RESEARCH ARTICLE

# Assessment of heat treatment of dairy products by MALDI-TOF-MS

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The formation of the Amadori product from lactose (protein lactosylation) is a major parameter to evaluate the quality of processed milk. Here, MALDI-TOF-MS was used for the relative quantification of lactose-adducts in heated milk. Milk was heated at a temperature of 70, 80, and 100°C between 0 and 300 min, diluted, and subjected directly to MALDI-TOF-MS. The lactosylation rate of α-lactalbumin increased with increasing reaction temperature and time. The results correlated well with established markers for heat treatment of milk (concentration of total soluble protein, soluble α-lactalbumin and β-lactoglobulin at pH 4.6, and fluorescence of advanced Maillard products and soluble tryptophan index; $r = 0.969–0.997$). The method was also applied to examine commercially available dairy products. In severely heated products, protein pre-purification by immobilized metal affinity chromatography improved spectra quality. Relative quantification of protein lactosylation by MALDI-TOF-MS proved to be a very fast and reliable method to monitor early Maillard reaction during milk processing.

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## 1 Introduction

Milk and dairy products are usually heated prior to retail and consumption in order to ensure microbiological safety. As a consequence, milk proteins undergo various modifications and damage. The most prominent in this context is the reaction of milk proteins with the milk sugar lactose [1]. In the Maillard reaction, lactose mainly binds to the ε-amino groups of lysine residues of the milk proteins, which are the most abundant amines in milk. First, a Schiff Base or a lactosylamine is formed, which is further converted to the Amadori product lactulosylamine [2]. During prolonged heating, further glycation products can be formed such as $N^{\varepsilon}$-carboxymethyllysine, oxalic acid monolysinylamide, pyrraline, or pentosidine [3–6]. Furthermore, heating in the presence of lactose can induce protein oxidation leading to the formation of methionine sulfoxide, aminoadipic semialdehyde, or dityrosine [7–10]. Finally, protein modifications can occur, which are formed independently from the presence of lactose, such as lysinoalanine, histidinoalanine, N-terminal pyrrolidone, or partial protein hydrolysis [11–13]. Mainly, the formation of early Maillard products leads to a considerable loss of bio-available lysine, which can reach 20% in some dairy products [2, 14]. As a consequence, the nutritional value of milk proteins can be largely decreased. Therefore, it is very important to assess and monitor protein damage during milk processing. Several methods have been

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**Abbreviations: ALA,** α-lactalbumin; **DHAP,** 2,5-dihydroxyacetophenone; **DHB,** 2,5-dihydroxybenzoic acid; $F_{AMP}$, fluorescence of advanced Maillard products; **FAST,** fluorescence of advanced Maillard products and soluble tryptophan; $F_{Trp}$, fluorescence of tryptophan; **HCCA,** α-cyano-4-hydroxy cinnamic acid; **IMAC,** immobilized metal affinity chromatography

suggested for this purpose. The direct measurement of lactulosyllysine in milk after enzymatic hydrolysis by ion exchange chromatography and post column derivatization with ninhydrin has been described [15]. Nevertheless, lactulosyllysine is usually assessed after its conversion to furosine during acidic protein hydrolysis [16]. As the Amadori product, however, is not quantitatively converted into furosine, an empirical conversion factor has to be determined in order to convert furosine concentration to lactulosyllysine concentration [17]. Since a lactulosyllysine standard is not readily available, the relative concentration of the Amadori product as furosine is usually given instead of an absolute quantification of lactulosyllysine [10, 14, 18].

The lactosylation of milk proteins can also be detected directly by protein MS, most commonly by LC-ESI-MS [19, 20]. The advantage of this method is that proteins can be measured directly with minimal sample workup, thus minimizing artifact formation and analysis time.

In this study, an improved MALDI-TOF-MS method was developed, which allows the detection of protein lactosylation in heated milk samples. Furthermore, relative quantification was applied to assess the lactosylation rate dependent on the heat impact.

