Analytical Methods

Determination of the native forms of vitamin B₁ in bovine milk using a fast and simplified UHPLC method

Anatol Schmidt*, Herbert Pratsch, Maximilian G. Schreiner, Helmut K. Mayer

Department of Food Science and Technology, Food Chemistry Laboratory, BOKU – University of Natural Resources and Life Sciences Vienna, Muthgasse 11, A-1190 Vienna, Austria

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Abstract

The aim of this study was to develop a high-throughput UHPLC method for the determination of vitamin B₁ active compounds; thiamin, thiamin monophosphate and thiamin diphosphate in bovine milk. In order to sustain the native vitamin B₁ phosphorus esters, sample preparation is crucial. Various acids as well as commonly used enzymes and their enzyme mixtures were compared. Method accuracy was confirmed using certified reference material as well as comparison with the corresponding CEN method, and was found to be satisfactory. Studied milk samples showed significant amounts of thiamin monophosphate, which can make up to 53.9% of the total vitamin B₁ content in commercial milk, and up to 78% in raw milk. Moreover, a tremendous variation of the total content of vitamin B₁ was observed between single cows, which ranged from 0.24 mg/L up to 0.54 mg/L of total vitamin B₁.

1. Introduction

Thiamin as vitamin B₁ is a necessary cofactor for various life-supporting enzymes. The active cofactor for those enzymes is the vitamer thiamin diphosphate (TDP), also called thiamin pyrophosphate. TDP is necessary for the performance of enzymes linked to the carbohydrate metabolism, and is thus inevitable linked to the pentose-phosphate pathway and the tricarboxylic acid cycle (Frank, Leeper, & Luisi, 2007). For humans, failing to match the daily required intake of vitamin B₁ active compounds leads to severe illnesses such as beriberi or the Wernicke-Korsakoff syndrome (Bettendorf & Wins, 2009). Other B₁ vitamers (similar compounds, exhibiting vitamin activity) are thiamin monophosphate (TMP) and thiamin, also referred to as free thiamin (Thia). Thiamin triphosphate is considered to be a messenger molecule and does not act as an enzymatic cofactor (Bettendorf & Wins, 2009; Makarchikov, 2009). Only microorganisms and plants possess the ability to synthesise thiamin. Higher life forms, such as animals and humans are only able to perform a pyro-phosphorylation of Thia to TDP, and therefore have to match their demand of TDP precursors by dietary intake (Bettendorf & Wins, 2009). The origin of TMP is controversial; it is the final product of the biosynthesis of Thia, in organisms being capable of, such as yeast (Nosaka, 2006), but it can also be a degradation product from TDP (Makarchikov et al., 2003). Humans match their TDP demand by the formation of TDP from Thia by pyro-phosphorylation (thiamin pyrophosphotransferase), and not by a consecutive phosphorylation of TMP (Bettendorf & Wins, 2009). In the case when TMP is the sole precursor for TDP, it is first dephosphorylated to Thia by non-specific enzymes and then subsequently pyro-phosphorylated to TDP (Pourcel, Moulin, & Fitzpatrick, 2013). Even if TMP might not be present in the nourishment, endogenous synthesis by the intestinal flora has to be considered (Roth-Maier, Wild, Erhardt, Henke, & Kirchgeßner, 1999), as some microorganisms of the human intestinal flora are able to synthesize TDP from TMP (Makarchikov, 2009). Although Thia, TMP and TDP can act as either precursors or the actual active cofactor for vitamin B₁ depending enzymes, the metabolism of those vitamers as well as their function is still not comprehensively understood (Bettendorf & Wins 2009; Makarchikov, 2009). Hence, adequate instruments are needed in order to investigate these unknowns. Particular rich...
sources of vitamin B1 for humans are yeast and cereals (Jakobsen, 2008). Milk and dairy products are consumed by a large number of humans on a daily basis. One hundred millilitres of bovine milk can provide up to 18% of the daily required intake of vitamin B1 (Claeys et al., 2014).

Foodstuffs in general, tend to be highly complex matrices, mostly containing a mixture of fat, proteins and carbohydrates. In order to quantify a chosen target-analyte in a specific matrix, sample preparation procedures have to be chosen wisely. Especially milk contains a challenging mixture of app. 87% water, 3.9% fat, 4.9% protein, 4.9% carbohydrates and 0.7% ash (Corbin & Whittlet, 1965). In order to convert Thia phosphate esters to so called free Thia, and to release such compounds from the milk matrix, acid hydrolysis and enzymatic digestion are widely used (Akalin, Gönç, & Dinkci, 2004; EN 14122:2014; Jakobsen, 2008; Lalic, Denic, Sunaric, Kocic, & Trutic, 2013). These treatments aim to break down the sample matrix as well as to convert all Thia phosphate esters to free Thia, thus making it impossible to study the native composition of B1 vitamers of the sample (Lynch & Young, 2000). Therefore, simpler sample preparation approaches, excluding enzymatic digestion and thus preserving the full spectrum of possible B1 vitamers have been suggested. Such approaches omit enzymatic digestions and solely focus on the precipitation of proteins in the sample by altering the pH value using acids or alkalis (Albalá-Hurtado, Veciana-Nogués, Izquierdo-Pulido, & Mariné-Font, 1997; Lynch & Young, 2000; Márquez-Silleró, Cámares, & Valcárcel, 2013). Nowadays, Thia and other possible vitamin B1 precursors are analysed by chromatographic separation, such as HPLC. These methods mostly employ the alkaline oxidation of Thia or TMP and TDP to the highly fluorescent thiochrome (Tcr) or thiochrome phosphate esters (TcrMP, TcrDP), thus enhancing sensitivity and selectivity of the analytical method (Akalin et al., 2004; Jakobsen, 2008; Lalic et al., 2013; Lynch & Young, 2000).

