Betaines and related ammonium compounds in chestnut (*Castanea sativa* Mill.)

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**Abstract**

Chestnut fruits, being poor of simple sugars and consisting mainly of fibers and starch, are among the constituents of Mediterranean diet. While numerous studies report on content of proteins and amino acids in chestnut, no one has appeared so far on betaines, an important class of nitrogen compounds ubiquitous in plants for their protective action in response to abiotic stress. In this study, we analyzed by HPLC–ESI-tandem mass spectrometry, in fruits and flours of varieties of chestnut cultivated in Italy, the composition of betaines and ammonium compounds intermediates of their biosynthesis. Besides the parent amino acids, the compounds quantified were choline, glycerophosphocholine, phosphocholine, glycine betaine, N-methylproline, proline betaine (stachydrine), β-alanine betaine, 4-guanidinobutyric acid, trigonelline, N,N,N-trimethyllysine. Interestingly, some uncommon derivatives of pipecolic acid, such as N-methylpipecolic acid, 4-hydroxypipecolic acid and 4-hydroxy-N-methylpipecolic acid were identified for the first time in chestnut samples and characterized by MS^n tandem mass spectrometry.

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**1. Introduction**

The fruits of chestnut are among the constituents of Mediterranean diet, a nutritional model which utilizes foods rich of fibers and carbohydrates with low glycemic index, able to reduce the risk of cardiovascular diseases and maintain health status (Jenkins et al., 2002). Chestnut belongs to the family Fagaceae, the same as oak and beech, and genus Castanea which includes several species. In Europe only one species, the *Castanea sativa* Mill., is exploited, particularly in the Mediterranean basin, for fruit and wood production. Fruits of chestnut are rarely eaten raw but, more frequently, roasted or boiled. A flour can be prepared from dried and milled fruits, which is utilized for bread and cake preparations or as a sauce thickener.

From composition point of view, chestnut fruit is described as a product poor of simple sugars, mainly consisting of fibers and starch (De Vasconcelos, Bennett, Rosa, & Cardoso, 2007, 2010), with low glycemic index and free from gluten, a protein found in wheat which can induce severe gut damages in people with celiac disease. As for nitrogen constituents, literature data are mainly focused on protein and free amino acid content (De Vasconcelos, Bennett, Rosa, & Cardoso, 2009, 2010) but no study has appeared so far on the occurrence and quantification of betaines in chestnut. Betaines are quaternary ammonium compounds, ubiquitous in the vegetal world, produced by specific biosynthetic pathways involving the exhaustive nitrogen methylation of amino and imino acids. These substances tend to accumulate in the cytoplasm and intercellular
fluids where they exert protective functions on the structures of proteins, nucleic acids, and cell membranes (Kavi Kishor et al., 2005; Street, Bolen, & Rose, 2006) in response to plant abiotic stresses, such as reduced availability of water, high soil salinity, hypoxia, cold, freezing. Plants express characteristic patterns and levels of betaines according to their species. As an example, proline betaine and 4-hydroxyprolinebetaine occur at high levels in citrus plants (Servillo, Giovane, Balestrieri, Cautela, et al., 2011; Servillo, Giovane, Balestrieri, Bata-Csere, et al., 2011), proline betaine and pipecolic acid betaine in alfalfa (Wood et al., 1991) and glycine betaine occurs at high levels in wheat, spinach and beet (Zeisel, Mar, Howe, & Holden, 2003). Some betaines are of particular interest in human diet for the maintenance of health status. Glycine betaine has the biological role in humans of methyl donor for the formation of methionine from homocysteine (Craig, 2004; McKeever, Weir, Molloy, & Scott, 1991), thus counteracting accumulation of homocysteine in plasma, which constitutes a risk factor for cardiovascular diseases (Shai et al., 2004). Also, choline, a quaternary ammonium compound abundant in plants, from which, by oxidation, glycine betaine derives, is an important nutrient (Zeisel & Blusztajn, 1994) acting as biosynthetic precursor of phospholipids and the neurotransmitter acetylcholine. More recently, proline betaine (stachydrine), occurring at high levels in citrus fruit juice, has attracted noticeable interest as a nutraceutical substance and a biomarker for citrus juice consumption (Heinze and et al., 2010). Stachydrine ameliorates high-glucose induced endothelial cell senescence and SIRT1 downregulation (Servillo et al., 2013), attenuates norepinephrine-induced cardiomyocyte hypertrophy (Zhang, Shan, et al., 2014), and interferes with the endoplasmic reticulum stress mediated apoptosis, ultimately leading to suppression of renal tubular epithelial cell apoptosis (Zhang, Lu, et al., 2014). On the basis of these results, it seemed of interest to investigate the content of betaines and some of their precursors in various cultivars of chestnut by employing a specific and sensitive mass spectrometric technique, which we successfully used to study betaines in citrus plant genus (Servillo et al., 2012; Servillo, Giovane, Balestrieri, Bata-Csere, et al., 2011). Chemical structures of betaines and related biosynthetic intermediates examined in this study, grouped on the basis of precursor amino acids, are reported in Fig. 1.

