soluble tryptophan (FAST) index have been also used.[19] Another possibility of distinguishing raw from reconstituted milk consists in measuring δD and δ^{18}O values using stable isotope ratio mass spectrometry.[20] However, the methods described above are often time-consuming and/or expensive or require tedious sample pretreatment procedures. As recently reported, a valid alternative to study the effects of lactosylation on the major milk proteins can be offered by specific mass spectrometry (MS) techniques such as matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF-MS) and electrospray ionization (ESI-MS).[21−24] The main advantages of MALDI are rapidity, sensitivity, and simplicity of the experiments, especially if compared with electrophoretic and chromatographic methodologies. On the other hand, ESI-MS could give more detailed structural information even if a previous chromatographic separation is often necessary to analyze complex mixtures and avoid ion suppression. Recently, MALDI has been successfully used for the detection of glycation and lactosylation of whey intact proteins in milk models,[25,26] heat-treated milk,[27] stored UHT milk,[28] and milk powder for infants.[29,30] However, to the best of our knowledge, MALDI applications for the analysis of fresh milk adulterated with powdered milk or whey proteins have not been reported so far.

In this work, a fast and sensitive MALDI-TOF MS method for the detection of the adulteration of powdered milk in liquid (raw and commercial) milk samples using specific peptide markers has been developed. Whey and casein fractions of milk samples were digested in solution and analyzed by MALDI using α-cyano-4-chlorocinnamic acid (ClCCA) as a matrix,[31] which enhances the detection of more acid peptides (such as those lactosylated) compared to traditional matrices such as α-cyano-4-hydroxycinnamic acid (CHCA) or 2,5-dihydroxybenzoic acid (DHB). Analyses were carried out on liquid milk samples adulterated with powdered milk at different percentages, and diagnostic peptides were detected down to a 1% of adulteration level.

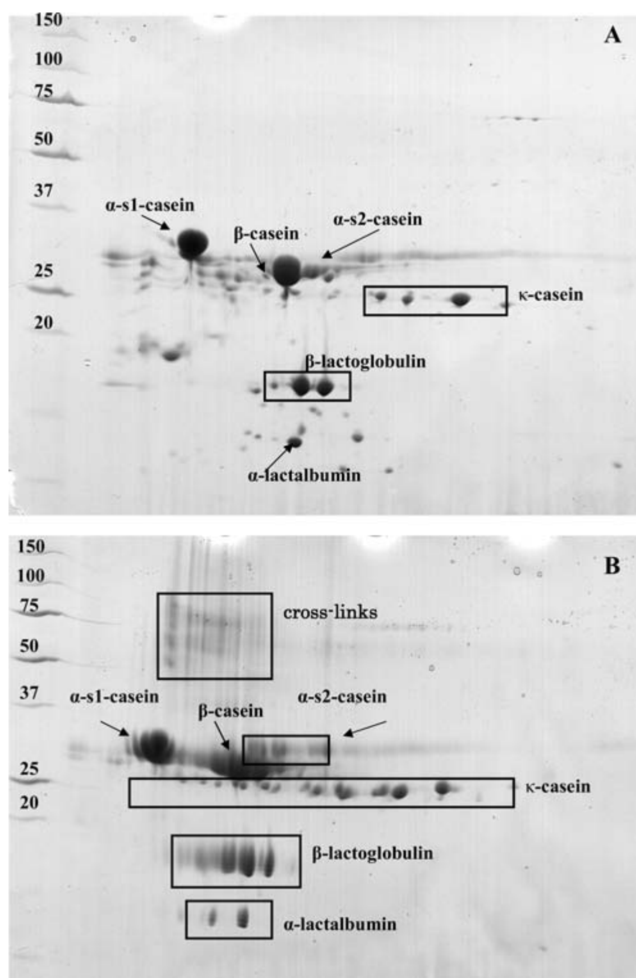

Figure 1. 2-DE gels relevant to (A) a raw and (B) a powdered milk sample. The samples were separated by using IPG strips (pH 4−7) in the first dimension and on a gradient polyacrylamide gel 10−17% in the second dimension.

■ EXPERIMENTAL PROCEDURES

Materials. Dithiothreitol (DTT), iodoacetamide, ammonium bicarbonate, urea, and porcine trypsin were obtained from Sigma-Aldrich (St. Louis, MO, USA). Trifluoroacetic acid (TFA) was from Fluka (St. Louis, MO, USA). All reagents used for two-dimensional (2D) gel electrophoresis (2-DE) were from Amersham Bioscience (Bucks, UK). CHCA, ACTH 18−39 fragment, angiotensin I, and [Glu][1]-fibrino peptide (GFP) were obtained from Sigma-Aldrich. Acetonitrile, water, chloroform, and methanol were of LC-MS grade; ClCCA was synthesized according to the procedure reported below and recrystallized four times before use.

Synthesis of ClCCA. ClCCA was synthesized according to a standard Knoevenagel condensation using cyanoacetic acid and *p*-chlorobenzaldehyde. Ammonium acetate was used as a catalyst. Two grams of cyanoacetic acid (1 equiv), 0.9 equiv of benzaldehyde, and 0.15 equiv of ammonium acetate were refluxed under stirring in sufficient amounts of toluene (ca. 50 mL). After quantitative separation of the reaction water by a Dean−Stark apparatus (ca. 3 h), the reaction mixture was cooled to 50 °C and filtered. The crude product was washed with sufficient amounts of distilled water and purified by recrystallization performed according to standard protocols.[32] In particular, recrystallization was carried out in 70% acetonitrile/30% water. A saturated solution of the matrix in 70% acetonitrile/30% water was heated until boiling. When the solid dissolved completely, the solution was cooled to room temperature and then on ice since a precipitate was observed. Finally, the precipitate was filtered and the procedure repeated in triplicate.