In order to mitigate possible health hazards and increase shelf life, milk is treated thermally and occasionally mechanically. The applied range of used temperatures and exposure time vary considerably, resulting in a broad range of possible heat loads (Sakkas, Moutafi, Moschopoulou, & Moatsou, 2014). As vitamin B1 and therefore its vitamers are considered to degrade under heat treatment, the impact of such treatment on the B1 vitamer content and composition in milk samples is of great importance.

Simplified sample preparation, together with UHPLC separation technology can enable high resolution and cost effective high-throughput analysis of the native spectra of vitamin B1 active compounds in milk. Hence, the aim of this study was to establish a simple and rapid UHPLC method for the simultaneous analysis of possible vitamin B1 active compounds, namely Thia, TMP and TDP in milk. Moreover, the developed method was employed to study commercial milk samples treated with different thermal preservation techniques, as well as non-commercial raw milk samples obtained from single cows, in order to evaluate possible deviation between individuals regarding their B1 vitamer composition.

2. Material and methods

2.1. Reagents and standards

For the calibration of vitamin B1, the following standards were purchased from Sigma-Aldrich (St. Louis, USA): thiamin hydrochloride (99%, 337.27 g/mol), thiamin monophosphate chloride dihydrate (99%, 416.82 g/mol) and thiamin diphosphate (95%, 480.77 g/mol). For the enzymatic assay, clara-diatase (No. 86959), acid phosphatase from potato (No. P3752), z-amylase from Aspergillus oryzae (No. A9875), and papain from Papaya latex (No. P3375) from Sigma-Aldrich were used. All other chemicals were of analytical grade, and solvents used for chromatography of HPLC grade. Ultrapure water from SG Ultra Clear UV system (Siemens Water Technologies, Warrendale, PA, USA) was used throughout all experiments. Sample preparation was performed with stock solutions of acetic acid (100%), hydrochloric acid (32%) and perchloric acid (70%) from Roth (Karlsruhe, Germany), and trichloroacetic acid (100%) from Sigma-Aldrich. Sodium hydroxide, solid (99%) and potassium ferricyanide(III) (99%) from Roth as well as ortho-phosphoric acid (85%) from Merck (Darmstadt, Germany) were used for thiochrome oxidation. For sample preparations, sodium acetate trihydrate (99.5%) from Merck, and sodiumacetate trihydrate (99.5%) from Sigma-Aldrich for mobile phase adjustment were used.

2.2. Sample collection

Different commercial and raw milk samples as well as raw milk obtained from single cows were studied. Commercial milk samples were obtained from different Austrian retailer markets located in the area of Vienna (Austria) and represent a comprehensive market survey of commercially available drink milk, containing from 0.5 up to 3.8% of fat and 3.3 to 3.5% of protein, as stated on the package. Raw milk samples and raw milk from single cows were obtained from a dairy facility in St. Veit im Mühlkreis (Upper Austria, Austria). The samples were collected by a veterinarian from healthy individuals from Simmental cattle held under the same conditions. Samples were frozen within a maximum of 3 h after collection and stored at –18 °C until analysis. Certified reference material (dried milk powder, CRM® BD-600) was obtained from Sigma-Aldrich.

2.3. Sample preparation

Preparation of milk samples was performed as follows. Dried milk powder was dissolved in UHQ water, resulting in a concentration of 1 g of dried sample per 10 mL. Frozen milk samples were reconstituted at 42 °C for 15 min in a water bath (Boitz & Mayer, 2016). Aluminium foil was used to ensure light protection during all consecutive manipulation steps. If not stated otherwise, samples were kept at 4 °C. For the evaluation of various acids used for protein precipitation, 10 mL of each liquid sample was mixed with either acetic acid (2.5 mol/L or 5.0 mol/L), hydrochloric acid (1 mol/L), trichloroacetic acid (4%) and perchloric acid (7%), brought to the corresponding pH level and filled to a volume of 30 mL with ultrapure water. Samples were then incubated for 10 min at 20 °C under continuous shaking.

For enzymatic digestions, the protocol follows the relevant CEN method (EN 14122:2014). The selection of used enzymes reflects recent publications (EN 14122:2014; Jakobsen, 2008) and was carried out as follows: 5 mL of pasteurized milk were mixed with 35 mL of 0.1 mol/L HCl, resulting at a pH of 1.2 and hydrolyzed for 15 min at 121 °C using a table autoclave (CertoClave, Traun, Austria). Afterwards, the sample was cooled down to 25 °C, and the required pH adjusted, using 2.5 mol/L sodium acetate and brought to a volume of 50 mL. Subsequently, the required enzyme was added and incubated under constant shaking for 9 h at a temperature of 45 °C for acid phosphatase, papain, z-amylase and 25 °C for clara-diatase, respectively. The used amount of enzyme were 0.5 g for clara-diatase, 0.6 g for papain and 0.05 g for acid phosphatase as well as z-amylase. Resulting in 75.000 U of clara-diatase, 3.000 U of papain, 150 U of acid phosphatase and 100 U of z-amylase. For enzyme mixtures, 0.05 g z-amylase (100 U) and 0.05 g acid phosphatase (150 U), as well as 0.05 g acid phosphatase (150 U) and 0.6 g of papain (3.000 U) were mixed. Samples solely consisting of UHQ water were employed for the evaluation of possible contamination of the used enzymes by target analytes. Following acidic protein precipitation or enzymatic digestion,