2. Materials and methods

2.1. Reagents

Glycine betaine, N-methylproline, trans-4-hydroxyproline, choline, phosphocholine chloride calcium salt tetrahydrate, L-α-glycerophosphorylcholine, (3-carboxypropyl)trimethylammonium chloride (γ-aminobutyric acid betaine), N-methylnicotinic acid (trigonelline), 4-guanidinobutyric acid, ε-N,N,N-trimethyllysine hydrochloride, pipecolic acid, 1-methylpyperidine-2-carboxylic acid hydrochloride, 1-methylpyperidine-3-carboxylic acid, and 1-methylpyperidine-4-carboxylic acid hydrochloride were from Sigma–Aldrich (Milan, Italy). N,N-dimethyl-L-proline (stachydrine) and 4-hydroxy-L-prolinebetaine (betonicine) were purchased from extrasynthese (Genay, France). Pipecolic acid betaine, β-alanine betaine (also known as homobetaine or propiobetaine), N-methyl-4-hydroxyproline acid and N,N-dimethyl-4-hydroxyproline acid (4-hydroxyproline acid betaine) were synthesized as described by Servillo et al. (2012), Boc-4-hydroxyproline-2-carboxylate was purchased from Sigma–Aldrich and used to prepare 4-hydroxyproline acid by reaction with trifluoroacetic acid. The standard mixture of amino acids, containing Ala, Arg, Asp, Glu, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Val and Cys at 2.5 mM concentration in 0.01 M HCl, was from Pierce company (Milan, Italy).

Fig. 1. Chemical structures of substances analyzed in this study deriving from (1) serine, (6) β-alanine, (8) proline, (11) hydroxyproline, (14) pipecolic acid, (21) γ-aminobutyric acid, (24) lysine and (26) nicotinic acid. (2) choline, (3) phosphocholine, (4) glycerophosphorylcholine, (5) glycine betaine, (7) β-alanine betaine, (9) N-methylproline, (10) N,N-dimethylproline, (12) N-methylhydroxyproline, (13) 4-hydroxyproline betaine, (15) N-methylpipecolic acid, (16) pipecolic acid betaine, (17) 4-hydroxyproline acid, (18) 3-hydroxyproline acid, (19) 4-hydroxy-N-methylpipecolic acid, (20) 4-hydroxy-N,N-dimethylproline acid, (22) γ-aminobutyric acid betaine, (23) 4-guanidinobutyric acid, (25) ε-N,N,N-trimethyllysine, (27) trigonelline.
AccQ Fluor reagent for amino acid analysis was from Waters (Milan, Italy). 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. Fresh fruit and chestnut flour samples

Fourteen different batches of about 6 kg of fresh chestnut fruits, all of Italian origin, were sampled in three different Italian regions (Calabria, Campania and Piedmont). All analytical determinations (total proteins, percent of dry matter, content of free amino acids, betaines and related compounds) were conducted on samples from the three regions obtained using 40 ground fruits, deprived of pericarp and endocarp, taken from individual batches. As for flours, 4 lots made with chestnuts of Italian origin, provided by three different Italian companies, were analyzed. All analyses were conducted in triplicate.