Samples. Raw cow's milk samples were obtained from different local farmers. Pure commercial milk samples of different brands and various types (UHT, pasteurized) were purchased from local supermarkets. Different brands of milk powder, infant milk powder, and milk protein concentrate have been tested. The procedure has been repeated at least in triplicate for all samples. Moreover, liquid cow's milk samples (raw and commercially pasteurized) intentionally adulterated with powdered milk or milk protein concentrate from 10 to 1% were prepared.

Sample Preparation. *2-DE.* Milk samples were centrifuged at 6000 rpm for 30 min at 4 °C, and the film of fat formed at the top of the solution was removed. The resulting solution was diluted in water (1:6). For milk powder, an aqueous solution (0.15 g/mL) was centrifuged at 6000 rpm for 30 min at 4 °C, and the lipid film was removed. The skimmed milk powder solution was diluted 1:3 in water.

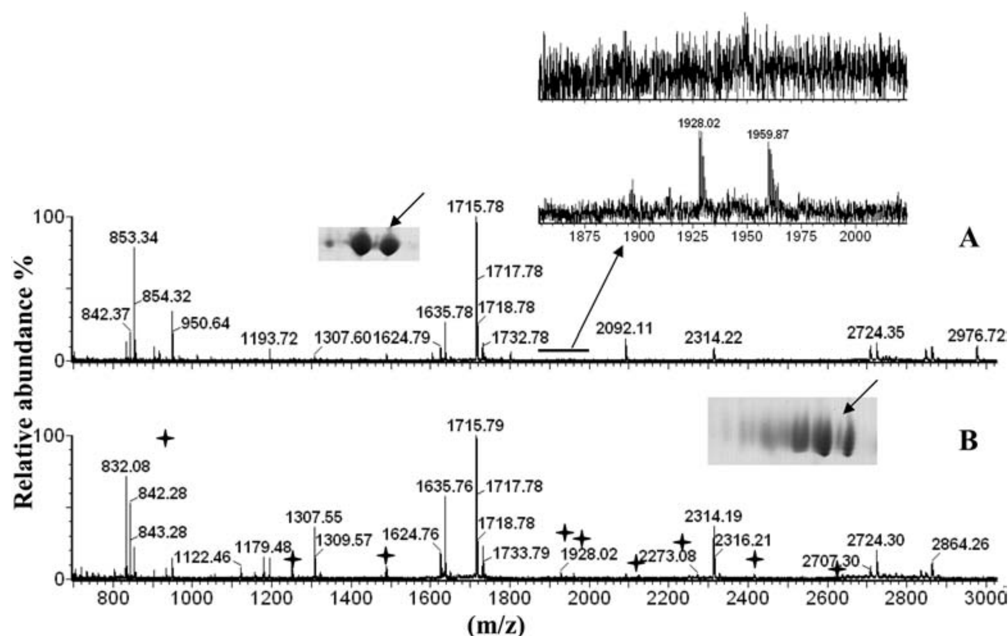

Figure 2. MALDI-TOF MS spectra relevant to one of the 2-DE gel spots of β-lactoglobulin obtained from (A) a raw and (B) a powdered milk sample.

Thus, 20 μL of fresh milk and milk powder (corresponding to 80 and 85 μg of total proteins, respectively) were dissolved in a rehydration buffer containing 8 M urea, 2% CHAPS, 2% Ampholine pH 4−7, 100 mM DTT, and traces of Bromophenol blue. IPG Dry Strips pH 4−7 gradient were left overnight in 250 μL of rehydration buffer.

Isoelectric focusing was performed using a Multiphor II electophoresis system (Pharmacia Biotech) in two steps: 500 V for 5 h and then 3500 V for 17 h. After the IPG electrophoresis, the strips were equilibrated for 25 min in a solution containing 1.5 M Tris-HCl, pH 8.8, 6 M urea, 30% glycerol (w/v), 2% SDS, 64 mM DTT, 135 mM iodoacetamide, and traces of Bromophenol blue.

The equilibrated IPG Dry Strips were loaded on top of the SDS-PAGE gradient gel 10−17%,[33] and electrophoresis was carried out overnight at a constant voltage of 100 V at 15 °C. The gels were stained in Blue silver[34] and destained in water.

In-Gel Digestion. The protein bands revealed by Blue silver staining were cut and digested by porcine trypsin (enzymatic activity >10000 UN/mg) using a specific protocol. At first, the bands were cut in small pieces that were covered with 200 μL of 200 mM NH_4HCO_3 in 40% of ACN, and the samples were incubated at 37 °C for 30 s in a thermoshaker. The step was repeated until the gel pieces were decolorized. The gel fragments were dried in speed vacuum for 15 s. Porcine trypsin (0.05 μg/μL) and 50 μL of 40 mM NH_4HCO_3 in 9% of ACN were added, and the samples were incubated at 37 °C overnight. After the incubation, the liquid fraction, containing peptides, was collected and acidified with TFA, mixed 1:1 (v/v) with the matrix CClCA, and then 1 μL of the sample/matrix solution was spotted, dried, washed on target twice, and analyzed by MALDI-TOF-MS.

Preparation of Caseins and Whey Fractions. Five milliliters of liquid milk or dried milk reconstituted in water (5 mg/mL) was treated with 250 μL of acetic acid to precipitate caseins. After centrifugation at 5000 rpm for 30 min, the casein pellet was stored and the remaining solution (containing whey proteins) was subjected to delipidation by adding an equal volume of ethyl ether. Then, the whey fraction was filtered through PVDF filters (0.45 μm porosity), and both fractions were dried under vacuum. Afterward, the fractions were redissolved in water (1 mg/mL) and subjected to in-solution digestion. The same procedure was followed for adulterated milk samples.

In-Solution Digestion. Each fraction was directly digested in solution as follows: 10 μL of whey or casein solution was mixed with 40 μL of 400 mM NH_4HCO_3 in 8 M urea. Then 5 μL of DTT (50 mM) was added, and the mixture was incubated for 40 min at 60 °C and chilled at room temperature. After 5 μL of iodoacetamide (150 mM) was added, the mixture was incubated in the dark at room temperature for 45 min. One hundred and twenty microliters of water and 20 pmol of porcine trypsin were added, and the solution was incubated overnight at 37 °C. The digestion was stopped with 10% trifluoroacetic acid (TFA). The tryptic digests were mixed 1:1 (v/v) with the matrix CClCA, and then 1 μL of the sample/matrix solution was spotted, dried, washed on target twice, and analyzed by MALDI-TOF-MS.

