



Short communication

A new UHPLC method for the quantitation of furosine as heat load indicator in commercial liquid milk

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ABSTRACT

The concentration of the artificial compound furosine in liquid milk can be used as an indicator to assess the heat load applied onto the product, which allows discrimination between differently processed milk (pasteurized, Extended Shelf Life (ESL) milk, Ultra-High Temperature (UHT) treated milk). In this work, a new UHPLC method for the rapid determination of furosine in 2 min only (total runtime 6 min) is presented, using PDA detection at 280 nm. The method was validated for the analysis of liquid milk samples regarding linearity, instrumentation precision, limits of detection and quantification, repeatability of sample work-up and recovery. Moreover, the method was compared to an existing RP-HPLC method. Selected commercial liquid milk samples ($n=36$) were analyzed, showing lowest furosine amounts for pasteurized milk, whereas highest amounts were found in UHT milk. For ESL milk, quite diverse concentrations were analyzed ranging from 11.70 up to 115.16 mg furosine 100 g⁻¹ protein, illustrating the different processing technologies of this category with lowest amounts for filtered ESL milk, followed by directly heated, and indirectly heated ESL milk samples, respectively.

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1. Introduction

Furosine is a routinely used heat load indicator for the early stage of Maillard reaction in human serum and foods, e.g., carrots and dairy products (Boitz and Mayer, 2015; Mayer et al., 2010; Wellner et al., 2011; Wu et al., 1995). During production of liquid milk, the applied heat load leads to chemical, nutritional and organoleptic changes of the final product, depending on its extent (pasteurization vs. ultra-high temperature treatment – UHT). Specific compounds extrinsic or intrinsic to milk, so called time-temperature integrators (TTIs), offer the possibility to assess the heat load applied to the product. These TTIs can be divided in type I- and type II-indicators (Claeys et al., 2002). Furosine is categorized as a type II-indicator, describing the formation of compounds which are almost not present in liquid milk prior to heating; these indicators are suitable for the assessment of milk products with higher heat load. On the contrary, type I-indicators include denaturation and inactivation processes of compounds

naturally occurring in milk (e.g., β -lactoglobulin), suitable for the evaluation of low-heated products (Mayer et al., 2010). As shown previously (Mayer et al., 2010), furosine is an appropriate quality indicator for liquid milk. Especially for Extended Shelf Life (ESL) milk, comprising a wide quality range in the final product due to different production technologies including a mechanical filtration technique (microfiltration/deep bed filtration) followed by a pasteurization step, or thermally (through a gentle direct or more intense indirect heat treatment), furosine serves as a useful TTI to distinguish between the different heat loads applied in this kind of milk.

Furosine is closely related to the early stage of Maillard reaction, also known as non-enzymatic browning reaction, associated with every heat treatment of dairy products, or resulting as a consequence of storage (Erbersdobler and Somoza, 2007; Ferrer et al., 2000; Wellner et al., 2011). The initial step is a reaction of a reducing sugar (in dairy products especially lactose) with the ϵ -amino group of amino acids, in particular lysine. Subsequently, the formed Schiff's base, lactosyl-lysine is converted via an Amadori rearrangement to the more stable protein-bound lactulosyl-lysine (ϵ -N-deoxylactulosyl-L-lysine), which is biologically unavailable (Mehta and Deeth, 2016; Van Boekel, 1998). Thus, the Maillard reaction reduces the nutritional quality of the product

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by reducing the biological value of the protein. Approximately 40% of the Amadori product is converted into furosine by acid hydrolysis, while pyridosine was reported to be built only in small amounts (Finot et al., 1969; Van Boekel, 1998). However, the hydrolysis rate is reproducible if constant conditions are ensured. Thus, furosine acts as a direct marker of lysine reaction products, which are not only of technological and analytical relevance, but also important for the nutritional value of the dairy product (Erbersdobler and Somoza, 2007).

Reported methods for the determination of furosine cover capillary zone electrophoresis (Tirelli, 1998) as well as a front-face fluorescence method (Kulmyrzaev and Dufour, 2002), and recently, a stable isotope dilution assay coupled with tandem mass spectroscopy was reported for the simultaneous detection of several Maillard reaction products including furosine (Troise et al., 2015). However, the most established method includes a hydrolysis of the milk sample with HCl, followed by a solid-phase extraction and finally, quantification with an ion-pair RP-HPLC system using UV detection at 280 nm, as recommended by the IDF (FIL/IDF, 2004; Standard 193). Due to the positive correlation of furosine to the protein content of sample, the amount is given in mg furosine per 100 g protein, requiring the measurement of the protein content in the sample.