2.3. Total proteins and dry matter

Total proteins were determined according to the Kjeldahl procedure using a selenium catalyst. The determination of dry matter (total solids) was performed by oven drying the samples under vacuum at 105°C until constant weight was obtained.

2.4. Amino acid sample preparation and HPLC analysis

The amino acids were extracted from fresh chestnut fruits or chestnut flours using 0.1% formic acid in water in the ratio (w/w) 1:10, under stirring for 1 h. The extract clarified at 18,000 g for 60 min was filtered first through 5 µm and then 0.45 µm Millipore filters, and finally 1 mL was applied on a cation-exchange column (100 x 6 mm AG50W×8-H⁺ Bio Rad). After column washing with 50 mL of Milli-Q water, amino acids were eluted with 3.0 M NH₃ (about 10 mL) and collected. The eluate was evaporated to dryness, recovered with 0.1 M HCl (10 mL), and filtered through 0.45 µm filter. Quantification of free amino acids was performed by reverse phase (RP) HPLC. A Waters chromatograph model 2690 equipped with fluorescence detector model 474 was employed. Samples (10 µL) were derivatized with the Waters ACCQFluor reagent according to the method of Van Wandel and Cohen (1997). Quantification was made using the peak area of the fluorescence emission intensity by excitation at 350 nm and recording fluorescence emission at 395 nm. Amino acids were identified on the basis of their retention times and quantified by comparison of the corresponding peak area with the respective calibration curve.

2.5. Analysis of betaine by HPLC–ESI-MS/MS

Betaines and related compounds were extracted from the fresh chestnut fruits or flours using 0.1% formic acid in water in the ratio (w/w) 1:5 or 1:10 according to Servillo, Giovanne, Balestrieri, Cautela, et al. (2011). The suspension was kept under stirring for about 3 h and finally centrifuged at 18,000 g for 60 min. The clarified extract was filtered first through 5 µm and then 0.45 µm Millipore filters and stored frozen until used. The analyses were performed by HPLC–ESI-MS/MS according to Servillo, Giovanne, Balestrieri, Bata-Csere, et al., 2011 with an Agilent 1100 series liquid chromatograph using a Supelco Discovery C8 column, 250 x 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 20 µL of standard solution or sample were injected. Betaines were identified on the basis of their retention times and MS² fragmentation patterns. Quantification of each betaine was generally obtained by comparison of the peak area of its most intense MS² 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) mode using the following MS² transitions: 104.1 → 60 for choline, 118.1 → 59 for glycine betaine, 132.1 → 73 for β-alanine betaine, 146.1 → 87 for γ-aminobutyric acid, 144.1 → 84 for N,N-dimethylproline (stachydrine), 130.1 → 84 for N-methylproline, 132.1 → 86 for 4-hydroxyproline, 146.1 → 100 for 4-hydroxy-N-methylproline, 130.1 → 84 for pipoceolic acid, 146.1 → 100 for 4-hydroxy-pipoceolic acid, 160.1 → 114 for N,N-dimethyl-4-hydroxy-pipoceolic acid, 144.1 → 98 for 1,2-N-methyl-pipoceolic acid, 158.1 → 72 for pipoceolic acid betaine (homostachydrine), 146.1 → 87 for 4-guanidinobutyric acid, 189.1 → 130 for ethano-l NS-trimethyllysine, and 138.1 → 110 for trigonelline. 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 71/min. The ion charge control (ICC) was applied with target set at 30,000 and maximum accumulation time at 20 ms. Each extract was analyzed in triplicate and the mean concentration of each compound was calculated and expressed in mg/kg of dry product. The concentrations of each compound were determined by comparison with the relative calibration curve. Standard stock solutions of each analyte were prepared at 2 mg/L. Additional calibration levels (0.2; 0.1; 0.05; 0.02, 0.002 and 0.001 mg/L) were prepared by serial dilution with water containing 0.1% formic acid. The calibration curves were built using these standard solutions. The linear regression analysis was carried out by plotting the peak areas of the monitored fragment ions versus the concentrations of the analyte standard solutions. The linearity of the instrumental response was assessed by correlation coefficients (r²) >0.99 for all analytes.