Mass Spectrometry. MALDI-TOF-MS analyses were performed on a Micromass M@LDI-LR time-of-flight mass spectrometer (Waters MS Technologies, Manchester, UK) equipped with a nitrogen UV laser (337 nm wavelength), a precision flat target plate sample introduction system bearing a microtiter target plate, reflectron optics, and a fast dual microchannel plate (MCP) detector.

Positive ion spectra were acquired in reflectron mode. The following voltages were applied: pulse, 2610 V; source, 15000 V; reflectron, 2000 V; MCP, 1900 V. The laser firing rate was 5 Hz, and, unless otherwise specified, 60 laser shots were used for each well. The 60 resulting spectra were averaged, background subtracted, and smoothed by a Savitzky−Golay algorithm.

For peptide analysis, the explored mass range was m/z 600−4000, and external mass calibration was performed using a peptide mixture composed of angiotensin I, the fragment ACTH 18−39, and GFP.

Protein Identification. The raw files relevant to tryptic digests were searched against the nonredundant database Swiss-Prot utilizing Mascot (Matrix Science Ltd., London, UK) or MS-Fit (Protein Prospector, University of California, San Francisco, CA, USA) database search engines. The search was performed against Other Mammalia (or *Bos taurus*) database, setting the following modifications: carbamidomethylation of cysteines as a fixed modification and oxidation of methionines as a variable modification for in-solution digestion and variable oxidation of methionines for in-gel digestion. Three missed cleavages were accepted, and various fixed and variable modifications were set up to take into account possible variations (e.g., oxidation, deamidation, lactosylation, glycation) likely occurring during the heating processes (see Results and Discussion).

RESULTS AND DISCUSSION

2-DE Analysis of Fresh and Powdered Milk. Samples were analyzed by 2-DE gel, under reducing conditions, to observe non-disulfide cross-links. Figure 1 shows 2-DE gels relevant,

Table 1. Main Protein Modifications Observed in Powdered Milk Samples (See Text for Details)[a]

m/z modified peptide	modification	sequence	protein
2559.31	lactosylation	HPIKHQGLPQEVLNENLLR	α_{s1}-casein
1661.76	lactosylation	HIQKEIVPSER	α_{s1}-casein
1661.76	deamidation	VPQLEIVPNSAEER	α_{s1}-casein
1580.78	dephosphorylation	VPQLEIVPNSAEER	α_{s1}-casein
1871.93	dephosphorylation	YKVPQLEIVPNSAEER	α_{s1}-casein
1267.71	glycation	KTKLTEEEK	α_{s2}-casein
1137.54	glycation	TKVIPYVR	α_{s2}-casein
1915.99	lactosylation	VLPVPQKAVPYPQR	β-casein
1649.78	CML[b]	VLPVPQKAVPYPQR	β-casein
1279.64	lactosylation	FFSDKIAK	κ-casein
1887.99	lactosylation	IAKYIPIQYVLSR	κ-casein
1928.01	lactosylation	LIVTQTMKGLDIQK	β-lactoglobulin
1489.79	lactosylation	ALKALPMHIR	β-lactoglobulin
1959.87	lactosylation	TPEVDDEALEKFDK	β-lactoglobulin
2637.27	lactosylation	VYVEELKPTPEGDLEILLQK	β-lactoglobulin
2415.21	lactosylation	IDALNENKVLVLDTDYKK	β-lactoglobulin
2272.17	lactosylation	TPEVDDEALEKFDKALK	β-lactoglobulin
897.37	lactosylation	IIAEK	β-lactoglobulin
1895.92	lactosylation	IPAVFKIDALNENK	β-lactoglobulin
2125.08	lactosylation	TKIPAVFKIDALNENK	β-lactoglobulin
2125.08	lactosylation	IDALNENKVLVLDTDYK	β-lactoglobulin
1121.46	deamidation	WENGECAQK	β-lactoglobulin
1249.56	deamidation	WENGECAQKK	β-lactoglobulin
1663.75	CML	LIVTQTMKGLDIQK	β-lactoglobulin
1223.72	CML	ALKALPMHIR	β-lactoglobulin
948.56	aminoadipic semialdehyde	LIVTQTMK	β-lactoglobulin
1731.79	oxidation	LSFNPTQLEEQCHI	β-lactoglobulin
2739.36	oxidation	VAGTWYSLAMAASDISLLDAQSAPLR	β-lactoglobulin
2878.35	oxidation	ALKALPMHIRLSFNPTQLEEQCHI	β-lactoglobulin
1633.76	lactosylation	EQLTKKCEVFR ILDKVGINYWLAHK	α-lactalbumin
1994.05	lactosylation	ALCSEKLDQWLCEKKL	α-lactalbumin
2217.03	lactosylation	EQLTKCEVFR ILDKVGINYWLAHK	α-lactalbumin
1220.55	oxidation	LDQWLCEKL	α-lactalbumin
1843.77	oxidation	FLDDDLTDDIMCVKK	α-lactalbumin
1801.85	oxidation	FLDDDLTDDIMCVKK	α-lactalbumin
1817.86	oxidation	FLDDDLTDDIMCVKK	α-lactalbumin
1833.85	oxidation	LDQWLCEKL	α-lactalbumin

[a]The probable site of modification is underlined. [b]CML, carboxymethyllysine.