## 2 Materials and methods

### 2.1 Materials

Bovine α-lactalbumin (ALA) and bovine β-lactoglobulin were purchased from Sigma-Aldrich (Taufkirchen, Germany). BSA solution was obtained from Perbio Science (Bonn, Germany), α-cyano-4-hydroxycinnamic acid (HCCA) and 2,5-dihydroxybenzoic acid (DHB) from Fluka (Taufkirchen, Germany), and 2,5-dihydroxyacetophenone (DHAP) from Bruker Daltonics (Bremen, Germany). All chemicals were of highest purity available. The magnetic beads immobilized metal affinity chromatography (IMAC)-Cu-kit was purchased from Bruker Daltonics. Protein quantification was carried out using the Bio-Rad DC Protein Assay from Bio-Rad (Munich, Germany).

### 2.2 Milk samples

Raw milk was obtained from a local dairy farm. Pasteurized, high-temperature milk (heated to a minimum temperature of 85°C until peroxidase negative (approx. 30–60 s)), ultra-high-temperature milk (heated to a minimum temperature of 135°C in a continuous flow-through process (approx. 3–6 s)), sterilized milk (sterilized by in-bottle heating at 105°C (approx. 20 min)), and condensed milk (sterilized after partial dehydration), as well as three liquid and one powdered infant formula were purchased in local supermarkets. The infant formulas from three different German manufacturers were labeled as follow-on formulas with adapted protein content. The powdered sample was mixed with water (2 mg in 12 mL). All samples were defatted by centrifugation at 3850 rpm for 60 min at 4°C and removal of the upper fat layer.

### 2.3 Heating of raw milk

The heating experiment was carried out in triplicate. Aliquots of 1.5 mL of raw milk were heated in capped plastic tubes in an oven at temperatures of 70, 80, or 100°C, and after defined periods of time, samples were drawn and cooled down on ice. Raw milk served as the control.

### 2.4 Precipitation of caseins and denatured whey proteins

For the precipitation of caseins and denatured whey proteins, 0.3 mL of the heated milk or the control was mixed with 1.5 mL of 0.4 M sodium acetate buffer pH 4.6. After 5 min at room temperature, samples were centrifuged at 3850 rpm and 4°C for 60 min. To remove the residual protein precipitates from the solution, the centrifugate was filtered through a polyvinylidene fluoride filter (0.45 μm). The filtrate was directly used for the quantification of soluble proteins according to Lowry and of soluble ALA and β-lactoglobulin (see in Sections 2.6 and 2.8).

### 2.5 MALDI-TOF-MS analysis

MALDI-TOF-MS experiments were performed in duplicate. The raw and heated milk samples as well as the dairy products were diluted 1:100 with water. For the analysis of ALA, the diluted sample was mixed 1:1 with a matrix consisting of equal volumes of an HCCA solution and a solution of DHB. For the HCCA solution, 20 mg of HCCA were dissolved in 1 mL of ACN/5% formic acid (70:30). The DHB solution was prepared by dissolving 20 mg of DHB in 1 mL of ACN/0.1% TFA (70:30). For the analysis of β-lactoglobulin, the diluted dairy products were added in a ratio of 1:1 to a 4:1 mixture of a saturated solution of DHAP in 50% ACN/0.1% TFA and 10 μM diammonium hydrogen citrate in 50% ACN/0.1% TFA. An aliquot of 1 μL was spotted onto a stainless steel target and air dried. The MALDI-TOF-MS analysis was carried out on a Bruker Autoflex (Bruker Daltonics), equipped with a nitrogen laser ($\lambda = 337$ nm). Laser-desorbed positive ions were analyzed after delayed extraction (350 ns) and acceleration by 20 kV in the linear mode. External calibration was performed using a mix of bovine ALA, β-lactoglobulin variants A and B, as well as chicken lysozyme. For each displayed mass spectrum, 300 individual spectra obtained from several positions on a spot were averaged.

 

### 2.6 Quantification of soluble proteins according to Lowry

The quantification of proteins soluble at pH 4.6 according to Lowry [21] was carried out with a protein quantification kit from Bio-Rad and measured at 630 nm with a Bio-Rad Microplate Reader. A solution of BSA in 0.4 M sodium acetate buffer pH 4.6 in the range from 0.1 to 1.6 g/L served as an external calibration standard.