2.6. Statistical analysis

The mean values of the concentrations of analytes and their standard deviations were calculated from the results of analyses carried out in triplicate.

3. Results and discussion

3.1. Quantification of betaines by LC–ESI-MS/MS

HPLC analysis performed on aqueous extracts of chestnut fruits and flours allowed determination of betaines and related compounds in about 30 min by using a 25 cm C8 column in isocratic condition with 0.1% formic acid in water as eluent, at flow rate of 100 µL/min. Most of the betaines and other metabolites investigated in this study eluted well separated in such chromatographic conditions. For the sake of clarity, results of our study are reported by grouping the quaternary ammonium compounds and their biosynthetic intermediates, when present, on the basis of the precursor amino acid. The levels of the compounds examined in this study, expressed as mean values and standard deviations of results on samples of the same type, that is fruits or flours, are reported in Table 1.

3.2. Serine derivatives

Serine is the amino acid precursor of ethanolamine from which choline is formed by methylation. Glycine betaine is biosynthesized from choline through two oxidative steps. In the first, choline is oxidized into betaine aldehyde from which, by further oxidation, glycine betaine is finally generated. Glycine betaine is almost ubiquitous in vegetal species. In some foods such as spinach, beet,
and wheat, the substance is present at high levels (De Zwart et al., 2003; Slow et al., 2005; Zeisel et al., 2003). In our chromatographic conditions, glycine betaine eluted at the retention time of 15.4 min and was quantified by using the MS2 strongest precursor to product ion transition 118.1 → 59, while 118.1 → 58 was used as the confirmatory transition. A representative HPLC–ESI-MS2 chromatogram of a chestnut fruit extract, where mass spectrometric data were collected in multiple reaction monitoring (MRM) mode, is shown in Fig. 2a. Besides, glycine betaine (5), choline (2), e-N,N,N-trimethyllysine (25), β-Alanine betaine (7), phosphocholine (3) and glycerophosphorylcholine (4) are well represented in the chestnut extracts (Fig. 2a). e-N,N,N-Trimethyllysine and choline coeluted in the chromatographic conditions used, instead other betaines appeared well resolved in the chromatogram.

Table 1
Concentrations of the free amino acids precursors of betaines, total proteins, betaines and related ammonium compounds in Italian chestnut products. Concentrations are expressed as mg/kg of dry matter. NQ = not quantified. Total protein levels are expressed as g/100 g of dry matter. Numbers in brackets cross-reference to Fig. 1.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Fresh chestnuts</th>
<th>Chestnut flours</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amino acids precursors of betaines</td>
<td>Min</td>
<td>Max</td>
</tr>
<tr>
<td>Serine (1)</td>
<td>270</td>
<td>780</td>
</tr>
<tr>
<td>Proline (8)</td>
<td>210</td>
<td>730</td>
</tr>
<tr>
<td>Hydroxyproline (11)</td>
<td>1.8</td>
<td>8.9</td>
</tr>
<tr>
<td>Pipecolic acid (14)</td>
<td>0.2</td>
<td>5.0</td>
</tr>
<tr>
<td>γ-Aminobutyric acid (21)</td>
<td>810</td>
<td>1550</td>
</tr>
<tr>
<td>Lysine (24)</td>
<td>110</td>
<td>810</td>
</tr>
<tr>
<td>Total Proteins</td>
<td>2.8</td>
<td>5.8</td>
</tr>
<tr>
<td>Betaines and ammonium compounds</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Choline (2)</td>
<td>17.0</td>
<td>33.0</td>
</tr>
<tr>
<td>Glycine betaine (5)</td>
<td>0.8</td>
<td>2.1</td>
</tr>
<tr>
<td>β-Alanine betaine (7)</td>
<td>0.5</td>
<td>2.7</td>
</tr>
<tr>
<td>Phosphocholine (3)</td>
<td>5</td>
<td>12</td>
</tr>
<tr>
<td>Glycerophosphocholine (4)</td>
<td>4</td>
<td>10</td>
</tr>
<tr>
<td>N,N-Dimethylproline (10)</td>
<td>0.1</td>
<td>0.7</td>
</tr>
<tr>
<td>N-Methylproline (9)</td>
<td>0.2</td>
<td>0.9</td>
</tr>
<tr>
<td>4-Hydroxypipeolic acid (17)</td>
<td>NQ</td>
<td>NQ</td>
</tr>
<tr>
<td>N-Methylpipolic acid (15)</td>
<td>0.1</td>
<td>0.6</td>
</tr>
<tr>
<td>4-Hydroxy-N-methylpipolic acid (19)</td>
<td>NQ</td>
<td>NQ</td>
</tr>
<tr>
<td>γ-Guanidinobutyric acid (23)</td>
<td>0.2</td>
<td>0.6</td>
</tr>
<tr>
<td>e-N,N,N-Trimethyllysine (25)</td>
<td>0.4</td>
<td>1.0</td>
</tr>
<tr>
<td>Trigonelline (27)</td>
<td>0.3</td>
<td>0.8</td>
</tr>
</tbody>
</table>