for instance, to (A) a raw and (B) a powdered milk sample, respectively. All of the major proteins (α_{s1}-casein (α_{s1}-CN), α_{s2}-casein (α_{s2}-CN), β-casein (β-CN), k-casein (κ-CN), β-lactoglobulin A (β-LgA) and B (β-LgB), and α-lactalbumin (α-LA)) were clearly observed in both samples. In particular, α-lactalbumin and β-lactoglobulin migrated according to their molecular weights (MWs) and isoelectric points (p*I* values), whereas caseins migrated at higher MWs and p*I* values, because they are likely present as aggregates (high MWs) and they are highly phosphorylated. In fact, each phosphate group attached to amino acids could shift the protein position closer to the acidic side of the gel. As far as κ-casein spots are concerned, a complex pattern due to glycosylated and phosphorylated variants was observed, as was reported.[35] In addition, the gel relevant to powdered milk showed important differences in the high MW region, suggesting the formation of protein aggregates or complexes, and in the low MW region where "stacked spots" of α-lactalbumin and β-lactoglobulin appeared, suggesting their chemical modifications. Moreover, further acidic forms of proteins due to deamidation were expressed for caseins and β-lactoglobulin. The presence of the above-mentioned differences between raw and powdered milk is certainly related to the heat treatment used during powdered milk processing. This assumption is also supported by previous studies on UHT milks stored at different temperatures and times.[28,36] In fact, Holland et al.[28] reported that UHT milk stored for 2 months at 4 °C did not show significant changes; on the contrary, in the same sample stored at 40 °C remarkable protein modifications were observed.

MALDI MS of Proteins from 2-DE Gel. As already mentioned, a detailed inspection of the 2-DE gel relevant to powdered milk revealed a number of additional spots in the vertical stacks of α-lactalbumin and β-lactoglobulin and, to a lesser extent, α_{s2}-casein. According to previous studies[25,28] each extra spot should be correlated to an additional lactose adduct on the protein. To confirm this assumption and to investigate the possible thermally induced chemical modifications, 2-DE gel spots relevant to both raw and powdered milk samples were subjected to tryptic in-gel digestion followed by MALDI-TOF MS analysis. It has to be emphasized that a key role to reach a high sensitivity was played by the matrix CClCA, which allowed a higher ionization efficiency for less basic peptides

dx.doi.org/10.1021/jf302999s | J. Agric. Food Chem. 2013, 61, 1609−1617

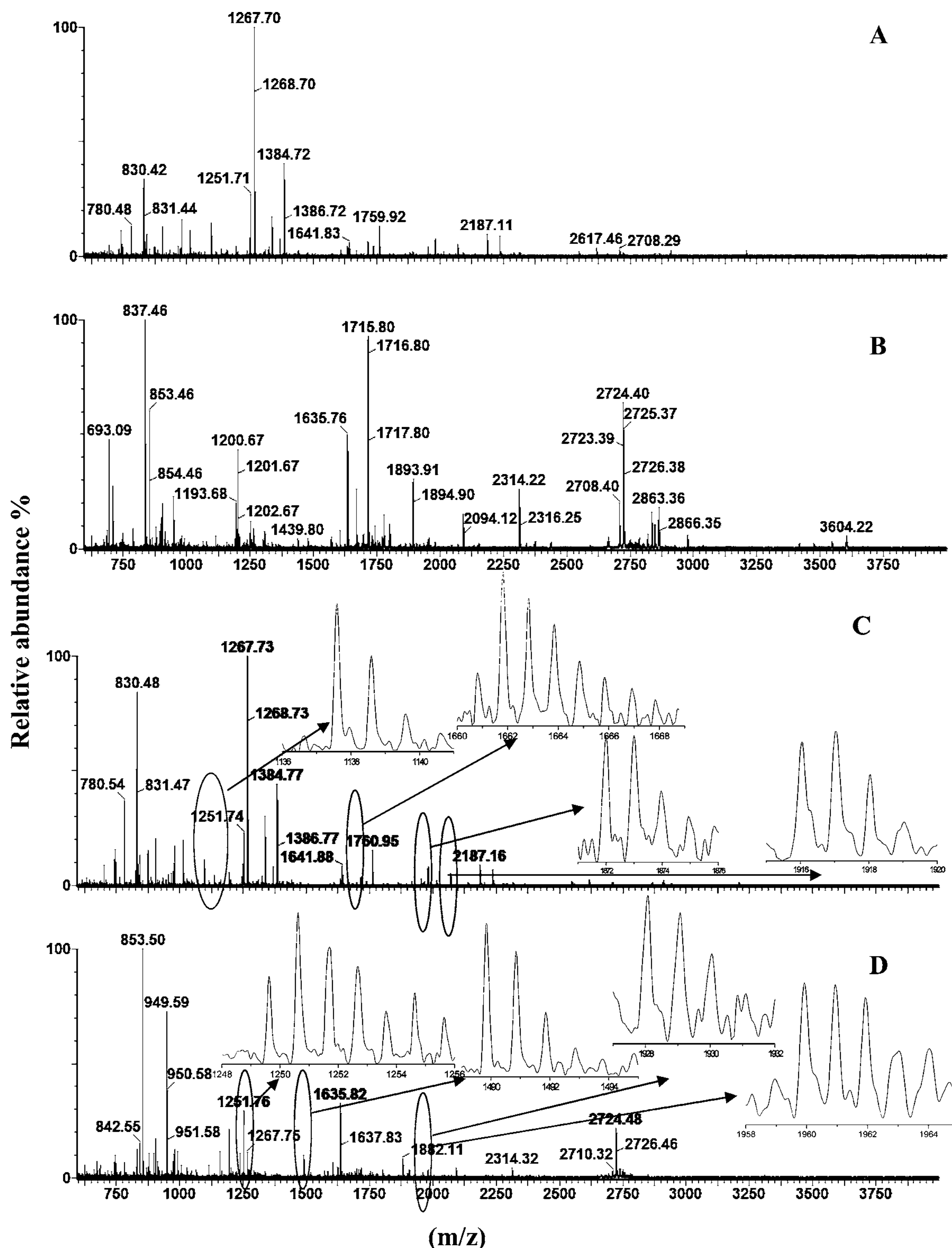

Figure 3. MALDI-TOF spectra relevant to tryptic digests of (A) caseins and (B) whey protein fraction from fresh raw milk and of (C) caseins and (D) whey protein fraction from powdered milk.