### 2.7 Determination of the fluorescence of advanced Maillard products and soluble tryptophan index

The determination of the fluorescence of advanced Maillard products and soluble tryptophan (FAST)-index was carried out according to Birlouez-Aragon *et al.* [22]. The filtrate, obtained after the precipitation of the caseins and denatured whey proteins, was diluted 1:5 with 0.1 M sodium acetate buffer pH 4.6. Measurements were performed on a Kontron fluorometer SFM 23 (Kontron, Eching, Germany). Sensitivity was ''medium'' and adjusted to 3. For measuring the fluorescence of tryptophan ($F_{Trp}$), the samples were excited at a wavelength of 290 nm and emission was measured at 340 nm. For the fluorescence of advanced Maillard products ($F_{AMP}$), an excitation wavelength of 330 nm and an emission wavelength of 420 nm were chosen. The FAST-index was calculated as follows: FAST-index = $F_{AMP}/F_{Trp} \times 100$.

### 2.8 Quantification of soluble ALA and β-lactoglobulin

ALA and β-lactoglobulin soluble at pH 4.6 were quantified by HPLC on a system from Jasco (Gross-Umstadt, Germany) equipped with an AS-1555 autosampler, a PU-1580 pump, an LG-1580-02 ternary gradient unit, a DG-1580-53 3-line degasser, and an FP-920 fluorescence detector. Proteins were separated on a C8 column (Machery-Nagel, Dueren, Germany, CC 125/3 Nucleosil 100-C8 ec) and a C8 guard cartridge at a flow rate of 0.8 mL/min. The injection volume was 20 μL. The following gradient was used: solvent A, ACN; solvent B, 0.1% TFA in water; 5 min 20% A; within 20 min increase to 60% A; within 2 min decrease to 20% A; hold at 20% A for 20 min. Fluorescence detection was carried out at an excitation wavelength of 280 nm and an emission wavelength of 350 nm. At these wavelengths, maximal signal intensities were obtained with the fluorescence detector used. The slight differences to the wavelengths published for the analysis of $F_{Trp}$ in the FAST index [22] are probably caused by the application of different instruments. Recorded chromatograms were analyzed by Jasco Borwin software. External calibration was performed using standard solutions of ALA (0.005−0.3 g/L) and β-lactoglobulin (0.005–0.6 g/L).

### 2.9 IMAC of dairy products

IMAC of dairy products from the supermarket was carried out using commercially available magnetic bead kits with immobilized copper ions. According to the manufacturer's instructions, 5 μL of the magnetic bead solution was suspended three times in 50 μL of copper-binding solution. Five microliters of sample were added, and after three washing steps with 100 μL of washing solution, the proteins were released from the beads by incubation in elution solution. The eluted proteins were measured by MALDI-TOF-MS as described in Section 2.5 for the diluted dairy samples using the HCCA-DHB-matrix. Each sample was cleaned up and analyzed in duplicate.

### 2.10 Statistical data evaluation

Statistical data evaluation was performed with the software Microsoft Excel 2003.

## 3 Results

### 3.1 Analysis of milk after thermal treatment

This study attempted to develop a MS method for the simple and fast evaluation of the early stage of the Maillard reaction during thermal treatment of milk by relative quantification of the Amadori product lactulosyllysine. For this purpose, defatted milk was heated at three temperatures in a range commonly applied in the milk industry (70, 80, and 100°C) and subsequently directly analyzed using MALDI-TOF-MS. Heating experiments on a laboratory scale are far less efficient than industrial processes, since the heat transfer to the milk samples in the oven takes considerably longer than using industrial heating techniques. Therefore, longer heating times were applied in our experiments in order to achieve a similar heat impact as during industrial processing.