Fig. 2. Representative MS2 extracted ion chromatograms conducted in MRM mode. HPLC analysis was performed by using a 25 cm C8 column in isocratic condition with 0.1% formic acid in water as eluent, at flow rate of 100 μL/min. (a) Chestnut fruit extract. (b) Standard mixture of the analyzed compounds. The MS2 transitions followed were 104.1 → 60 for choline (2), 189.1 → 130 for e-N,N,N-trimethyllysine (25), 132.1 → 73 for β-alanine betaine (7), 184.1 → 86 for phosphocholine (3), 118.1 → 59 for glycine betaine (5) and 258.1 → 104 for glycerophosphorylcholine (4). Retention times in minutes were 13.0 (2), 13.1 (25), 13.8 (7), 15.2 (3), 15.4 (5) and 15.8 for (4).
HPLC–ESI-MS/MS analysis of a standard solution of the same metabolites is shown in Fig. 2b. The average glycine betaine levels were 1.5 mg/kg of dry matter for fresh chestnut fruits and 3.3 mg/kg of dry matter for chestnut flours (Table 1). In contrast, choline resulted to be the most abundant quaternary ammonium compound in the metabolic profile of chestnut (Table 1). Choline eluted at the retention time of 13.0 min and was quantified through the MS2 transition at m/z 104 (MS2) and at m/z 98 (MS3). Phosphocholine (PCho), the first intermediate in the biosynthetic route leading from choline to lecithin, eluted at retention time of 15.2 min. Its quantification was achieved by using the MS2 strongest transition at m/z 184.1 → 125 as the confirmatory transition. Phosphocholine levels were in the range of 5–12 mg/kg of dry matter for fresh chestnut fruits and 3–15 mg/kg of dry matter for flours. Glycerophosphorylcholine (GPC), like phosphocholine (PCho), was present in all samples of chestnut although at levels slightly lower than phosphocholine. Its quantification was achieved by using the MS2 transition at m/z 258.1 → 104, since in our instrumental conditions only the MS2 fragment at m/z 104 (corresponding to choline) was produced, the presence of glycerophosphorylcholine (GPC) was confirmed in MS3 by isolating the MS2 fragment at m/z 104, from which only the MS3 fragment at m/z 60 was formed, as expected for choline (data not shown). Glycerophosphorylcholine...
levels resulted in the range of 4–10 mg/kg of dry matter in chestnut fruits and in the range of 2–14 mg/kg of dry matter for flours.

3.3. β-Alanine derivatives

β-Alanine is straightforwardly produced in prokaryotes by decarboxylation of aspartic acid. Instead, in plants, it is biosynthesized by rather complex routes starting from the polyamines spermidine and spermine (Terano & Suzuki, 1978) or from propanoate (Rathinasabapathi, 2002). This non-proteinogenic amino acid is the direct precursor of β-alanine betaine by successive methylation steps. We found that β-alanine betaine, rather uncommon in plants, was present in all chestnut samples examined. Results showed that the content of β-alanine betaine was in the range of 0.5–2.7 mg/kg of dry matter for both fruits and flours (Table 1). β-Alanine betaine eluted at the retention time of 13.8 min (Fig. 2b). Its quantification was achieved by using the strongest MS² transition 132.1 → 73, while 132.1 → 60 was used as the confirmatory transition. The occurrence in chestnut of β-alanine betaine is rather surprising as it was reported so far in stress tolerant herbaceous plants such as those of the Plumbaginaceae family (Hanson et al., 1994). β-Alanine betaine is believed to be a more suitable osmoprotectant than glycine betaine under hypoxic conditions (Hanson et al., 1994; Rathinasabapathi, Sigua, Ho, & Gage, 2000) because the biosynthesis of β-alanine betaine, differently from glycine betaine, does not require oxidative steps but occurs only through methylation steps of β-alanine (Rathinasabapathi et al., 2000).

