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Occurrence of Pipecolic Acid and Pipecolic Acid Betaine (Homostachydrine) in *Citrus* Genus Plants

Luigi Servillo,*[,†] Alfonso Giovane,[†] Maria Luisa Balestrieri,[†] Giovanna Ferrari,[‡] Domenico Cautela,[§] and Domenico Castaldo[#,⊥]

[†]Dipartimento di Biochimica e Biofisica, II Università degli Studi di Napoli, via L. De Crecchio 7, 80138, Naples, Italy

[‡]Dipartimento di Ingegneria Industriale e ProdAl scarl, Università degli Studi di Salerno, via Ponte Don Melillo, 84084 Fisciano (SA), Italy

[§]Stazione Sperimentale per le Industrie delle Essenze e dei Derivati dagli Agrumi (SSEA), Azienda Speciale C.C.I.A.A., Reggio Calabria, via Tommaso Campanella 12, 89125 Reggio Calabria, Italy

[#]Ministero dello Sviluppo Economico (MiSE), via Molise 2, Roma, Italy

ABSTRACT: The presence of pipecolic acid and pipecolic acid betaine, also known as homostachydrine, is herein reported for the first time in *Citrus* genus plants. Homostachydrine was found in fruits, seeds, and leaves of orange, lemon, and bergamot (*Citrus bergamia* Risso et Poit). As homostachydrine was not commercially available, as a comparative source, extracts of alfalfa leaves (*Medicago sativa* L.) were used, in which homostachydrine is present at high concentration. Then, the results where confirmed by comparison with an authentic standard synthesized and purified starting from pipecolic acid. The synthesized standard was characterized by a ESI-MS/MS study using a 3D ion-trap mass spectrometer. When subjected to MS/MS fragmentation in positive ion mode, homostachydrine, unlike its lower homologue proline betaine (also known as stachydrine), showed a pattern of numerous ionic fragments that allowed unambiguous identification of the compound. For the quantitation in the plant sources, high sensitivity and specificity were achieved by monitoring the transition (158→72), which is absent in the fragmentation patterns of other major osmolytes commonly used as markers for studies of abiotic stress. As for the metabolic origin of homostachydrine, the occurrence in citrus plants of pipecolic acid leads to the hypothesis that it could act as a homostachydrine precursor through direct methylation.

KEYWORDS: *pipecolic acid betaine, homostachydrine, pipecolic acid, citrus fruit, betaine biosynthesis, food analysis, citrus fruit composition*

INTRODUCTION

The cytoplasmic accumulation of betaines is a common biochemical and physiological plant response to stress phenomena, which are induced in large part by adverse environmental conditions, such as sudden changes in temperature, high salinity, dryness of the soil, and drought.[1,2] These substances, biochemically inert in the cell, are synthesized from some specific amino acids such as serine, alanine, methionine, the nonprotein amino acid γ-aminobutyric acid, and some cyclic amino acids such as proline and piperidine-2-carboxylic acid (pipecolic acid). The biosynthesis of betaines in the cytoplasm under abiotic stress conditions is mainly due to the action of methyltransferases, which utilize *S*-adenosylmethionine as methyl group donor.[3−5] The betaines resulting from the amino acid methylation are permanently charged compounds, highly soluble in water. They are also commonly defined as compatible osmolytes because they do not negatively interfere with the cellular metabolism even at molar concentrations. On the contrary, they exert a protective action on the proteins and nucleic acid structures, contributing to maintain osmotic homeostasis during variations of soil osmotic potential (water stress) or specific ionic effects (salt stress).[6,7]

In the *Citrus* genus, osmoregulation in stress conditions is mainly accomplished through accumulation of proline and its methylated derivatives such as *N*-methylproline (hygric acid), 4-hydroxy-L-prolinebetaine (betonicine), and *N*,*N*-dimethyl-L-proline (stachydrine).[8−10] Also present at much lower concentrations are other osmolytes such as *N*-methylnicotinic acid (trigonelline) and choline. Despite the presence of moderate levels of γ-aminobutyric acid, γ-aminobutyric acid betaine was never detected.[11−13]

Pipecolic acid and its betaine can be considered as the higher homologues of proline and proline betaine and, for this reason, they are also called homoproline and homostachydrine, respectively. Their presence has been reported at moderate levels in some vegetal species.[14−18] However, the presence of pipecolic acid betaine has never been reported so far in woody plants, but it has been detected at noticeable concentrations in the *Medicago* and *Achillea* genera,[19,20] in which it was found that pipecolic acid betaine accumulates, under stress conditions, together with proline betaine, which is the most represented betaine in the *Citrus* genus.[8,10,11,21] Moreover, it was also reported that some halophilic plants and sand dune plants[15] accumulate both pipecolic acid and proline, which is the supposed precursor of proline betaine in the *Citrus* genus. On