(i.e., lactosylated peptides) compared to the traditional matrix CHCA.[31]

The most likely modifications occurring in proteins subjected to heating processes are oxidation (+16, +32 Da), generation of N^{ε}-carboxymethyllysine (CML, +58 Da), lysine aldehyde formation (−1 Da), deamidation (+0.98 Da), glycation (+162 Da), lactosylation (+324 Da), loss of water (−18 Da), and, in some cases, hydrolytic degradation leading to protein cleavage. Therefore, in the database search, all of these potential modifications have been considered.

Figure 2 shows, for instance, the MALDI-TOF MS spectra relevant to one of the 2-DE gel spots of β-lactoglobulin obtained from (A) a raw and (B) a powdered milk sample. The spectra showed a similar profile with the same major ions attributable to peptides arising from β-lactoglobulin. However, a closer examination of the spectra revealed differences in the

dx.doi.org/10.1021/jf302999s | J. Agric. Food Chem. 2013, 61, 1609−1617

isotopic profiles of specific ions. These differences could be explained by deamidation or lysine aldheyde occurrence in the powder milk sample. Furthermore, in the same sample, the appearance of new signals likely due to lactosylation or oxidation or related to carboxymethyllysine formation were observed. For instance, the m/z ion 2637.27, observed only in the powdered milk spectrum, could correspond to the addition of a lactose residue on lysine 63[38] of the native peptide (m/z ion 2313.19) that, in fact, has been detected in the raw milk sample. The m/z ion 1121.48 shows an isotopic pattern different from the theoretical one and could be due to the deamidation of asparagine. This specific modification has been recently observed and explained by means of MS/MS analyses.[28] In the same way, the careful inspection of the MALDI-TOF MS spectra relevant to all of the most interesting 2-DE gel digested spots revealed the occurrence of different significant modifications. Table 1 reports the m/z values of the modified peptides, the possible modification, the relevant sequence, and the originating proteins. The proposed attributions on modified peptides are made on the basis of searching database outputs and by means of comparison with literature data.[28,36,39] As expected, an elevated number of modifications is observed for whey proteins due to the higher reactivity of their Lys residues toward lactose and to a diverse response of their structure to unfolding processes caused by thermal treatment, which should result in a higher exposition of internal amino acids to lactosylation.[40]

As already mentioned, in the high MW region of the 2-DE gel relevant to powdered milk, the presence of protein aggregates or complexes was observed; their presence could be explained by the formation of lysinoalanine cross-links between proteins.[41] The loss of phosphate from phosphoserine in caseins can occur via a β-elimination mechanism to form dehydroalanine. This residue can react with the ε-amino group of a lysine residue, producing lysinoalanine cross-links. This mechanism is supported by the presence of some dephosphorylated serines (see Table 1) in heated samples. In any case, detection of cross-linked phosphorylated peptides is very difficult, and no peaks in the spectra of the tryptic digests of the complexes could be assigned to cross-linked peptides. Another interesting experimental evidence is that lactosylation was observed on both whey proteins and caseins. In fact, according to previously reported data, whey proteins are more sensitive to the Maillard reaction because of their small size and the surface location of lysine residues; however, due to the limited number of available lysines also caseins could be involved in lactosylation processes[37] in dried milk samples.

In-Solution Digestion. The proposed approach proved to be a useful tool for the detection and characterization of protein modifications occurring in milk powder. However, the aim of the present work was the development of a fast and sensitive method to detect adulterations of raw and commercial liquid milk by means of powdered derivatives. Thus, the direct in-solution digestion of milk samples followed by MALDI-TOF MS analysis was performed in the attempt to speed the whole procedure, trying to preserve a greater amount of analytical information.

At first, the in-solution tryptic digestion was performed on whole milk samples (both liquid and powdered). However, this straight approach was discarded because the relevant MALDI-TOF MS spectra were dominated by casein-related peptides, whereas whey proteins were almost absent.

Thus, a preliminary procedure (see Experimental Procedures) to separate caseins from whey fractions, which were then subjected to independent processing and analysis, was adopted.

Table 2. Peptide Markers in Different Samples after In-Solution Digestion

m/z modified peptide	skim milk powdered	infant milk powdered	pasteurized milk
2559.31	X		
1661.76	X	X	
1580.78	X	X	
1871.93	X	X	
1137.54	X	X	X
1915.99	X	X	
1649.78	X		
1279.64			
1887.99			
1928.01	X		
1489.79	X		
1959.87	X	X	
2637.27			
2415.21			
2272.17	X		
897.37	X	X	X
1895.92			
2125.08	X		
1121.46			X
1249.56	X		X
1663.75			
1223.72	X		
948.56	X	X	
1731.79	X	X	X
2739.36	X		
2878.35			
1633.76	X	X	X
1994.05			
2217.03			
1220.55			
1843.77			
1801.85	X	X	X
1817.86	X	X	
1833.85			

Figure 3 shows the MALDI-TOF MS spectra relevant to the in-solution tryptic digests of (A) casein and (B) whey fractions of raw milk, respectively, and (C) casein and (D) whey fractions of powdered milk, respectively. As apparent, all of the main peptides relevant to whey proteins of both raw and powdered milk samples were observed in this case. The comparison of these data with the results previously obtained by 2-DE gel revealed that most of the modified peptides, specific to powdered milk, were still detectable with the new rapid approach. In fact, in powdered milk, the most abundant peptides (m/z 1928.01, 1489.79, 1959.87, 1249.56) arising from modified β-lactoglobulin together with other peptides originating from modified caseins (m/z 1661.76, 1580.78, 1871.93, 1137.54, 1915.99) were observable in the whey and casein fractions, respectively (see insets). Moreover, as far as peptide relative intensities are concerned, the comparison of the spectra relevant to the whey fraction of raw (Figure 3B) and powdered (Figure 3D) milk evidenced further differences. In fact, the acidic peptides (pI values comprised between 4.21 and 4.68) at m/z 1715.80, 2313.26, and 2723.39 (β-Lg) and at m/z 1892.92 (α-LA) are almost absent in the spectrum relevant to the whey fraction of powdered milk. This evidence could be the consequence of degradation or aggregation phenomena due to the heat treatment

