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The betaine profile of cereal flours unveils new and uncommon betaines

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ABSTRACT

We report the LC-ESI-MS/MS determination of betaines in commercial flours of cereals and pseudocereals most utilized in human nutrition. Results showed that glycine betaine, trigonelline, proline betaine, N^{ε}-trimethyllysine were metabolites common to all examined flours, whereas an uncommon betaine, valine betaine, and glutamine betaine were present only in flours of barley, rye, oat, durum wheat, winter wheat, Triticum dicoccum and Triticum monococcum. Valine betaine and glutamine betaine, the latter never reported before in plants and animals, are not evenly distributed in the Poaceae family, but their presence or absence in flours depends on the subfamily to which the plant belongs.

Interestingly, we also report for the first time the occurrence of pipecolic acid betaine (homostachydrine) and its precursor 1,2-*N*-methylpipecolic acid in rye flour. These two metabolites were not detected in any other cereal or pseudocereal flour, suggesting their potential role as markers of rye flour occurrence in cereal-based foods.

1. Introduction

Cereals belong to the large grass family of Poaceae, which represents the most important staple food in human nutrition in both developed and developing countries. The main food sources among the Poaceae subfamilies are the Pooideae, Panicoideae and Oryzoideae. The Pooideae subfamily comprises the genus Triticum, including the widely-exploited *Triticum durum* (durum wheat) and *Triticum aestivum* (winter wheat) species, the genus Hordenum including the important *Hordenum vulgare* (barley) species, the genus Avena to which belongs *Avena sativa* (oat), and the genus Secale to which belongs *Secale cereale* (rye). The Panicoideae subfamily includes maize (*Zea mays*), sorghum (*Sorghum bicolor*), and millet (*Panicum miliaceum L.*). The only important species the Oryzoideae subfamily is rice (*Oryza sativa L.*). Among the non-Poaceae plants utilized for human nutrition, known as pseudocereals, the principal crops are buckwheat, amaranth and quinoa.

Some cereals, such as rice, barley and oats are usually consumed as whole grains, while others, such as wheat and corn, are mainly used as flours obtained industrially by milling followed by complete or partial removal of bran (refined flours). Flours without bran removal are commercialized as wholemeal flours. Refined flours are characterized by improved taste and higher digestibility but the poor content of bioactive substances lowers their nutritional value. Therefore, wholemeal flours, although less palatable, are particularly appreciated by a growing number of consumers who recognize elements that enrich their daily diet, such as vitamins, phenolic acids, alkilresorcinols, flavonoids, lignans (Bartłomiej, Justyna, & Ewa, 2012).

As for betaines in cereals, there are only few studies limited to some flours and derivatives, such as bread and pasta, although broad and in-depth survey have been conducted on their content in various foods (Bruce, Guy, Rezzi, & Ross, 2010; De Zwart et al., 2003; Likesa, Madla, Zeiselb, & Craigc, 2007; Slow et al., 2005; Zeisel, Mar, Howe, & Holden, 2003). Chemically, betaines are quaternary ammonium compounds, originating from the exhaustive methylation of amino or imino acids through specific biosynthetic pathways in which S-adenosylmethionine is the methyl group donor. Betaines are ubiquitous in plants for their defensive roles against abiotic stresses, as those induced by drought, cold, freezing, hypoxia, high salinity of soil. The protective role of the betaines is accomplished through the accumulation in cells or intracellular fluids where they stabilize the structures of nucleic acids, proteins and membranes (Burg & Ferraris, 2008; Street, Bolen, & Rose, 2006). According to the species, plants show characteristic types

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and levels of betaines. Indeed, citrus plants express high levels of proline betaine and 4-hydroxyproline betaine (Servillo, Giovane, Balestrieri, & Bata-Csere et al., 2011; Servillo, Giovane, Balestrieri, & Cautela et al., 2011), alfalfa is rich in proline betaine and pipecolic acid betaine (Wood et al., 1991), whereas spinach, beet and wheat are rich in glycine betaine (Zeisel et al., 2003). Betaines play important roles also in animals. In this regard, glycine betaine functions both as a compatible osmolyte accumulated to high levels in tissues and as a major supplier of labile methyl groups. In humans (and other animals), glycine betaine can be both synthesized endogenously, through the two-step oxidation of choline, and obtained from the diet. Instead, the sources of other betaines, such as trigonelline and proline betaine, are only dietary. Glycine betaine is important for the maintenance of human health as it is essential in the conversion of homocysteine to methionine by functioning as donor of methyl groups in a reaction catalyzed by the betaine-homocysteine methyltransferase (BHMT) (Craig, 2004; Lawson-Yuen & Levy, 2006), thus lowering the plasma levels of homocysteine, a risk factor of cardiovascular disease (Shai et al., 2004). Among other betaines, proline betaine exerts beneficial health effects counteracting the high-glucose induced endothelial senescence via SIRT1 signaling pathway (Servillo et al., 2013) and ameliorating the hypertrophy of cardiomyocytes induced by norepinephrine (Zhang, Lu et al., 2014; Zhang, Shan et al., 2014). Similarly, trigonelline has been shown to counteract oxidative stress in diabetic hypertensive nephropathy (Ghule, Jadhav, & Bodhankar, 2012). However, there are reasons for concern on the risks represented by high dietary levels of exogenous betaines. As matter of fact, studies have shown that both proline betaine and trigonelline are not metabolized and are BHMT inhibitors. This can lead to higher homocysteine levels, thus increasing the risk for cardiovascular disease. Furthermore, doses of proline betaine and trigonelline in rats caused not only an increase in homocysteine but also an increase of glycine betaine excretion by blocking its reabsorption in the kidney. Indeed, it has been suggested that these betaines could in fact be harmful. (Slow, Lever, Lee, George, & Chambers, 2004).