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the basis of these studies, taking into account what was hypothesized (Figure 1) for proline betaine formation in this

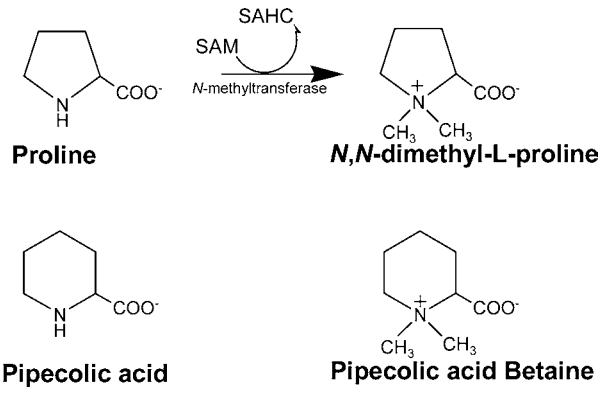

Figure 1. Chemical structures of proline, proline betaine, pipecolic acid, and pipecolic acid betaine.

plant genus,[22−24] we sought to investigate the presence of pipecolic acid betaine with a sensitive mass spectrometry technique, which we previously employed to quantitate proline derivatives in citrus fruits.[10,11]

MATERIALS AND METHODS

Reagents. L-Proline, *N*-methyl-L-proline, 4-hydroxy-L-proline, γ-aminobutyric acid betaine, choline, *N*-methylnicotinic acid (trigonelline), and methyl iodide were from Sigma-Aldrich (Milan, Italy). *N*,*N*-Dimethyl-L-proline (stachydrine) and 4-hydroxy-L-proline betaine (betonicine) were purchased from Extrasynthese (Genay, France). The 0.1% solution of formic acid in water used for the LC-ESI-MS analyses was from Sigma-Aldrich. The standard mixture of L-amino acids, containing Ala, Arg, Asp, Glu, His, Iso, Leu, Lys, Met, Phe, Pro, Ser, Thr, Tyr, Val, and Cys at 2.5 mM concentration in 0.01 M HCl, was from Pierce. Milli-Q water was used for all of the preparations of solutions and standards. AccQ FLuor reagent was from Waters (Milan, Italy).

Synthesis and Purification of Pipecolic Acid Betaine. For the conversion of pipecolic acid into its betaine, we used, with some modifications, the procedure proposed by Chen and Benoiton,[25,26] which is based on a heterogeneous phase reaction employing methyl iodide as methylating agent in the presence of $KHCO_3$. In short, about 200 mg of pipecolic acid was dissolved in 20 mL of methanol, and 1 g of $KHCO_3$ was added; subsequently, 10 mL of methyl iodide (CH_3I) was added. The mixture was stirred for 12 h at room temperature. The addition of methyl iodide (10 mL) and $KHCO_3$ (1 g) was repeated twice more. Finally, the mixture was centrifuged and the supernatant collected and evaporated to dryness at 40 °C in a rotavapor. The residue, containing the pipecolic acid betaine, was dissolved in 10 mL of Milli-Q grade water and applied on a 10 cm column, filled with a mixed-bed resin of Dowex-1-OH^- and Biorex-70-H^+ (1:1 v/v) able to retain amino acids but not betaines.[27] 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. The pipecolic acid betaine was finally eluted with 30 mL of 6 M NH_4OH and evaporated to dryness under a stream of air.

Plant Materials. Citrus fruits (≈3 kg) and fully expanded leaves (≈50 g) were taken from a single tree of each species or cultivar. Trees were 10−20 years old. The sampling (fruits and leaves from orange and lemon) was conducted in February and April 2011 at SSEA Citrus Arboretum in Reggio Calabria (Calabria, Italy). Bergamot fruits and leaves were harvested in the "Pellaro" area near Reggio Calabria (Calabria, Italy).

Citrus Seed Extracts. Citrus fruits were first washed with water to remove dust and pollutants from the exocarp. Then flavedo was separated manually from albedo (endocarp), which contained seeds and the edible part the fruit. The recovered seeds were washed with water (Milli-Q grade), drained, and finally dried on filter paper. Subsequently, 10 g was homogenized in a blender with 90 mL of 0.2% formic acid in Milli-Q grade water. The homogenate was then kept under stirring for about 3 h and finally centrifuged at 18000*g* for 30 min. The supernatant was stored in 20 mL vials at −20 °C.

Citrus Leaf Extracts. The citrus leaves were washed with distilled water and dried with filter paper. Then 25 g of product, finely chopped, was homogenized in a blender with 100 mL of 0.2% formic acid in Milli-Q grade water and then kept under stirring for 3 h. The homogenate was finally centrifuged at 18000*g* for 30 min, and the supernatant was stored in 20 mL vials at −20 °C.