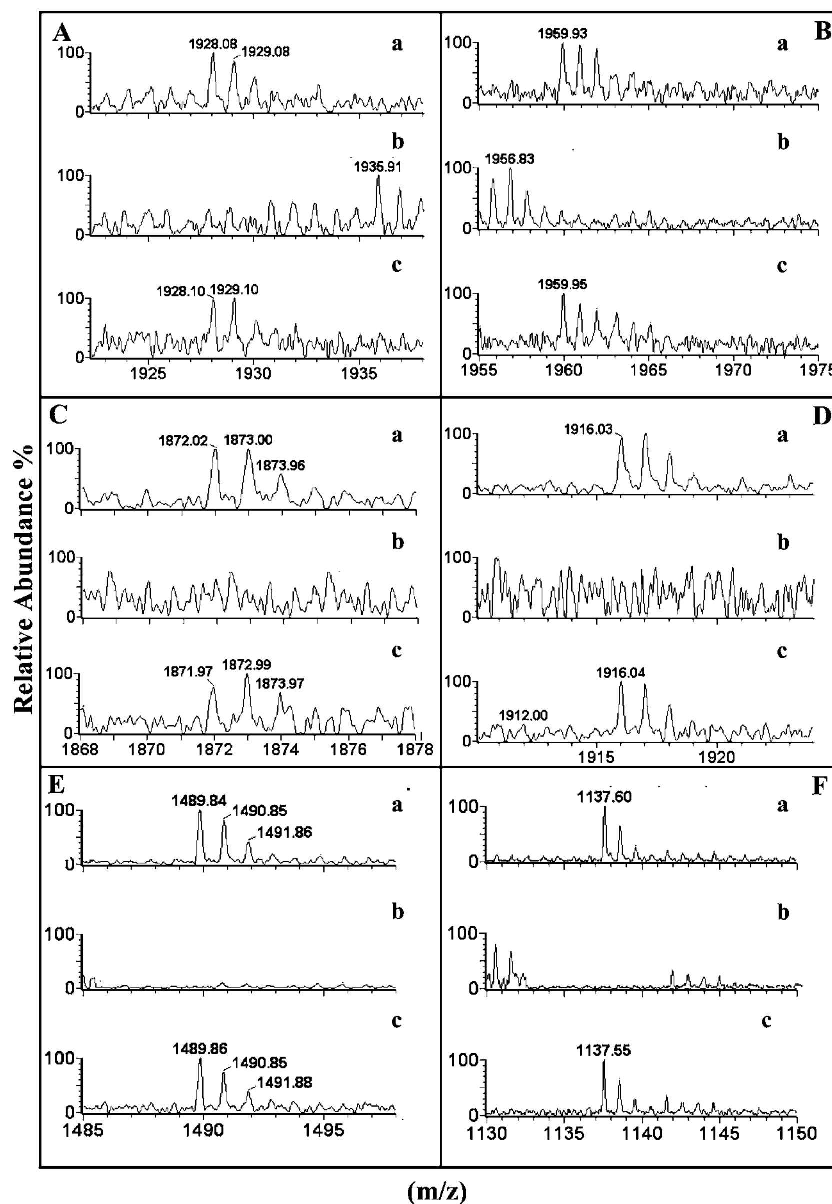

Figure 4. MALDI-TOF MS spectra relevant to the analysis of (a) powdered milk, (b) raw liquid milk, and (c) raw liquid milk (98%) adulterated with powdered milk (2%), respectively: (A–F) most significant m/z windows.

together with an ion suppression effect, typically observed in the case of acidic compounds. Furthermore, in the same spectrum some peptides previously attributed to caseins were observed. The presence of these peptides might be explained as arising from caseins cross-linked to β-lactoglobulin (see above).

The fast procedure was then tested on different samples (n = 3 for each kind), that is, pasteurized milk, skim milk powder, and infant powdered milk. The relevant results are given in Table 2. As expected, some few modification sites were identified also in pasteurized milk, likely due to the processing conditions. In fact, pasteurization is a relatively mild heat treatment (15 s at a temperature minimum of 72 °C), which ensures the death of pathogenic bacteria without affecting the final product. As far as infant milk powder is concerned, many more peptides were obviously detected, whereas the highest number of modified peptides was observed in skim milk powder. This evidence was expected because infant products are very carefully controlled in process parameters and quality of the ingredients to decrease the protein degradation phenomenon;[42] accordingly, a minor number of modified peptides have been detected in these products.

Analysis of Adulterated Samples. To assess if and to what extent the present approach is able to individuate the adulteration of liquid milk with powdered milk, different mixtures at decreasing percentages of powdered milk starting from 10 to 1% have been prepared, subjected to the fast pretreatment procedure and analyzed by MALDI-TOF. From the analysis of spectra relevant to adulterated samples, the m/z ions 932.56, 1018.53, 1249.56, 1594.69, and 1862.98 were detected up to the adulteration of 5%, whereas the m/z ions 1137.54, 1871.98, 1916.03, 1959.87, 1489.79, and 1928.01 were still visible at 1% of adulteration.

Figure 4 shows, as an example, the most significant m/z windows of the MALDI-TOF MS spectra relevant to the analysis of (a) powdered milk, (b) raw liquid milk, and (c) raw liquid milk (98%) adulterated with powdered milk (2%), respectively.

dx.doi.org/10.1021/jf302999s | J. Agric. Food Chem. 2013, 61, 1609–1617

It should be said that the official method used to assess adulterations in pasteurized milk[14] consists of the HPLC determination of furosine (few milligrams of furosine per 100 g of total protein, 8 mg/100 g being the legal limit) that, however, is naturally present in milk at a concentration of 3−5 mg. The method here proposed provides an alternative way of looking at the lactosylation level, overcoming some limitations of the official method such as the difficulty in detecting the fraud with powder derivatives from free lactose milk. The detection of lactose adducts to a 1% level of adulteration correlates with the furosine measurements in terms of sensitivity.