Optimization of MS conditions showed that a MALDI matrix composed of a mixture of HCCA and DHB greatly favors the ionization of ALA, while signals of other milk proteins are less intensive (data not shown). Thus, modifications of ALA can be easily detected using this matrix. Figure 1 depicts mass spectra in the range of the signal of ALA, recorded from raw milk and milk, which was heated at 80°C. In the heated samples, two new signals were detected, with a mass difference of 324 Da and 648 Da to the native protein, respectively, corresponding to single and double lactosylation of the protein. Both signals increased with prolonged heating time. At the same time, the signal of the native protein became less intense, which can be explained by the formation of the lactosylated protein and possibly other modifications. Moreover, partial denaturation and coaggregation of ALA with other milk proteins take place

 

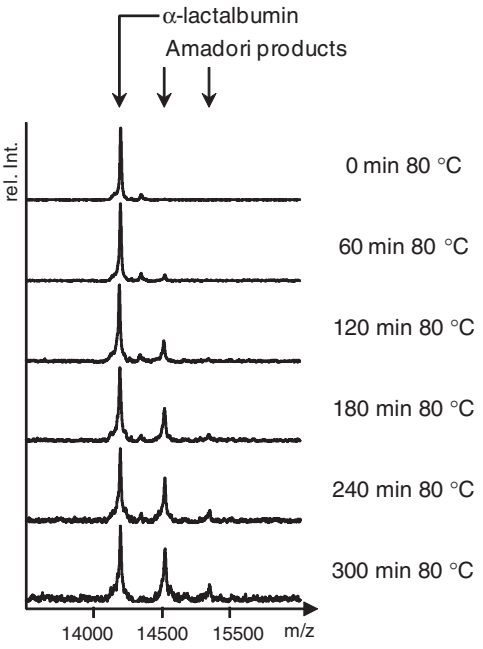


**Figure 1.** MALDI-TOF-MS spectra of heated milk. Defatted raw milk was heated at 80°C up to 300 min and directly analyzed using MALDI-TOF-MS. The mass range of ALA as well as mono- and dilactosylated ALA is shown ($\Delta m/z = 324$ and 648).

during heating, thus reducing the intensity of the monomeric form.

### 3.2 Relative quantification of the Amadori product of ALA

The relative amounts of detected Amadori products were determined according to a method of Kislinger *et al.* by relating the sum of the intensities of glycated ALA to the sum of the intensities of the native and the modified proteins [23]. The intensities of double lactosylated ALA were multiplied by two in order to take into account that the signal represents twice the amount of Amadori product in comparison with the monolactosylated species: Relative content [%] = [Int. (monolactosylated ALA) + 2 × Int. (double lactosylated ALA)]/[Int. (native ALA) + Int. (monolactosylated ALA) + 2 × Int. (double lactosylated ALA)] × 100.

Figure 2 shows that the relative content of the Amadori product increased with prolonged heating as well as with the increasing temperature. Whereas relative contents between 17 and 52%, respectively, were obtained at the temperatures of 70 and 80°C after 120 min, a maximum of 77% was reached after heating the milk at 100°C for the same time. It should be noted that the shape of the curves is influenced by the relatively slow heat transfer in the oven, which leads to a lag in the initiation of the Maillard reaction.

In order to substantiate relative quantification performed by the new MS method, the results were correlated with

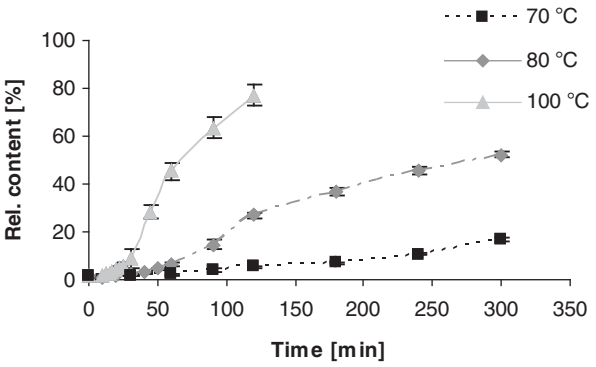


**Figure 2.** Relative content of the Amadori product of ALA depending on heat duration and temperature. Defatted raw milk was heated at a temperature of 70, 80, and 100°C, respectively, and directly analyzed by MALDI-TOF-MS. The mean values of an independent triplicate ± standard deviation are shown.