Citrus Juices. The juices of lemon, orange, and bergamot were obtained manually with a squeezer starting from 3 kg of each type of fruit. The juice yield was about 40% in each case. The pulp was removed by centrifugation at 18000*g* for 30 min. The supernatant was stored in 50 mL vials at −20 °C until used.

Alfalfa (Medicago sativa L.) Extracts. The seeds of *Medicago sativa* L. (Garisenda variety, N. reg. 1416 Reg ref: EMG 41) were provided by the Agricultural Consortium of Reggio Calabria and produced by the Juffray Drilland Co. (France). The seeds were germinated in styrofoam flats containing a mixture of peat moss and sand (1:2). After 10 days, the seedlings were transferred on a vermiculite−sand (2.1, v/v) bed and received an adequate amount of fertilizers. Leaves were harvested for analyses after 4 weeks. The leaves (200 g) were washed with distilled water, dried with filter paper, homogenized (1:1 w/v) with 0.2% formic acid in a mixer, and then kept under stirring for 3 h. The homogenate was centrifuged at 18000*g* for 30 min. The supernatant was recovered and stored in 20 mL vials at −20 °C.

Preparations of Standards. The standard stock solutions of L-Proline, *N*-methyl-L-proline, *N*,*N*-dimethyl-L-proline, 4-hydroxy-L-proline betaine, γ-aminobutyric acid betaine, choline, *N*-methyl nicotinic acid, and pipecolic acid betaine were prepared at a concentration of 2000 ng/mL. Additional calibration levels (400, 200, 100, 50, and 25 ng/mL) 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 demonstrated by a correlation coefficient (r^2) of >0.99 for all analytes.

Sample Preparations for HPLC ESI-MS/MS Analyses. The determination of pipecolic acid betaine and other betaines in the samples was performed by HPLC ESI-MS/MS according to the method of Servillo et al.[10,11] by subjecting the centrifuged samples to a passage on a (5 × 1 cm) column filled with Bio-Rad AG 50WX8-(H^+) resin. In brief, the column, after loading 1 mL of extract, was washed with 5 volumes of Milli-Q water and then one step eluted with 10 mL of 12% ammonia solution, followed by 5 mL of water. The pooled eluates were dried in a rotavapor and reconstituted with 1 mL of 0.1% formic acid in water.

HPLC ESI-MS/MS and FIA ESI-MS/MS Analyses of Pipecolic Acid Betaine. The optimization of the instrumental parameters for pipecolic acid betaine analyses was performed by continuous infusion of 5 μM standard solution in 0.1% formic acid. The mass cutoff and the fragmentation amplitude were optimized to obtain the most efficient MS/MS transitions from the positively charged precursor ion $[M + H]^+$ to the fragment ions. Successively, the substance was analyzed by HPLC ESI-MS/MS, as described for the dosage of proline derivatives.[10] Briefly, the chromatography, isocratically conducted with 0.1% formic acid in water, was performed with a Supelco Discovery-C8 column, 100 × 3.0 mm, particle size = 5 μm, at flow rate of 100 μL/min. Aliquots of 20 μL of standard solutions or samples were injected. An Agilent 1100 series liquid chromatograph equipped with an online degasser and an automatic injector was employed. The ESI-MS/MS analyses were performed, both for FIA and for HPLC, with an Agilent LC-MSD SL quadrupole ion trap, in positive ion mode, 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