Conclusions. A simple and sensitive procedure for the detection of the very challenging adulteration of powdered milk in liquid (raw and commercial) milk samples has been developed, based on in-solution digestion of whey and casein fractions followed by MALDI-TOF MS analysis using the new matrix CClCA. Several modified peptides specific for powdered milk were detected and identified, and six of them were still detectable to an adulteration level of 1%. The results obtained with the in-solution digestion were also in good agreement with those obtained with the reliable but time-consuming 2D gel followed by in-gel digestion approach. Future work should address increasing the number of marker peptides by performing analyses in negative ion mode using new alternative matrices.[43−45] Moreover, the developed approach will also be applied on control cheese samples such as mozzarella[46] made with raw fresh or adulterated milk.

■ AUTHOR INFORMATION

Corresponding Author

*Fax: +39-080-5442026. Phone: +39-080-5443589. E-mail: cosimadamiana.calvano@uniba.it.

Funding

Partial funding from Universita' degli Studi di Bari "Aldo Moro" is acknowledged.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We gratefully acknowledge Antonio Trani for his technical support in carrying out HPLC analyses for furosine determination. We also acknowledge Dr. Giovanni Ruppi for helpful discussion.

■ REFERENCES

(1) Cozzolino, R.; Passalacqua, S.; Salemi, S.; Malvagna, P.; Spina, E.; Garozzo, D. Identification of adulteration in milk by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. *J. Mass Spectrom.* **2001**, *36*, 1031−1037.

(2) http://old.politicheagricole.it/SicurezzaAlimentare/ConoscereGliAlimenti/040_Latte.htm

(3) Goswami, T. K.; Gupta, S. K. Detection of dilution of milk with the help of glass transition temperature by differential scanning calorimetry (DSC). *Afr. J. Food Sci.* **2008**, *2*, 7−10.

(4) http://www.pnragrobio.unina.it/SIFORTI.html

(5) De La Fuente, M. A.; Juarez, M. Authenticity assessment of dairy products. *Crit. Rev. Food Sci* **2005**, *45*, 563−585.

(6) Fox, P. F. Heat-induced changes in milk preceding coagulation. *J. Dairy Sci.* **1981**, *64*, 2127−2137.

(7) Singh, H.; Creamer, L. K. Heat stability of milk. In *Advanced Dairy Chemistry − 1. Proteins*; Elsevier Applied Science: London, U.K., 1992; pp 621−656.

(8) Mauron, J. The Maillard reaction in food; a critical review from the nutritional standpoint. *Prog. Food Nutr. Sci.* **1981**, *5* (1−6), 5−35.

(9) van Boekel, M. A. J. S. Effect of heating on Maillard reactions in milk. *Food Chem.* **1998**, *62*, 403−414.

(10) Anema, S. G.; Li, Y. Association of denatured whey proteins with casein micelles in heated reconstituted skim milk and its effect on casein micelle size. *J. Dairy Res.* **2003**, *70* (1), 73−83.

(11) Pompei, C.; Rossi, M.; Mare, F. Protein quality in commercial milk-based infant formulas. *J. Food Qual.* **1988**, *10*, 375−391.

(12) Finot, P.-A. Historical perspective of the Maillard reaction in food science. *Ann. N.Y. Acad. Sci.* **2005**, *1043*, 1−8.

(13) Singh, H. Modification of food proteins by covalent crosslinking. *Trends Food Sci. Technol.* **1991**, *2*, 196−200.

(14) Erbersdobler, H. F.; Somoza, V. Forty years of furosine-forty years of using Maillard reaction products as indicators of the nutritional quality of foods. *Mol. Nutr. Food Res.* **2007**, *51*, 423−430.

(15) Resmini, P.; Pellegrino, L.; Masotti, F.; Tirelli, A.; Prati, F. Detection of reconstituted milk powder in raw and in pasteurized milk by HPLC of furosine. *Sci. Tec. Latt.−Casearia* **1992**, *43* (3), 169−186.

(16) Montilla, A.; Gómez-Ruiz, J.; Olano, A.; del Castillo, M. A GC-FID method for analysis of lysinoalanine. *Mol. Nutr. Food Res.* **2007**, *51*, 415−422.

(17) Madkour, M. A. M.; Moussa, B. M. I. The determination of milk powder added to whole milk. *Nahrung* **1989**, *33* (1), 77−82.

(18) Rehman, Z. U.; Saeed, A.; Zafar, S. I. Hydroxymethylfurfural as an indicator for the detection of dried powder in liquid milk. *Milchwissenschaft* **2000**, *55* (5), 256−257.

(19) Birlouez-Aragon, I.; Sabat, P.; Gouti, N. A new method for discriminating milk heat treatment. *Int. Dairy J.* **2002**, *12*, 59−67.

(20) Lin, G. P.; Rau, Y. H.; Chen, Y. F.; Chou, C. C.; Fu, W. G. Measurements of δD and δ^{18}O stable isotope ratios in milk. *J. Food Sci.* **2003**, *68* (7), 2192−2195.

(21) French, S. J.; Harper, W. J.; Kleinholz, N. M.; Jones, R. B.; Green-Church, K. B. Maillard reaction induced lactose attachment to bovine beta-lactoglobulin: electrospray ionization and matrix-assisted laser desorption/ionization examination. *J. Agric. Food Chem.* **2002**, *50*, 820−823.

(22) Czerwenka, C.; Maier, I.; Pittner, F.; Lindner, W. Investigation of the lactosylation of whey proteins by liquid chromatography-mass spectrometry. *J. Agric. Food Chem.* **2006**, *54*, 8874−8882.

(23) Leonil, J.; Molle, D.; Fauquant, J.; Maubois, J. L.; Pearce, R. J.; Bouhallab, S. 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. *J. Dairy Sci.* **1997**, *80*, 2270−2281.