**Table 1.** Correlation coefficients (*r*) of the results obtained with the new MS method and conventional techniques used for the evaluation of thermal treatment of dairy products[a)]

| Heat indicators | | *r* | Type |
|---|---|---|---|
| AP-α-lactalbumin[b)] | 70, 80, 100°C | 0.969 | exp. |
| AP-β-lactoglobulin[b)] | 70°C | 0.995 | exp. |
| | 80°C | 0.982 | exp. |
| | 100°C | 0.983 | exp. |
| AP-protein[b)] | 70°C | 0.984 | exp. |
| | 80°C | 0.978 | exp. |
| | 100°C | 0.984 | exp. |
| AP-FAST index | 70°C | 0.991 | lin. |
| | 80°C | 0.992 | lin.[c)] |
| | | 0.995 | exp.[d)] |
| | 100°C | 0.997 | lin.[c)] |
| | | 0.996 | exp.[d)] |

a) The mathematical relation between the heat indicators is given: exp., exponential; lin., linear. AP: Amadori product of α-lactalbumin determined by MALDI-TOF-MS.
b) Soluble at pH 4.6.
c) During mild heat treatment.
d) During prolonged heat treatment. Values of severely heated samples resulting in maximal protein denaturation were not considered for the calculations (see Section 4).

those obtained by conventional and well-established techniques for the evaluation of thermal treatment of milk. For this purpose, the concentration of soluble protein at pH 4.6 according to Lowry and the concentrations of soluble ALA and β-lactoglobulin at pH 4.6 – all indicators of protein denaturation – as well as the FAST-index, reflecting the formation of fluorescent substances during the Maillard reaction, were measured. Good coefficients of correlations between 0.969 and 0.997 were obtained (Table 1), suggesting that the MS approach presented here is a valid alternative to classical parameters allowing a

 

simple and fast assessment of the thermal treatment of dairy products.

### 3.3 Direct analysis of dairy products

As a next step, several commercially available dairy products were analyzed. Again, signals corresponding to the Amadori products of ALA could be detected in all samples except the control raw milk (Fig. 3A). Whereas only the monolactosylated species was detected in the high-temperature milk, additional signals with a mass shift of 648 Da to the native protein were observed in the two ultra-high-temperature milk samples, suggesting the addition of two lactose molecules. Moreover, the triple and quadruple lactosylation of ALA was observed in the infant formulas, reflecting the higher glycation rates in these samples. Furthermore, signals with a mass difference of 162 Da and multiples thereof indicate the reaction of lysine residues in the protein with a hexose. The sterilized milk and the condensed milk, however, yielded spectra with a poor signal intensity of ALA, probably due to severe protein denaturation and consequent loss of the monomeric protein.

As mentioned above, spectra with relatively weak signals for β-lactoglobulin were obtained when using the described MS protocol. In this context, the use of DHAP as matrix instead of an HCCA-DHB mixture allowed a significantly increased sensitivity. Nevertheless, although the control and the high-temperature milk showed intensive peaks, β-lactoglobulin could be only poorly recovered in the more severely heated samples and was even absent in two infant formulas and the condensed and sterilized milk sample (Fig. 4). This clearly demonstrates that β-lactoglobulin is much more sensitive toward heat denaturation and quickly coaggregates with other milk proteins, which leads to the decrease in signal intensity of the monomeric protein. In a consequence, ALA represents the more appropriate target protein for the evaluation of thermal treatment by this MS technique.

### 3.4 Analysis of dairy products after metal affinity chromatography

For improving spectra quality and detection limits, milk samples were cleaned up before MALDI-TOF-MS analysis by metal affinity chromatography. In order to remove interfering substances that might hinder protein ionization during MS analysis, milk proteins were bound to copper ions immobilized on magnetic beads and eluted after a washing step, followed by matrix addition and determination by MALDI-TOF-MS.

Figure 3B shows that sample purification by metal affinity chromatography clearly improved spectra quality and hence a considerable improvement of the $S/N$ ratio was achieved. In particular, the signals of native and modified ALA from the condensed and sterilized milk as well as from one infant formula were noticeably more intensive.