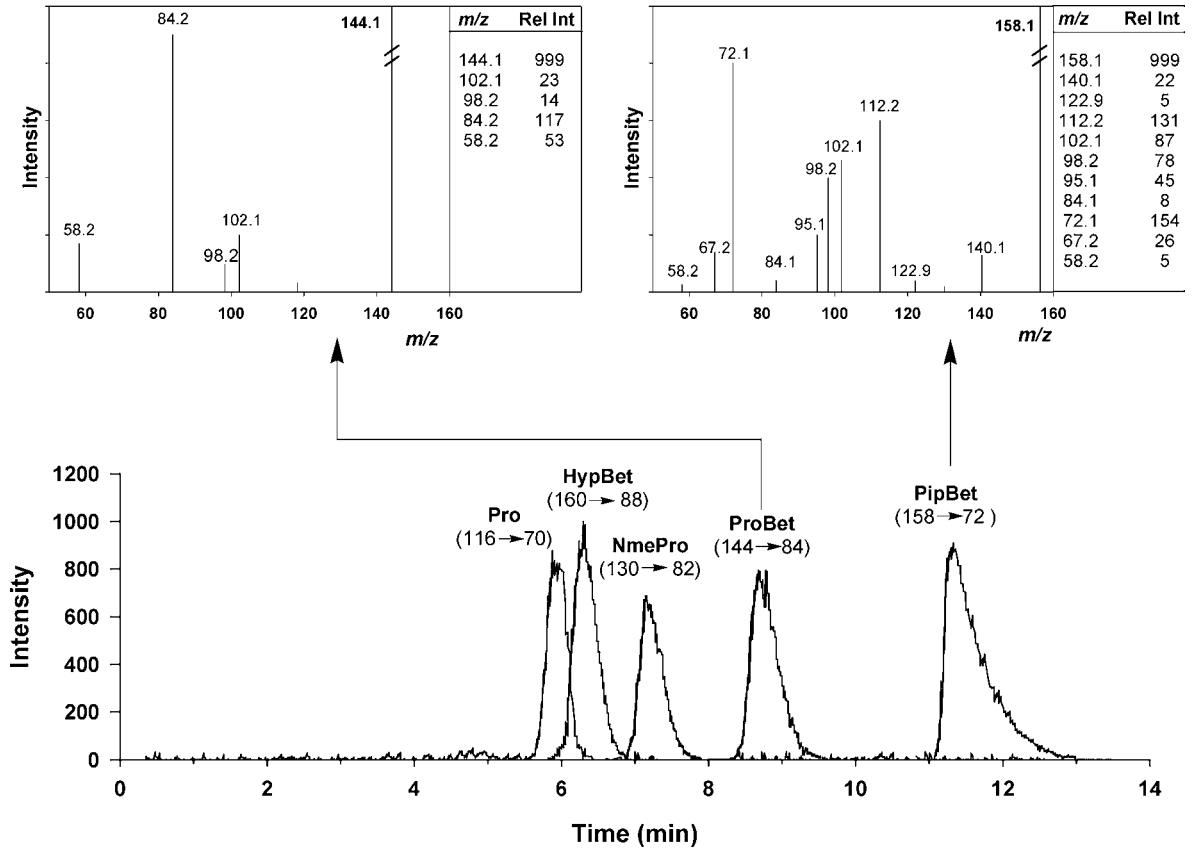

Figure 2. Chromatographic separation of some proline-derived compounds and pipecolic acid betaine by HPLC ESI-MS/MS. For each compound, the extracted ion chromatogram (EIC) at the indicated transition (MS^2) is reported. The insets indicated by the arrows above proline betaine and pipecolic acid betaine peaks report the respective fragmentation patterns and the fragment relative intensities. Pro, proline; HypBet, 4-hydroxy-L-proline betaine; NmePro, *N*-methyl-L-proline; ProBet, proline betaine; PipBet, pipecolic acid betaine.

with target set at 30000 and maximum accumulation time at 20 ms. The measurements were performed from the peak area of the extracted ion chromatogram (EIC). The quantitation was achieved by comparison with the calibration curves obtained with standard solutions. The retention time (min) and peak areas of the monitored fragment ions were determined by Agilent software Chemstation version 4.2.

RESULTS AND DISCUSSION

Pipecolic Acid Betaine Synthesis and MS/MS Characterization. As pipecolic acid betaine was not commercially available, we first synthesized it according to the Chen and Benoiton method.[25,26] The purified substance was subjected to mass spectrometry analysis to find the best instrumental conditions for detection and quantitation. In particular, amplitude and cutoff, which are the main parameters for an ionic trap mass spectrometer to achieve the most effective collision-induced dissociation (CID) of the parent ion toward its ionic fragments, were optimized. This study was performed in positive ion mode by direct infusion of 5 μM pipecolic acid betaine solution in 0.1% formic acid. The pipecolic acid betaine and proline betaine fragmentation patterns are shown in Figure 2. It is interesting to note that pipecolic acid betaine shows a MS/MS spectrum richer in fragments than proline betaine. This wealth of fragments is of great importance for the unambiguous MS/MS identification of the substance in a complex matrix. However, pipecolic acid betaine and proline betaine also show a common feature, that is, the resistance of the parent ions to MS/MS fragmentation. Both substances, in fact, in the optimized MS/MS instrumental conditions show the relative abundance of parent ions by far higher than that of fragment ions (Figure 2). Harsher MS/MS fragmentation conditions did not improve the fragment yield but brought about uniform intensity decrease of both parents and fragments.