(24) Morgan, F.; Molle, D.; Henry, G.; Venien, A.; Leonil, J.; Peltre, G.; Levieux, D.; Maubois, J. L.; Bouhallab, S. Nonenzymatic lactosylation of bovine β-lactoglobulin under mild heat treatment leads to structural heterogeneity of the glycoforms. *Biochem. Biophys. Res. Commun.* **1997**, *236*, 413−417.

(25) Carulli, S.; Calvano, C. D.; Palmisano, F.; Pischetsrieder, M. MALDI-TOF MS characterization of glycation products of whey proteins in a glucose/galactose model system and lactose-free milk. *J. Agric. Food Chem.* **2011**, *59*, 1793−1803.

(26) Meltretter, J.; Seeber, S.; Humeny, A.; Becker, C.; Pischetsrieder, M. Site specific formation of Maillard, oxidation, and condensation products from whey proteins during reaction with lactose. *J. Agric. Food Chem.* **2007**, *55*, 6096−6103.

(27) Fenaille, F.; Parisod, V.; Visani, P.; Populaire, S.; Tabet, J.; Guy, P. Modifications of milk constituents during processing: a preliminary benchmarking study. *Int. Dairy J.* **2006**, *16*, 728−739.

(28) Holland, J. W.; Gupta, R.; Deeth, H. C.; Alewood, P. F. Proteomic analysis of temperature-dependent changes in stored UHT milk. *J. Agric. Food Chem.* **2011**, *59*, 1837−1846.

(29) Cozzolino, R.; Passalacqua, S.; Salemi, S.; Malvagna, P.; Spina, E.; Garozzo, D. Identification of adulteration in milk by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. *J. Mass Spectrom.* **2001**, *36*, 1031−1037.

(30) Galvani, M.; Hamdan, M.; Righetti, P. G. Two-dimensional gel electrophoresis/matrix-assisted laser desorption/ionisation mass

spectrometry of a milk powder. *Rapid Commun. Mass Spectrom.* **2000**, *14*, 1889−1897.
(31) Calvano, C. D.; De Ceglie, C.; Monopoli, A.; Zambonin, C. G. Detection of sheep and goat milk adulterations by direct MALDI-TOF-MS analysis of milk tryptic digests. *J. Mass Spectrom.* **2012**, DOI: 10.1002/jms.2995.
(32) Jaskolla, T. W.; Lehmann, W.-D.; Karas, M. 4-Chloro-α-cyanocinnamic acid is an advanced, rationally designed MALDI matrix. *Proc. Natl. Acad. Sci. U.S.A.* **2008**, *105*, 12200−12205.
(33) Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **1970**, *227*, 680−685.
(34) Candiano, G.; Bruschi, M.; Musante, L.; Santucci, L.; Ghiggeri, G. M.; Carnemolla, B.; Orecchia, P.; Zardi, L.; Righetti, P. G. Blue silver: a very sensitive colloidal Coomassie G-250 staining for proteome analysis. *Electrophoresis* **2004**, *25*, 1327−1333.
(35) Holland, J. W.; Deeth, H. C.; Alewood, P. F. Proteomic analysis of κ-casein micro-heterogeneity. *Proteomics* **2004**, *4*, 743−752.
(36) Le, T. T.; Deeth, H. C.; Bhandari, B.; Alewood, P. F.; Holland, J. W. A proteomic approach to detect lactosylation and other chemical changes in stored milk protein concentrate. *Food Chem.* **2012**, *132*, 655−662.
(37) Pinto, G.; Caira, S.; Cuollo, M.; Fierro, O.; Nicolai, M. A.; Chianese, L.; Addeo, F. Lactosylated casein phosphopeptides as specific indicators of heated milks. *Anal Bioanal Chem* **2012**, *402*, 1961−1972.
(38) Meltretter, J.; Pischetsrieder, M. Application of mass spectrometry for the detection of glycation and oxidation products in milk proteins. *Ann. N.Y. Acad. Sci.* **2008**, *1126*, 134−140.
(39) Arena, S.; Renzone, G.; Novi, G.; Paffetti, A.; Bernardini, G.; Santucci, A.; Scaloni, A. Modern proteomic methodologies for the characterization of lactosylation protein targets in milk. *Proteomics* **2010**, *10*, 3414−3434.
(40) de la Fuente, M. A.; Singh, H. H. Y. Recent advances in the characterisation of heat induced aggregates and intermediates of whey proteins. *Trends Food Sci. Technol.* **2002**, *13*, 262−274.
(41) van Boekel, M. A. J. S. Heat-induced deamidation, dephosphorylation and breakdown of caseinate. *Int. Dairy J.* **1999**, *9*, 237−241.
(42) Pischetsrieder, M.; Henle, T. Glycation products in infant formulas: chemical, analytical and physiological aspects. *Amino Acids* **2012**, *42*, 1111−1118.
(43) Monopoli, A.; Cotugno, P.; Cortese, M.; Calvano, C. D.; Ciminale, F.; Nacci, A Selective N-alkylation of arylamines with alkylchloride in ionic liquids: scope and applications. *Eur. J. Org. Chem.* **2012**, *16*, 3105−311.
(44) Calvano, C. D.; De Ceglie, C.; D'Accolti, L.; Zambonin, C. G. MALDI-TOF mass spectrometry detection of extra-virgin olive oil adulteration with hazelnut oil by analysis of phospholipids using an ionic liquid as matrix and extraction solvent. *Food Chem.* **2012**, *134*, 1192−1198.
(45) Calvano, C. D.; Zambonin, C. G.; Palmisano, F. Lipid fingerprinting of Gram-positive lactobacilli by intact cells − matrix-assisted laser desorption/ionization mass spectrometry using a proton sponge based matrix. *Rapid Commun. Mass Spectrom.* **2011**, *25*, 1757−1764.
(46) Francolino, S.; Locci, F.; Ghiglietti, R.; Iezzi, R.; Mucchetti, G. Use of milk protein concentrate to standardize milk composition in Italian citric Mozzarella cheese making. *LWT−Food Sci. Technol.* **2010**, *43*, 310−314.