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Analysis of betaine levels in cereals, pseudocereals and their products

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ABSTRACT

Betaine has a range of health benefits and therefore has been recommended as a functional ingredient in dietary supplements. The main dietary sources of betaine are processed grains such as bread, biscuits, cereals, pasta and similar products. This study describes analysis of 54 samples of cereals and pseudocereals for betaine content by using HPLC – ELSD method. By using this approach betaine levels were identified, quantified and compared. Analysis of variance showed significant differences between analyzed samples (from <LOQ mostly in gluten free products to 328.5 mg/100 g DM in enriched plain biscuit with molasses). PCA analysis gave two large clusters, one for gluten-free samples and the second cluster containing all of the remaining samples. As a final result the average betaine levels in analyzed food samples were in the following order: buckwheat < millet < wheat < oats < rye < barley < amaranth < spelt.

1. Introduction

Although betaine is known as a non-essential nutrient, numerous studies in recent years reported a wide range of its health benefits (Craig, 2004; Schwahn et al., 2003). For these reasons betaine is used as functional ingredient and dietary supplement (Filipčev et al., 2016). Betaine is considered as GRAS ingredient in the US, while in Europe, it has an approval for use in foods by the European Commission (Commission Regulation EU 432, 2012). Chemically, betaine (N,N,N-trimethylglycine) is a zwitterionic compound at neutral pH with dual function in the human organism: as an osmolyte and as a methyl donor. Betaine participates in the methionine cycle primarily in the human liver and kidneys by acting as methyl group donor required for the formation of methionine and S-adenosylmethionine (SAM) (Craig, 2004; De Zwart et al., 2003; Ross, Zangger, & Guiraud, 2014; Schwahn et al., 2003). Choline and betaine are important sources of one-carbon units, in particular, during folate deficiency (Ueland, 2011). By providing the one-carbon units, betaine also enables the conversion of homocysteine to methionine, conserves methionine, detoxifies homocysteine, and produces S-adenosylmethionine which is currently used successfully to treat liver disease (Barak, Beckenhauer, & Tuma, 1996; Craig, 2004; Lever & Slow, 2010). Zhang, Wu, et al., 2016 reported that betaine inhibits hepatitis B virus (HBV) and the antioxidant activity of betaine was confirmed by the same author (Zhang, Zhang, et al., 2016). Recently, experiments in rats fed high fat diet and supplemented with 1% betaine resulted in anti-steatotic activity of betaine (Ahn et al., 2015).

As a dietary component of many foods, betaine is present at different concentrations, depending on the source and processing conditions. It has primarily been isolated from sugar beet, nowadays the major source of betaine in the Western diet are cereal based foods (De Zwart et al., 2003; Gao et al., 2016; Likes, Madl, Zeisel, & Craig, 2007; Ross et al., 2014). Slow et al. (2005) found high levels of betaine in grain products such as bread, pasta and

Abbreviations: HPLC-ELSD, high performance liquid chromatography-with evaporative light scattering detector; HPLC-UV, high-performance liquid chromatography with ultraviolet spectrometric detection; HILIC, Hydrophilic Interaction Chromatography; NP, normal-phase; RP-LC, reversed-phase liquid chromatography; ANOVA, analysis of variance; PCA, principal component analysis; SS, standard score; LOQ, the limit of quantitation; LOD, the limit of detection.

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flour, ranged from 360 μg/g in white bread to 7200 μg/g in cereal bran. Other sources of betaine are shellfish, shrimps, chicken, as well as plant sources such as beetroot and spinach (members of the beet family) (De Zwart et al., 2003; Filipčev, Brkljača, Krulj, & Bodroža-Solarov, 2015). A US Department of Agriculture database of the choline and betaine content of food has been developed by Zeisel, Mar, Howe, and Holden (2003). They found highest betaine concentration (mg/100 g) in: wheat bran (1339), wheat germ (1241), spinach (645), pretzels (237), shrimp (218) and wheat bread (201). Since most of betaine sources have rather complex matrices, the isolation and characterization of betaine could be a promising area of research.