The most useful fragments for pipecolic acid betaine determination in complex matrices are at m/z 112, 102, and 72. The highest specificity and sensitivity are accomplished by monitoring the fragment at m/z 72, which is absent in the fragmentation patterns of proline betaine and other methylated proline derivatives that occur in citrus juices.[10,11]

Chromatographic Analyses. In a previous study[10,11] was developed an isocratic chromatographic method that employed a Supelco Discovery-C8 column, 150 × 3.0 mm, 5 μm, and 0.1% formic acid solution in water, at flow rate of 100 μL/min as unique eluent. In this way, five proline derivatives and other betaines present in citrus juices were analyzed in about 10 min without using any gradient. The use of a gradient, in fact, has adverse effects both on analysis time, requiring column equilibration after each analysis, and on the detection response, the analyte ionization being in the ion source strongly dependent on the eluent composition. However, we observed that in these conditions pipecolic acid betaine showed a rather long retention time (>15 min) with a consequent peak broadening and sensitivity loss. For this reason in the present study we employed a shorter column (10 cm). In this new condition, many marker compounds of abiotic stress (i.e., methylated proline derivatives, choline, trigonelline, and γ-

aminobutyric acid betaine) were still well resolved and easily identified. Moreover, pipecolic acid betaine eluted at 11.4 min, resolved from the other methylated proline derivatives, which showed retention times lower than 9 min (Figure 2). Actually, elution times were 6.1 min for proline, 6.4 min for hydroxyproline betaine, 7.2 min for *N*-methylproline, and 8.8 min for proline betaine. For the sake of clarity, the elution peaks of γ-aminobutyric acid betaine (7.0 min), choline (8.0 min), and trigonelline (9.2 min) are not reported in Figure 2. It is worth noting for later considerations that one more methylene group in the ring noticeably increased the retention time of pipecolic acid betaine with respect to its lower homologue proline betaine. Conversely, the introduction of a hydroxyl group on the ring, as happens in the case of hydroxyproline betaine, drastically reduces the retention time on the octyl phase (C8) column. This clearly demonstrates that, also in the case of these highly hydrophilic substances, their retention mechanism relies on their different hydrophobic interaction with the column stationary phase.

Identification and Quantitation of Pipecolic Acid Betaine in Plant Extracts. As alfalfa (*M. sativa* L.) is well-known to contain high levels of methylated proline derivatives, particularly proline betaine and pipecolic acid betaine,[19,20,23] we first tested our analytical procedure on this plant species. Figure 3 reports a typical MS/MS EIC chromatogram of a leaf extract. Proline betaine was monitored through the transition 144→84 and pipecolic acid betaine through the transition 158→72. In particular, pipecolic acid betaine was easily recognized by the matching of retention time and fragmentation pattern with the authentic standard. In agreement with that reported by Wood et al.[19] and Bonham et al.,[20] both substances were present at comparable levels. On the contrary, the situation was completely different for the extracts of citrus plants. Figure 4 depicts the dramatic diversity of levels between the two betaines in a bergamot juice extract. Notwithstanding the noticeable difference of the retention times, the proline betaine peak completely overwhelms that of pipecolic acid betaine. However, the great resolving power of the mass spectrometry method, well represented by Figure 4, allows pipecolic acid betaine to be resolved and quantitated with confidence. It is worth noting that Wood et al.[27] detected the presence of an ion species at *m/z* 158, which produced major fragments at *m/z* 140, 112, 102 and 72 in the ESI mass spectra of *Achillea filipendulina* extracts. The authors hypothesized that the compound was a hydroxy derivative of dehydroprolinebetaine, but the lack of authentic standard made it impossible to sustain such a hypothesis. In our case, we can exclude this possibility