3.4. Proline derivatives

Low but significant levels of stachydrine (proline betaine or N,N-dimethylproline) were found in all chestnut samples examined (Table 1). Stachydrine (10) occurs at high levels in alfalfa and citrus genus plants where also its hydroxylated derivative, betonicine (4-hydroxy-N,N-dimethylproline), occurs. Stachydrine is believed to derive from proline methylation through the intermediate N-methylproline, while it is unclear if betonicine (13) is formed through stachydrine hydroxylation or by successive methylation of hydroxyproline. We examined the presence in the chestnut samples of all the possible precursors of stachydrine and betonicine. Proline was found at rather high levels, with mean values of 420 mg/kg of dry matter for flour and 470 mg/kg of dry matter for fresh fruits (Table 1). Also 4-hydroxyproline was detected in all samples but at much lower levels than proline, in the range of 2–10 mg/kg of dry matter (Table 1). Interestingly, N-methylproline (9), the immediate precursor of stachydrine, was found in all samples (Table 1). Instead, betonicine (4-hydroxy-N,N-dimethylproline) was always absent and, although a compound eluting at r.t. 20 min with m/z 146 was detected in all samples, mass spectrometric analyses ruled out that it was 4-hydroxy-N,N-dimethylproline (12) (see below).

3.5. Pipecolic acid derivatives

In plants in which stachydrine is present, frequently also homostachydrine (pipecolic acid betaine) occurs (Servillo et al., 2012; Wood, 1991). Homostachydrine probably derives from N-methylation of pipecolic acid. We examined the presence of the components of the entire pathway leading from pipecolic acid to its betaine. Homostachydrine was absent in all chestnut samples. However, both pipecolic acid and a substance showing the same mass spectrometric analyses ruled out that it was 4-hydroxyproline. As far as is known, Erythrochiton brasiensis is the unique vegetal species in which it was identified (Sargenti et al., 1993). Considering the absence of homostachydrine in all chestnut samples, in order to avoid false assignment for that unknown substance, we compared the three possible isomers of N-methylpipecolic acid by their chromatographic and mass spectrometric features to assess if one of them could be the unknown compound. The mixture of the three isomers was completely resolved in our chromatographic conditions (Fig. 3). The 1,2 isomer was that eluting later, with a retention time of 19.8 min, virtually coincident to the retention time of the unknown compound. As for MS² fragmentation patterns, both the positional isomers 1,3 and 1,4 showed the most intense fragment at m/z 144 as stachydrine but eluting at higher retention time (20 min) were detected. This unknown compound might be N-methylpipecolic acid (alias 1-methylpiperidine-2-carboxylic acid), supposed the homostachydrine precursor (Essery, McCaladin, & Marion, 1962; Wood, 1991). Little information is available in the literature on the occurrence of N-methylpipecolic acid in plants. As far as is known, Erythrochiton brasiensis is the unique vegetal species in which it was identified (Sargenti et al., 1993). Considering the absence of homostachydrine in all chestnut samples, in order to avoid false assignment for that unknown substance, we compared the three possible isomers of N-methylpipecolic acid by their chromatographic and mass spectrometric features to assess if one of them could be the unknown compound. The mixture of the three isomers was completely resolved in our chromatographic conditions (Fig. 3). The 1,2 isomer was that eluting later, with a retention time of 19.8 min, virtually coincident to the retention time of the unknown compound. As for MS² fragmentation patterns, both the positional isomers 1,3 and 1,4 showed the most intense fragment at m/z 126 likely due to the neutral loss of H₂O from the carboxyl group of the parent ion, and a less intense fragment at m/z 98, which correspond to the loss of CO + H₂O from the parent ion (Fig. 3a and b). Instead, the fragmentation pattern of 1,2 isomer showed only one intense MS² fragment at m/z 98 (Fig. 3c). This is not surprising as the easier formation of the highly stable immonium ion as a consequence of the carboxyl loss favors mostly its loss from position 2, closer to piperidine nitrogen atom, than from the other two positions. The unknown compound also showed the MS³ fragmentation pattern with only one intense fragment at m/z 98. Furthermore, the MS³ fragmentation patterns of the unknown compound and 1,2 isomer, obtained by isolating
the MS² fragment at m/z 98, resulted identical (Fig. 3c). All together, these data leave no doubt that the unknown compound is the 1,2 isomer, that is, N-methylpipecolic acid. Quantitative analysis on the chestnut samples revealed that its levels were in the range of 0.1–1 mg/kg (Table 1).