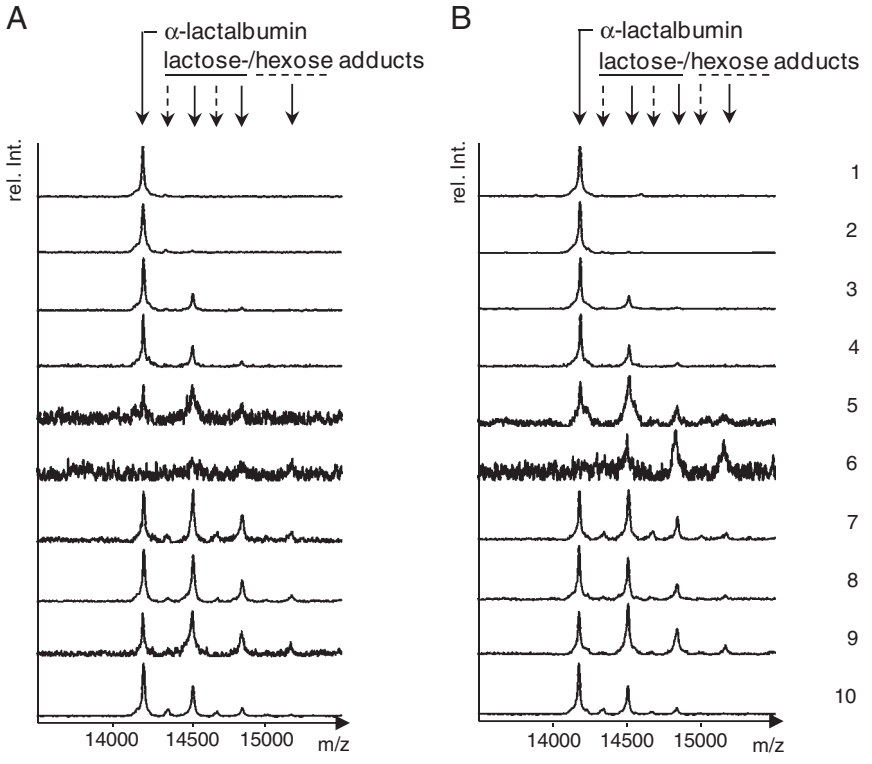


**Figure 3.** MALDI-TOF-MS spectra of commercially available dairy products. Samples were measured directly (A) or after IMAC (B). The mass range of ALA and its lactose and hexose adducts is shown. 1, raw milk; 2, high-temperature milk; 3, ultra-high-temperature milk I; 4, ultra-high-temperature milk II; 5, sterilized milk; 6, condensed milk; 7, infant formula I (liquid); 8, infant formula II (powdered); 9, infant formula III (liquid); 10, infant formula IV (liquid).

 

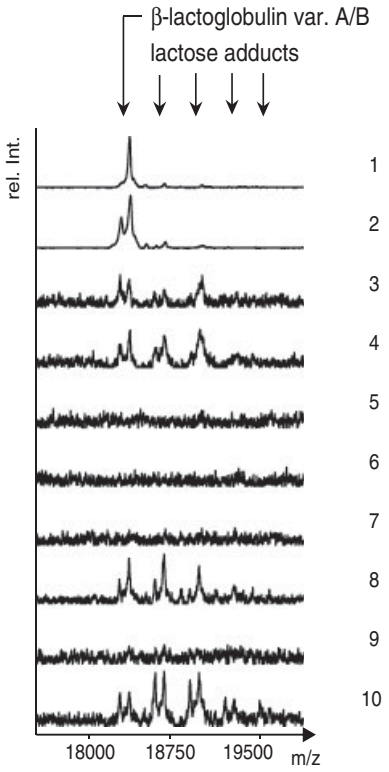


**Figure 4.** MALDI-TOF-MS spectra of commercially available dairy products obtained by direct measurement of the samples. The mass range of β-lactoglobulin and its lactose adducts is shown. 1, raw milk; 2, high-temperature milk; 3, ultra-high-temperature milk I; 4, ultra-high-temperature milk II; 5, sterilized milk; 6, condensed milk; 7, infant formula I (liquid); 8, infant formula II (powdered); 9, infant formula III (liquid); 10, infant formula IV (liquid).

However, the signals of β-lactoglobulin were not significantly influenced by the purification (data not shown), indicating that the denaturation of the protein is the limiting factor for the analysis.