The growing interest in betaines as natural compounds potentially beneficial to human health prompted us to undertake a comprehensive study about their distribution in cereals, which represent the most important sources of food for the contribution of proteins, vitamins, minerals and energy to the world population.

2. Materials and methods

2.1. Reagents

Glycine betaine, *N*-methylnicotinic acid (trigonelline), N^{ε}-trimethyllysine, piperidine-2-carboxylic acid (pipecolic acid), 1-methylpiperidine-2-carboxylic acid (alias 1,2-*N*-methylpipecolic acid), valine, glutamine, glutamic acid, and glutaminase from E. coli were from Sigma-Aldrich (Milan, Italy). Proline betaine (stachydrine) was purchased from Extrasynthese (Genay, France). Pipecolic acid betaine (homostachydrine) was synthesized as described (Servillo et al. (2012). Milli-Q water was used for all the preparations of solutions and standards. The solution of formic acid 0.1% in water used for the HPLC-ESI-MS/MS analyses was from Sigma-Aldrich (Milan, Italy).

2.2. Synthesis and purification of valine betaine (*N,N,N*-trimethylvaline), glutamine betaine (*N,N,N*-trimethylglutamine) and glutamic acid betaine (*N,N,N*-trimethylglutamic acid)

The preparation of betaines of valine, glutamine, and glutamic acid was performed following the procedure of Chen and Benoiton (1976), based on a heterogeneous phase reaction using methyl iodide in the presence of $KHCO_3$ as a methylating agent. Briefly, about 100 mg of valine (or glutamine or glutamic acid) was dissolved in 20 mL of methanol, added with 1 g of $KHCO_3$, 10 mL of methyl iodide, and stirred 12 h at room temperature. The addition of $KHCO_3$ (1 g) and methyl iodide (10 mL) was repeated twice more. Finally, the mixture was centrifuged and the supernatant was evaporated to dryness at 40 °C in a rotavapor. The residue, containing the betaine, was dissolved in 10 mL of Milli Q grade water and applied on 10 cm column filled with a mixed-bed resin of Dowex-1-OH^{8} and Biorex-70-H^{+} (1:1 v/v), able to retain amino acids but not betaines (Wood et al., 2002). The aqueous wash from this column was then applied to a 10 × 2 cm column with AG50WX8-H^{+} resin, and washed with 20 mL of water. Finally, the betaine was eluted with 30 mL of 6 M NH_4OH and evaporated to dryness under a stream of air.

2.3. Sample preparation of cereal flour extracts

All refined flours, purchased in local markets, were from plant species cultivated in European countries, except for quinoa, cultivated in Chile and Argentina. For each type of flour, three lots of different brands were employed for sample preparations. Each lot was constituted by two samples of the same brand. On the whole, 72 commercial samples of refined flours of 10 types of cereal species (durum wheat, winter wheat, rye, Triticum dicoccum, Triticum monococcum, oat, maize, millet, sorghum and rice) and 2 types of pseudocereal species (buckwheat and quinoa) were analyzed. Betaines and related compounds were extracted according the procedure described by Servillo et al. Servillo et al., 2015; Servillo, Giovane, Casale, & Balestrieri et al., 2016; Servillo, Giovane, Casale, & Cautela et al., 2016. Briefly, 500 mg of flour was placed in a 20 mL centrifuge tube containing a magnet bar and extracted with a solution containing 0.1% formic acid in water 1:5 or 1:10 w/w. The suspension was agitated for 3 h and finally centrifuged at 18,000*g* for 60 min. The clarified supernatant was filtered sequentially through a 5 μm and 0.45 μm Millipore filters and then stored frozen until used. All extracts were prepared in triplicate for each type of flour.

2.4. Analysis of betaines by HPLC-ESI-MS/MS

The analyses were performed by HPLC-ESI-MS/MS according to Servillo et al. (Servillo et al., 2015; Servillo, Giovane, Casale, & Balestrieri et al., 2016; Servillo, Giovane, Casale, & Cautela et al., 2016) with an Agilent 1100 series liquid chromatograph using a Supelco Discovery C8 column, 250 × 3.0 mm, particle size 5 μm. The chromatography was conducted isocratically with 0.1% formic acid in water at flow rate of 100 μL/min. Volumes of 10 μL of standard solution or sample were injected. Betaines were identified on the basis of their retention times and MS^2 fragmentation patters. Quantification of each betaine was generally obtained by comparison of the peak area of its most intense MS^2 fragment with the respective calibration curve built with standard solutions. HPLC-ESI-MS/MS analyses were performed with an Agilent LC-MSD SL quadrupole ion trap, in positive multiple reaction monitoring (MRM) using for each analyte the MS^2 transitions reported in Table 1. Matrix effect in quantitative determinations was assessed by the standard addition method. The mass spectrometer was operated utilizing nitrogen as the nebulizing and drying gas. The instrumental conditions were as follows: nebulizer pressure, 30 psi; drying temperature, 350 °C; drying gas 7 L/min. The ion charge control (ICC) was applied with target set at 30,000 and maximum accumulation time at 20 ms. The concentrations of each compound were determined by comparison with the relative calibration curve. Standard stock solutions of each analyte were prepared at