In the course of the chromatographic analyses of chestnut extracts, also two somewhat intense peaks at m/z 146 were observed. The one eluting at r.t. 15.9 min was identified as 4-guanidinobutyric acid (see below), the other, eluting at r.t. 14.3 min, showed a MS² fragmentation pattern that at first sight suggested it could be 4-hydroxy-N-methylproline, a proline derivative occurring in various plant species (Blunden, Patel, Adrian-Romero, & Melendez, 2004). Indeed, this possibility was discarded when, after having synthesized the substance, we found that it had a retention time slightly higher than the unknown compound and different MS² fragmentation pattern. In particular, the unknown compound showed two MS² fragments at m/z 55 and 74, which are absent in the fragmentation spectrum of 4-hydroxy-N-methylproline (Fig. 4a).

Therefore, we sought to see if the unknown compound was a hydroxylated derivative of pipecolic acid. In plants, only two of such derivatives are reported to occur, they are 4-hydroxy-pipecolic acid, particularly abundant in Acacia species (Clark-Lewis & Mortimer, 1959) and 5-hydroxy-pipecolic of which dates are a rich source (Ali, Alhaj, Al-Khalifa, & Bruckner, 2014). In order to have reference standards, we prepared 4-hydroxy-pipecolic acid from the hydrolysis of its t-BOC derivative, which was commercially available, and aqueous extract from ripe dates to obtain 5-hydroxy-pipecolic. In our chromatographic conditions, the two substances were badly resolved, being 4-hydroxy-pipecolic eluted at r.t. 13.6 min and 5-hydroxy-pipecolic at r.t. 14.0 min. However, their MS² fragmentation spectra were different (Fig. 4b and c) and, most importantly, the chestnut unknown substance perfectly coeluted with 4-hydroxy-pipecolic acid and showed identical MS² fragmentation pattern (data not shown).

Interestingly, one more derivative of pipecolic acid was found in all chestnut samples. Indeed, a substance eluting at r.t. 14.4 min showed m/z 160 and a MS² fragmentation pattern which suggested that it was 4-hydroxy-N,N-dimethylpipecolic acid. Actually, after having synthesized the substance by methylation of 4-hydroxy-pipecolic acid (Fig. 5), we found that it coeluted with the unknown peak and showed identical MS² fragmentation pattern (data not shown). It is worth to note that 4-hydroxy-N,N-dimethylpipecolic acid is the likely precursor of 4-hydroxy-N,N-dimethylpipecolic acid, an uncommon betaine reported to occur in Lamium species (Yuan, Patel, Blunden, & Turner, 1992). We sought to see if this betaine might occur also in chestnut, but our
effort was unsuccessful. Indeed, we did not find any compound in the chestnut extracts at m/z 174 coeluting with the synthesized trans-4-hydroxypippecolic acid betaine. In summary, we have observed that of the three pathways leading to the formations of the three betaines N,N-dimethylproline (stachydrine), N,N-dimethylpippecolic acid betaine (homostachydrine), and 4-hydroxy-N,N-dimethylpippecolic acid betaine, only the first pathway is complete in chestnut, whereas the other two end at level of the N-methylpippecolic acid and 4-hydroxy-N-methylpippecolic acid, respectively.