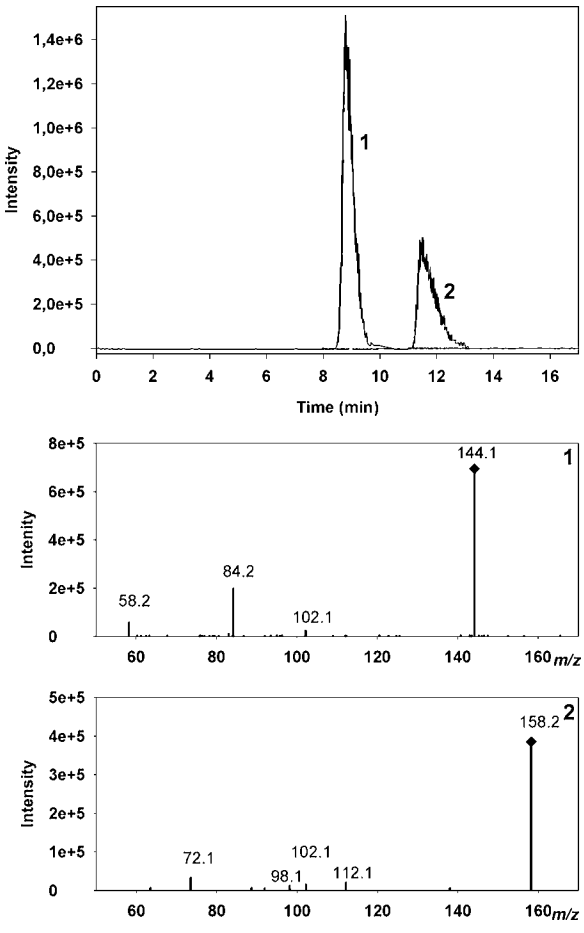

Figure 3. Chromatographic separation of a *Medicago sativa* leaf extract by HPLC ESI-MS/MS. The extracted ion chromatograms at the MS^2 transitions 144.1 → 84.2 (peak 1) and 158.2→72.1 (peak 2) are reported. (Inset 1) Fragmentation pattern of peak 1. (Inset 2) Fragmentation pattern of peak 2.

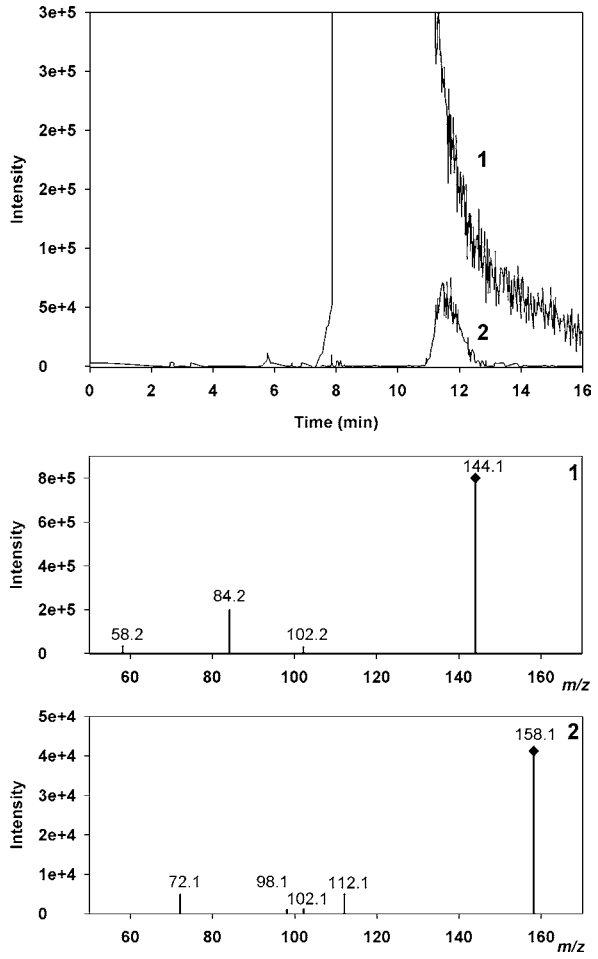

Figure 4. Chromatographic separation of a bergamot juice by HPLC ESI-MS/MS. The extracted ion chromatograms at the MS^2 transitions 144.1 → 84.2 (peak 1) and 158.1→72.1 (peak 2) are reported. (Inset 1) Fragmentation pattern of peak 1. (Inset 2) Fragmentation pattern of peak 2.

Table 1. Ranges of Pipecolic Acid Betaine (PipBet) and Pipecolic Acid (Pip) Levels in Citrus Plants Estimated by HPLC ESI-MS/MS

	bergamot			lemon			orange		
	leaves	seed	juice	leaves	seed	juices	leaves	seed	juice
PipBet (mg/kg)	8−10	1−2	1−2	1−3	1−2	max 0.5	max 1	1−2	max 0.5
Pip (mg/kg)	16−24	8−12	trace−3	6−8	4−8	trace−2	8−11	3−6	trace−2