Referring to dairy products and liquid milk, limits of TTIs are not regulated consistently within the EU. For instance, furosine thresholds are fixed in Italy for raw and pasteurized milk (Krause et al., 2003), whereas in Austria and Germany, only recommendations for pasteurized and UHT milk exist (Schlimme, 1995). The *Austrian Food Codex* suggests threshold limits for type I-indicator β -lactoglobulin, but regulatory limits for furosine are merely under discussion in Austria (Mayer et al., 2010). To close this lack, there is a strong need to update these recommendations including the quite heterogeneous products of ESL milk, and to assess the heat load applied for this new category of liquid milk (including filtered, directly or indirectly heated ESL milk). Threshold limits (as they already exist for β -lactoglobulin) for furosine would provide a meaningful tool for the quality assessment of liquid milk, especially for higher heated ESL milk. Due to the growing market share of ESL milk and the need for a comprehensive quality

assessment of differently processed milk types, and the consequential demand for faster chromatographic methods, the aim of this study was to adapt an existing RP-HPLC method (Mayer et al., 2010) onto an ultra-high performance liquid chromatography (UHPLC) system. These high-throughput systems, equipped with columns packed with sub-2 μm particles, improve the LC performance by enhancing resolution, analysis speed and sensitivity. Due to smaller particles in stationary phase, UHPLC systems have to overcome back pressures up to 1300 bar, whereas conventional HPLC systems work at 400 bar. Thus, a rapid analysis method for furosine in liquid milk was to be established to enable the assessment of actual heat load of the product and to differentiate between differently processed milk samples.

2. Material and methods

2.1. Standards and chemicals

Furosine standard (furosine dihydrochloride; >97%) for calibration was purchased from NeoMPS PolyPeptide Laboratories Group (Strasbourg, France). L-Lysine hydrochloride was purchased from Pierce (Rockford, Illinois, USA). Chemicals used for sample preparation were of analytical, and solvents for chromatographic analysis of HPLC-grade. Acetonitrile (ACN, 100%) and heptanesulphonic acid (>98%) were purchased from Sigma Aldrich (St. Louis, Missouri, USA). Hydrochloric acid (37%) and formic acid (FA, 98%) were purchased from Roth (Karlsruhe, Germany). Ultrapure water prepared using a SG Ultra Clear UV system (Siemens Water Technologies, Warrendale, Pennsylvania, USA) was used throughout all experiments. Stock solution for furosine (2 mmol L^{-1}) was prepared in 3 mol L^{-1} HCl. Further dilutions for calibration were prepared with diluting solution (5% ACN, 0.2% FA in ultrapure water) ranging from 0.25 to $8.0\text{ }\mu\text{mol L}^{-1}$.

2.2. Preparation of milk samples

Differently processed commercial Austrian liquid milk samples ($n = 36$) comprising different heat loads applied, were analyzed for their furosine concentrations including pasteurized ($n = 8$) as well

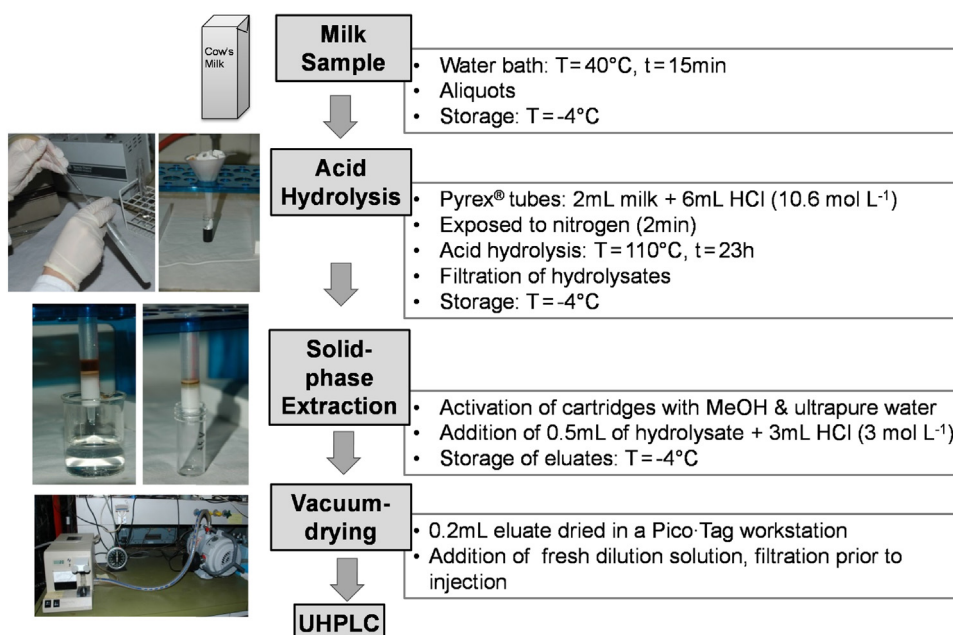


Fig. 1. Flow-chart showing the sample preparation of liquid milk for the chromatographic determination of furosine.

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