3.6. γ-Aminobutyric acid derivatives

γ-Aminobutyric acid (21) is present at high levels in chestnut (Table 1), for this reason we looked for the possible occurrence also of its betaine that might be produced by direct methylation as it happens for the formation of β-alanine betaine from β-alanine. Actually, it appeared reasonable to suppose that the same enzyme which methylates β-alanine could do the same also with γ-aminobutyric acid, which is the next higher homolog of β-alanine. Indeed, we did not detect the substance in any chestnut sample examined. However, it is interesting to note that another compound, related to γ-aminobutyric acid and with the same m/z 146 as γ-aminobutyric acid betaine, was detected in all chestnut samples. This substance turned out to be γ-guanidinobutyric acid (23) and, for all we know, its occurrence has never been reported before in chestnut fruits and flours. The biosynthesis of γ-guanidinobutyric acid (23) involves transamination of the amino group from arginine to the amino group of γ-aminobutyric acid (Irreverre, Evans, Hayden, & Silber, 1957; Tachikawa & Hosoya, 2011). Indeed, also arginine, like γ-aminobutyric acid, occurs at high levels in chestnut, with mean values of 900 mg/kg of dry matter for fruits and 1300 mg/kg of dry matter for flours. The reaction, catalyzed by an amidinotransferase enzyme, is:

\[
\text{Arginine} + \gamma \rightarrow \text{Guanidinobutyric acid} + \text{Ornithine}
\]

γ-guanidinobutyric acid has a MS² fragmentation pattern with numerous fragment ions which make reliable its identification also in a complex matrix. Its retention time was 15.9 min. Quantification was achieved by the MS² transition 146.1 → 87 while 146.1 → 104 was used as the confirmatory transition. The levels in chestnut samples were in the range of 0.2–1.7 mg/kg of dry matter (Table 1). It is worth noting that beneficial effects in healing of gastric lesions have been recently attributed to this compound (Hwang & Jeong, 2012).

3.7. Lysine derivatives

\(\varepsilon\)-N,N,N-Trimethyllysine is a particular betaine as it can be considered also an amino acid, being the α-amino group still present in its structure (Table 1). In mammals, \(\varepsilon\)-N,N,N-trimethyllysine is the precursor of γ-aminobutyric acid betaine, which, by hydroxylation, forms carnitine, a metabolite essential for the fatty acid transport and utilization in mitochondria. Recently, we showed that \(\varepsilon\)-N,N,N-trimethyllysine is not rare in vegetables, as it was supposed, but, on the contrary, it is ubiquitous in vegetal world (Servillo, Giovane, Cautela, Castaldo, & Balestrieri, 2014). Actually, we found the occurrence of \(\varepsilon\)-N,N,N-trimethyllysine also in all chestnut sample examined. \(\varepsilon\)-N,N,N-Trimethyllysine eluted at retention time of 13.1 min and its quantification was achieved by using 189.1 → 130, which was the MS² strongest precursor to product ion transition, while 189.1 → 84 was used as the confirmatory transition. The average \(\varepsilon\)-N,N,N-trimethyllysine levels resulted in the range 0.4–1.1 ppm (Table 1).

3.8. Nicotinic acid derivatives

Trigonelline (27), the betaine of nicotinic acid (26), is commonly found in cotyledons of seeds and has an important role as hormone which controls the cell cycle in plants by promoting preferential cell arrest in G2 phase (Tramontano, Hartnett, Lynn, & Evans, 1982). Recently, it has been reported that trigonelline possesses hypoglycemic properties and could assist in maintaining glycemic control in diabetes mellitus (Adams et al., 2014). In our chromatographic conditions, trigonelline eluted at retention time of 16.4 min and its quantification was achieved by using 138.1 → 110, which was the MS² strongest precursor to product ion transition, while 138.1 → 94 was used as the confirmatory transition. The average trigonelline levels resulted both for fruits and flours lower than 2 mg/kg of dry matter (Table 1).

Conflict of interest statement

The authors declare no conflicts of interest.

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