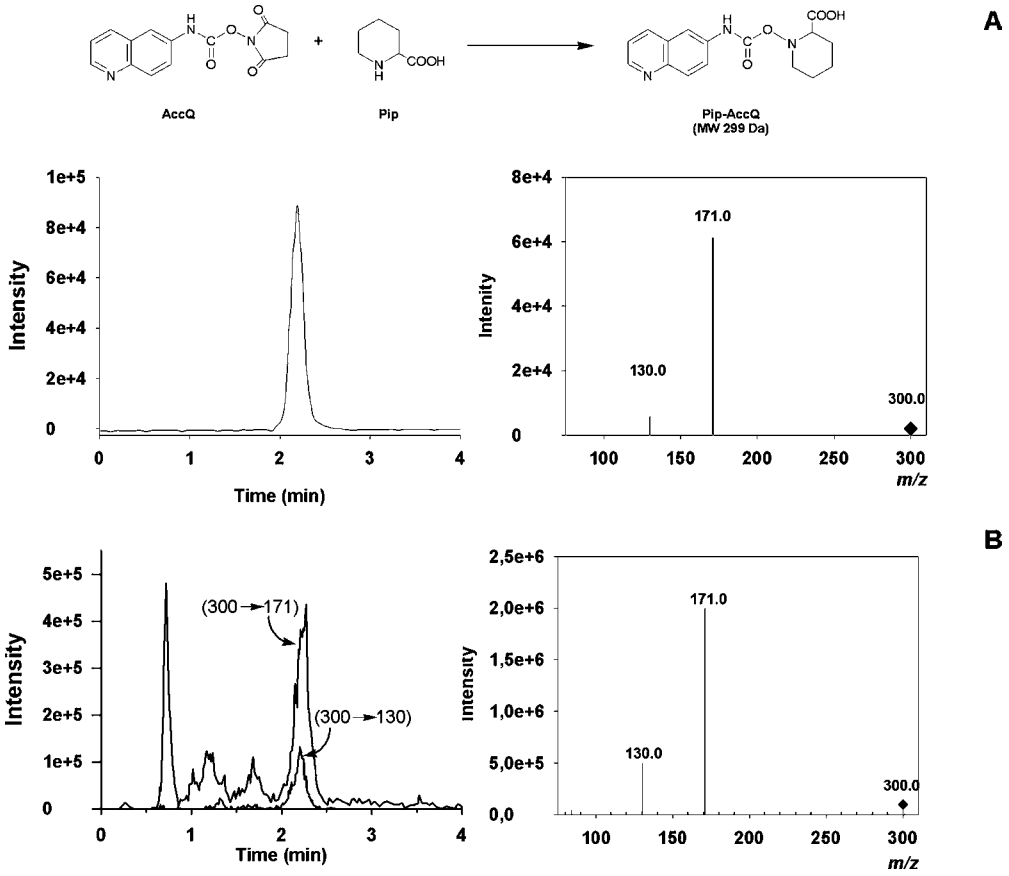

Figure 5. (A) Chromatogram of the derivative of pipecolic acid with AccQ (Pip-AccQ) standard solution while monitoring the MS^2 transition 300→130. On the right is reported the peak fragmentation pattern and, above, the pipecolic acid derivatization reaction with AccQ. (B) Chromatogram of a bergamot leaf extract derivatized with AccQ while monitoring the MS^2 transitions 300→130 and 300→171. On the right is reported the fragmentation pattern of the peak with retention time 2.3 min.

with certainty. First, the retention time and fragmentation pattern of the ion at *m/z* 158 in the citrus extracts were identical to those of the authentic pipecolic acid betaine standard. Furthermore, for the reason discussed above, a putative hydroxyproline derivative should have shown in our chromatographic procedure a much shorter retention time, close to that of hydroxyproline betaine.

Pipecolic acid betaine levels were examined in leaves, seeds, and juices of lemon, bergamot, and orange (Table 1). It is evident that pipecolic acid betaine, although at low levels, is present in all parts of each type of plant (Table 1). The highest levels were found in bergamot, whereas orange was the species with the lowest contents. Moreover, it also appears that leaves contain the highest and juices the lowest pipecolic acid betaine levels (Table 1).

Identification and Quantification of Pipecolic Acid in Plant Extracts. The occurrence of pipecolic acid betaine in citrus plants raises the question if it may originate from a biosynthetic pathway analogous to that proposed for proline betaine formation, that is, the methylation of the corresponding amino acid.[22,24] The most exhaustive literature data on the presence of pipecolic acid in vegetal matrices are reported by Fujita et al.,[16] who investigated the origin of D- and L-pipecolic acid in human physiological fluids. The authors reported the content of pipecolic acid in 17 edible plants, except the citrus plants, showing that some of them, such as common bean, broccoli, cabbage, and cauliflower, contained fair levels of pipecolic acid, in the range of 10−50 mg/kg, with higher contents of the L-isomer than the D-isomer. From an analytical point of view, the ESI-MS/MS determination of pipecolic acid in vegetal matrices presents several problems due to the simultaneous presence at much higher concentrations of many free amino acids and other substances that heavily interfere with the quantitation. Studies on the amino acid determination by ESI-MS/MS in biological fluids[28−30] have pointed out that some amino acids, particularly glutamic acid, glutamine, lysine, and pyro-L-glutamic acid, represent important interfering compounds in the ESI-MS/MS determination of pipecolic acid. More recently, we showed that *N*-methylproline, represented at moderate levels in citrus matrices, constitutes

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the main potentially interfering compound having the same molecular mass of pipecolic acid and, in our chromatographic conditions, the closest retention time to it.