Different extraction procedures have been performed for extraction of betaine from different food matrices. The most commonly used solvent for extraction of betaine is water (Bruce, Guy, Rezzi, & Ross, 2010; De Zwart et al., 2003; Hefni, McEntyre, Lever, & Slow, 2016; Ross et al., 2014; Slow et al., 2005). Hefni et al. (2016) reported strong impact of extraction conditions on the quantified betaine content in different foods, demonstrating the necessity of repeating the extraction procedure to obtain reliable results. In order to determine the betaine content in food, different methods have been developed. The most of them are based on using liquid chromatography. However, there is no a universal method which could be applied to all food matrices. Saarinen et al. analyzed betaine in chicken liver using a cation exchange column of Ca^{2+} type and refractive index detector although quantification is limited because of poor detection sensitivity (Saarinen et al., 2001). Considering its physicochemical properties, this quaternary amine could not be analyzed by the conventional reversed-phase high performance liquid chromatography, and could not be detected with UV detector without derivatization. De Zwart et al. (2003) derivatized wide range of foods commonly found in the western diet and betaine analysis was performed by high-performance liquid chromatography with standard ultraviolet spectrometric detection (HPLC-UV) using different columns. Slow et al. (2005) extracted betaine in different products grouped into 10 food categories: grains, fruit, vegetables, beverages-nonalcoholic, beverages-alcoholic, meat, seafood, dairy products, nuts and miscellaneous using water and dichloromethane, and the extracts were derivatized with 2-naphthacyl trifluoromethanesulfonate. Hefni et al. (2016) developed a simple HPLC-UV method for betaine determination in several different food matrices such as fresh spinach, whole wheat flour, wheat (*Triticum aestivum*) and beet (*Beta vulgaris*) after derivatization on strong cation exchange column. Bruce et al. (2010) and Ross et al. (2014) performed the analysis using LC-MS/MS coupled with an HILIC column. Bruce et al. (2010) developed LC-MS/MS method for the analysis of 47 plasma samples, 32 cereal flours and cereal fractions, and 51 cereal products. Additionally, Ross et al. (2014) analyzed betaine by liquid-chromatography with tandem mass spectrometry in a wide range of commercially available cereal foods and cereal fractions. Du Shin et al. (2012) proposed HILIC column in combination with evaporative light scattering detector (ELSD) for betaine analysis in *Fructus* Lycii. Recently, Hydrophilic Interaction Chromatography (HILIC) is an alternative to reversed-phase liquid chromatography (RP-LC). HILIC is a type of normal-phase (NP) chromatography because it has the same polar stationary phase, but can use large amounts of organic solvent (>80%) as the mobile phase compared to NP. As such, the HILIC column was more effective for the chromatographic separation of betaine (Buszewski & Noga, 2012).

The main aim of this study was to establish betaine content in 54 samples of cereals and pseudocereals. In order to analyze betaine levels we have modified a method previously used by Du Shin et al. (2012). This included a change from gradient to isocratic mode which resulted in shortened sample elution time. Additionally, optimization of pH value of mobile phase and vortex extraction time has been performed. By using this methodology betaine levels were analyzed and compared.

2. Materials and methods

2.1. Chemicals and reagents

Anhydrous betaine was used as an internal standard (98% purity, AlfaAesar GmbH&KG, Karlsruhe, Germany). Acetonitrile and methanol UHPLC grade were purchased from PanReac AppliChem (Barcelona, Spain). 10 mM ammonium acetate buffer was prepared using ammonium acetate (99% purity, Lach-Ner, Neratovice, Czech Republic) and the ultrapure water, which was produced by a Simplicity UV system from Millipore (Bedford, MA, USA). The pH was adjusted to target value by using concentrated acetic acid or a diluted ammonium hydroxide solution and finally buffer was filtered through a membrane of 0.45 μm (Millipore) into a measuring flask.

2.2. Sample collection and preparation

The majority of the food samples analyzed in this study have been obtained from the local market and food stores in Novi Sad (Serbia). Wheat grain (*Triticum aestivum*), all durum wheat (*T. durum*), triticale (*Triticosecale*), barley (*Hordeum vulgare*), and rye (*Secale cereale*) samples were obtained from the collection of samples of the Laboratory of the Institute of Food Technology. Amaranth grain and related samples were provided from the local producer. Ground and homogenized sample (2 g) was weighted and suspended in methanol (25 mL) and vortexed for 10 min. After a 30 min of ultrasonic extraction in an ultrasonic bath (ATU Ultrasonidos, Valencia, Spain), the sample was vigorously shaken and centrifuged for 10 min at 5000 r/min (Eppendorf Centrifuge 5804R, Eppendorf, Wien, Austria). Upper methanol layer (3 mL) was evaporated to dryness. Afterwards, the residue was reconstituted in 2 mL of water and filtered through a membrane filter (regenerated cellulose, pore size 0.22 μm, diameter 25 mm, Agilent Technologies, Santa Clara, USA).

2.3. Optimal chromatographic conditions

Betaine analysis was performed using a HPLC system (Agilent Technologies Inc., USA) equipped with a Kinetex®HILIC (Phenomenex, Aschaffenburg, Germany) column (2.6 μm, 100 × 2.1 mm) and ELSD detector (1290 Infinity ELSD, Agilent Technologies, USA). Separation was performed at a flow-rate 0.5 mL/min with a mixture of acetonitrile and 10 mM acetate buffer at pH 3.7 (80:20, v/v) following isocratic regime. Total run time was 10 min. Injection volume was 5 μl using autosampler injection mode. The injector was at room temperature. Detector parameters were as follows: evaporator temperature 40 °C; nebulizer temperature 55 °C; gas flow rate 1.60 standard liter per minute (SLM), a photomultiplier tube (PMT) gain 3.0.

2.4. Method performance

2.4.1. Calibration curve and linearity

A test for the general matrix effect is performed by means of 'standard additions' or the method of analyte additions according to guidelines for validation of analytical methods (Huber L., 2010). A calibration curve is prepared in the same biological matrix as the samples by spiking the matrix with known concentrations of the analyte. A calibration curve consists of a zero sample and five non-zero samples covering the expected range (0.05, 0.075, 0.1, 0.15, 0.20 mg/mL). The curve was constructed by plotting the peak