## 4 Discussion

The formation of Amadori products from lactose and milk proteins (protein lactosylation) is a major parameter to evaluate the quality of heat-processed milk. Therefore, the purpose of the present study was to develop a MALDI-TOF-MS method, that can be used to measure the protein lactosylation rates in heated milk.

Although direct methods have been described to quantify lactulosyllysine in milk [15], the Amadori product is usually determined after its conversion to furosine [16]. During acidic protein hydrolysis, Amadori products are not stable, but are degraded to furosine, pyridosine, and lysine [19]. Usually, furosine, which is formed in yields between 30 and 50% [17], is then analyzed by HPLC/UV [24]. The drawback of this method is that the conversion rate depends on the sugar moiety of the Amadori product as well as on the hydrolysis conditions [17]. As a consequence, an empirical conversion factor must be determined with an authentic standard of the Amadori product in order to achieve absolute quantification. Furthermore, Amadori products cannot be quantified in complex samples, where glycation can be caused by different sugar sources. Therefore, the furosine method is usually applied for the relative quantification giving the furosine content after hydrolysis instead of the concentration of the Amadori product.

Protein MS can be used to detect lactulosyllysine residues directly. The mono- or oligolactosylated proteins are detected by mass differences of $m/z$ 324 or multiples thereof compared with the native protein [19]. Direct protein analysis by MS has the advantage of minimal sample workup, without protein hydrolysis resulting in minimal artifact formation and fast analysis. Most commonly, LC-ESI-MS is used to detect lactosylation of intact milk proteins [20, 25]. The presence of lactulosyllysine on a protein can be deduced by the mass difference in the deconvoluted mass spectrum. MALDI-TOF-MS has been used successfully to analyze the protein profile of milk or to detect lactosylation of whey proteins in milk models [8, 26]. When applied after partial enzymatic digestion, MALDI-TOF-MS can be efficiently used to analyze structure and binding site of protein modifications formed during the heat treatment of milk [7, 25]. MALDI-TOF-MS, however, has also been applied to detect protein lactosylation in heated milk. Fenaille *et al.* were able to detect up to eight lactosylation residues in different milk proteins using ALA as protein marker [10]. Furthermore, mono-, di-, and trilactosylated ALA and lactoglobulin were detected in powdered milk by MALDI-TOF-MS [27]. Compared with LC-ESI-MS/MS, MALDI-TOF-MS is even faster and easier because diluted milk can be directly applied to the MALDI target and subjected to MS without chromatography or other sample workup.

Thus, a MALDI-TOF-MS method was first optimized for its application for relative quantification of protein lactosylation in heated milk. The most important limitation of MALDI-TOF-MS is a low-spectrum quality, which can be observed for complex or more severely heat-treated samples [19]. The quality of MALDI-TOF mass spectra of milk proteins is highly influenced by the composition of the MALDI matrix. As previously suggested, the 1:1 mixture of DHB and HCCA, used in the present study, greatly favors the detection of ALA [10]. For the analysis of β-lactoglobulin, DHAP gave the best results. However, the analysis of more severely heat-treated samples showed that β-lactoglobulin is more susceptible to heat denaturation, which interferes with MALDI analysis, most likely by the formation of aggregates with other milk proteins [28, 29]. Denaturation of ALA, however, was less pronounced and influenced mainly the peak heights, but not the determination of the lactosylation rate. Furthermore, the relatively low molecular weight of ALA compared with the most

 

abundant caseins results in a better mass resolution, thus allowing a baseline separation for the signals of ALA and its glycated forms. Therefore, ALA was determined as the best marker protein to assess the formation of Amadori products by MALDI-TOF-MS.

For further improvement of spectrum quality and, particularly, *S*/*N* ratio, proteins were pre-purified by IMAC prior to MALDI-TOF-MS. IMAC has been used before to profile the peptide fraction of milk [13]. For IMAC, copper is immobilized on magnetic beads, which binds selectively to the protein fraction of milk. Since copper can interact with several functional groups of proteins, such as carboxylic and phosphate groups, as well as histidine, cystein, and tryptophan residues, the protein recovery is usually very good [30]. Thus, IMAC is a very fast, easy, and efficient way to remove low molecular weight compounds from peptides and proteins in complex matrices, such as milk. Particularly for severely heated dairy products, IMAC considerably improved the spectrum quality.