Actually, glutamic acid, glutamine, and lysine should not interfere, having higher molecular mass than pipecolic acid. Instead, these substances easily undergo in-source CID, losing ammonia. Therefore, they are also detected at m/z 130, the same m/z value of pipecolic acid. Furthermore, when subjected to fragmentation, the three compounds produce the same intense fragment at m/z 84 as pipecolic acid does.[29,30] Another reason for concern arises from the consideration that in citrus matrices those amino acids are represented at high levels. The AIJN reports for citrus juices mean concentrations of 400 mg/L for Glu, 75 mg/L for Gln, and 70 mg/L for Lys.[31] For these reasons, after having unsuccessfully tried to detect with certainty pipecolic acid by direct HPLC ESI-MS/MS of plant extracts, we tested a procedure to overcome the presence of those interfering compounds. This procedure is based on the derivatization of the free amino acids in the samples with AccQ (6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate) according to the van Wandelen and Cohen method,[32] which is usually employed for the determination of the free amino acids by HPLC with fluorescence detection in the quality control of fruit juices. AccQ reacts with primary and secondary amino groups of amino acids, forming stable highly fluorescent derivatives. In particular, Lys, Gln, and Glu form derivatives with molecular masses of 317 Da (Lys-AccQ), 317 Da (Gln-AccQ), and 318 Da (Glu-AccQ). Interestingly, AccQ does not react with amide and tertiary amine groups; therefore, it is unable to derivatize pyroglutamic acid and *N*-methylproline. The derivatized pipecolic acid (Pip-AccQ), which has a molecular mass of 299 Da, was characterized by FIA ESI-MS/MS and HPLC ESI-MS/MS in positive ion mode. Because of the high hydrophobicity of Pip-AccQ, the retention time on the 10 cm C8 column was too long. Therefore, the analyses were isocratically conducted with a shorter column of the same type, that is, a Supelco Discovery-C8, 20 × 3.0 mm, 5 μm, using as eluent a mixture of 0.1% formic acid in water and methanol (75:25, v/v) at flow rate of 100 μL/min. In these conditions, Pip-AccQ elutes at 2.3 min. As expected, the EIC chromatogram shows transitions at m/z 300→171 and 300→130 (Figure 5 A). These two transitions are very specific as they can arise only from Pip-AccQ and not from the other interfering compounds mentioned above.

The analyses on citrus plant extracts were performed on samples subjected to a previous purification step on AG 50WX8-(H+) resin, according to the standard procedure for amino acid determination in citrus juices and then derivatized according to the van Wandelen and Cohen method.[32] Figure 5B reports the MS/MS EIC chromatogram obtained on a bergamot leaf extract for the 300→171 and 300→130 transitions. Pipecolic acid was detected in all samples examined (Table 1). In leaves, the highest pipecolic acid concentration was found for bergamot and the lowest, for lemon. In the juices the concentration resulted in each case much lower than in leaves.

It is well assessed that plants in the course of evolution have developed defense mechanisms toward abiotic stress based on the production of osmolyte compounds in patterns which are characteristic of the various genera. Proline and its methylated derivatives appear to characterize and to be of great physiological relevance in the *Citrus* genus. It has been hypothesized that the methylated proline derivatives are produced by the action of methyltransferase(s), which employ adenosylmethionine as a methyl donor, although such enzymes have never been isolated and characterized in citrus plants so far. Recently, we quantitated, with the same HPLC ESI-MS/MS procedure employed in this study, several osmolytes in fruit juices of numerous commercially important citrus plants such as yellow orange, blood orange, lemon, mandarin, bitter orange (*Citrus aurantium*), chinotto (*Citrus myrtifolia*), and grapefruit.[10,11] It was found that the most represented osmolytes in the juices, that is, proline, proline betaine, and hypdroxyproline betaine, showed in each case concentrations well above 100 mg/kg. Therefore, the levels of pipecolic acid betaine detected in this study for the first time in the citrus matrices are comparatively too low to infer a relevant role as osmolyte in the plant. However, the finding of pipecolic acid in the same matrices allows the hypothesis that pipecolic acid betaine might be a sort of byproduct of proline betaine biosynthesis. In fact, proline and pipecolic acid have very similar chemical structures (Figure 1); thus, it could occur that the same methyltransferase(s) which methylate proline recognize also pipecolic acid as a substrate. In this case, the much lower levels of pipecolic acid betaine than proline betaine, which we observed, could be ascribed to both the much lower concentration of pipecolic acid than proline and a likely lower affinity of the enzyme(s) for pipecolic acid than for proline. Obviously, the validity of this hypothesis requires further studies.

AUTHOR INFORMATION

Corresponding Author

*Phone: +390815665865. Fax: +390815665863. E-mail: luigi.servillo@unina2.it.

Present Address

⊥ Dipartimento di Ingegneria Industriale e ProdAl scarl, Università degli Studi di Salerno, via Ponte Don Melillo, I-84084 Fisciano (SA), Italy.

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