The optimized method was then tested for relative quantification of ALA-bound Amadori products. Kislinger *et al.* introduced a method for relative site- and product-specific quantification of protein-bound glycation products by MALDI-TOF-MS after partial enzymatic hydrolysis [23]. This method was applied, for example, to record product- and site-specific glycation kinetics. Furthermore, site-specific relative quantification was applied according to a similar protocol to commercial dairy products [7]. In the present study, the relative quantification of lactulosylysine modifications of intact proteins in heated milk was performed as a very fast and easy method to monitor the formation of early glycation products. Thus, it was possible to record the kinetics of adduct formation for raw milk heated at different temperatures. In the original approach, all modifications that were accessible by MALDI-TOF-MS were accounted for, when the modification rate of the Amadori product was determined [23]. In the present study, however, other modifications than the Amadori product were not detected so that only the integrals of lactosylated proteins were considered for relative quantification. In case when additional modifications are recorded in a sample, their integral should be included in the calculations as suggested by Kislinger *et al.* [23].

For electrospray ionization, it was shown that the binding of few sugar moieties to an intact protein did not influence ionization properties compared with the intact protein [31], but similar experiments are not reported for matrix-assisted ionization. However, since mono- or dilactosylation represents only a minor change in relation to the entire protein structure, it may be assumed that modified and unmodified ALA cause equivalent response in MALDI. Thus, it can be concluded that the relative concentrations obtained in this study do indeed reflect the ratio of lactosylated to unmodified ALA. Similar to the furosine method, however, MALDI-TOF-MS will not allow absolute quantification of lactulosyllysine in milk samples.

In order to evaluate the results, the samples were reinvestigated measuring several established markers for thermal treatment of milk: concentration of soluble protein at pH 4.6 according to Lowry, the concentrations of soluble ALA and β-lactoglobulin at pH 4.6, and FAST-index [21, 22, 32]. The former three methods record thermally induced denaturation of whey protein, whereas the FAST-index assesses protein denaturation as well as the formation of fluorescent Maillard products. Relative quantification of lactulosyllysine by MALDI-TOF-MS showed good correlation with all four established markers. Only in some very severely heat-treated samples, no correlation was found, because maximal denaturation of whey proteins had occurred so that further heating did not lead to further changes in total soluble protein, soluble ALA, and β-lactoglobulin, as well as FAST-index. On the other hand, the relative concentration of lactulosyllysine increased even further during more severe heat treatment. These results indicate that the three conventional methods are rather suitable for the early phase of the heating process, whereas the relative quantification of lactulosyllysine can be applied over a wider range of thermal treatment.

In addition to the milk samples, which had been heated under standardized laboratory conditions, commercially available milk products were analyzed by MALDI-TOF-MS. The spectra obtained for the dairy products were very similar to those of heated milk, indicating that the chosen heating conditions simulated industrial heating well. In some samples, such as the infant formulas, even higher lactolysation rates were found compared with the most severely heated milk samples of the model, most likely due to a higher lactose-to-protein ratio in these formulas. However, in strongly processed products, such as condensed milk, the results become less reliable, most likely because of heat-induced denaturation and coaggreagtion of the whey protein, which make them inaccessible to MALDI analysis. The effect was more pronounced for β-lactoglobulin than for ALA. These observations are in accordance with a study of Marsilio *et al.* [29], who reported the disappearance of some signals in the spectra of milk after heating. Thus, it can be concluded that – with some exceptions – the method can be applied to monitor industrial milk processing.

Compared with the furosine method, MALDI-TOF-MS analysis of early Maillard products is very easy and fast, even when IMAC extraction is applied. Furthermore, the present study confirms that MALDI-TOF-MS can provide reliable results, if Amadori products are formed in dairy products from different sugar sources as shown before for model reaction mixtures [33]. Problems may arise only in severely heat-treated samples, such as condensed milk, where whey proteins are largely coaggregated.

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