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Proline Derivatives in Fruits of Bergamot (*Citrus bergamia* Risso et Poit): Presence of *N*-Methyl-L-proline and 4-Hydroxy-L-Prolinebetaine

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The content of proline and various compounds deriving from its metabolism (4-hydroxy-L-proline, *N*-methyl-L-proline, *N,N*-dimethylproline, and 4-hydroxy-L-prolinebetaine) was determined in fruits and seeds of Bergamot (*Citrus bergamia* Risso et Poit), growing in the Calabria region (South Italy). A HPLC-ESI-tandem mass spectrometry method, which allowed rapid determination of L-proline, 4-hydroxy-L-proline, *N*-methyl-L-proline, *N,N*-dimethylproline, and 4-hydroxy-L-prolinebetaine in juice and extracts of bergamot fruit with minimum sample preparation and short analysis time (about 10 min), is presented. Proline and 4-hydroxy-L-proline levels in the samples were also determined by HPLC analysis with fluorescence detection and the results compared to those obtained with HPLC-ESI-tandem mass spectrometry. For the first time, the presence of *N*-methyl-L-proline and 4-hydroxy-L-prolinebetaine in the fruits of a plant of the *Citrus* genus is reported.

KEYWORDS: L-proline; 4-hydroxy-L-proline; *N*-methyl-L-proline; *N,N*-dimethylproline; 4-hydroxy-L-prolinebetaine; bergamot; *Citrus bergamia*

INTRODUCTION

Plants subjected to stress conditions such as sudden environment temperature changes, high salinity, drought, scarce soil nutrition, respond with the biosynthesis of a series of protecting metabolites known as osmolytes. Many osmolytes are chemically derived from some amino acids or iminoacids in which the nitrogen atom is completely methylated (betaines) (1, 2). Betaines are quaternary ammonium compounds, highly soluble in water, which tend to accumulate in the cytoplasm as "compatible solutes" having osmoprotective functions able not only to reduce the inhibitory effects of ions on enzyme activities (hypersalinity) but also to increase protein conformational stability and to prevent the dissociation of enzyme complexes (3). Moreover, these substances also show cryoprotectant activity, being able to reduce cellular damages of freeze-induced dehydration (4, 5).

The main amino acids involved in this specific process are serine, alanine, methionine, pipecolic acid, and proline. Serine is first metabolized into choline, which is then transformed into glycine betaine (3, 6) or choline *O*-sulfate. Alanine is converted into the osmolyte β -alanine betaine through three different *N*-methyltransferases (7). Methionine is converted into 3-dimethylsulfoniopropionate (8), an analogue of the β -alanine betaine. The nonprotein amino acid, pipecolic acid, derived from lysine metabolism, is converted into pipecolatebetaine in some species, notably *Medicago* and *Achillea* (9). Proline deserves

particular attention. This amino acid is naturally present at high concentrations in higher plants, including the *Citrus* genus. In citrus juices, proline content is between 200 and 900 mg/kg (10). The literature data show that under plant stress conditions proline accumulates in greater amounts than other amino acids. It has been widely reported that under stress conditions plants preferentially accumulate proline rather than other amino acids. It remains controversial whether proline acts as an osmoprotectant or rather its accumulation in response to stress conditions is a symptom of cellular injury (11).

It has been hypothesized that proline increase acts as a signal/regulator able to activate multiple responses in the adaptation process or that this amino acid regulates the accumulation of useable nitrogen (12). In Figure 1, the chemical structures of the main quaternary ammonium compounds derived from proline are reported.

The *N,N*-dimethylproline (also known as prolinebetaine or stachydrine) is thought to be a more potent osmoprotectant than proline itself. In some plants, 4-hydroxy-L-prolinebetaine (known as betonicine) is also present (13). The synthesis of prolinebetaine is thought to involve *N*-methylation of proline by methyltransferase, via the intermediate *N*-methylproline (14) (Figure 1).

Unfortunately, the determination of some compounds resulting from the metabolism of proline, i.e., *N*-methylproline, prolinebetaine, and 4-hydroxyprolinebetaine is not particularly easy. In fact, primary and secondary amino groups are missing in these substances (Figure 1); therefore, the usual sensitive derivatization reactions or postcolumn reactions of amino acids cannot be

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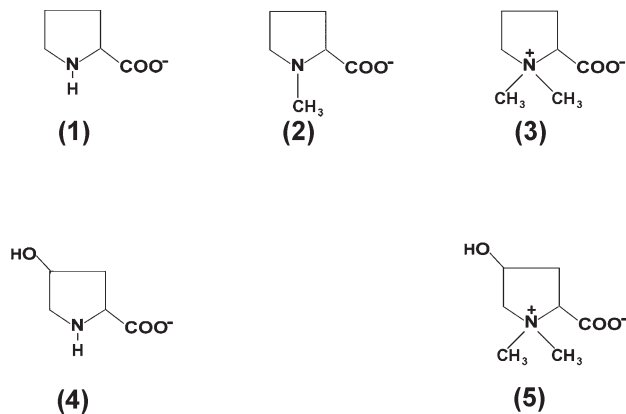


Figure 1. Chemical structures of the proline derived compounds analyzed in this study. **1**, L-proline; **2**, N-methyl-L-proline; **3**, N,N-dimethylproline; **4**, 4-hydroxy-L-proline; **5**, 4-hydroxy-L-prolinebetaine.

applied, and time-consuming chromatographic procedures with chemical conversion steps or precolumn derivatization must be employed (15–17).

In this investigation, we developed a rapid HPLC-tandem mass spectrometry procedure to analyze proline metabolites in bergamot fruit. The study was conducted on bergamot as this plant has great commercial importance in South Italy, where it is intensively cultivated, particularly in the Calabria region, for the production of the essential oil which is widely used in the perfume industry. In the past few years, likely due to variations of climatic and hydro-saline soil conditions, abnormal phenomena in the bergamot plant cycle that could be ascribed to vegetative stress have been frequently observed. Therefore, it seemed of interest to investigate the content of proline derived osmoprotectants in various parts of bergamot fruit, i.e., juice, seeds, peel (flavedo and albedo), and endocarp and to devise a rapid method for their determination.

MATERIALS AND METHODS

Reagents. L-Proline, N-methyl-L-proline, and 4-hydroxy-L-proline were from Sigma-Aldrich (Milan, Italy). N,N-Dimethylproline and 4-hydroxy-L-prolinebetaine were purchased from Extrasynthese (Genay, France). Milli-Q water was used for all the preparations of solutions and standards. The 0.1% solution of formic acid in water used for the LC/ESI/MS analyses was from Sigma-Aldrich.

Plant Materials. Bergamot fruits utilized in this study were harvested at the beginning (January 8th, 2010) and at the end (February 28th, 2010) of the production season in six different localities (Ravagnese; Pellaro; Macellaro; Arangea; Gallico Superiore and Reggio Calabria Airport) of the bergamot essential oil production area around the Reggio Calabria town (Italy). A total of 18 lots, each made up of about 4 kg of fruits, were sampled from the six different sites in order to evaluate the qualitative and quantitative distribution of the main osmolytes derived from the amino acid proline in the various tissue parts of the bergamot fruit, i.e., seeds, peel (flavedo and albedo) and endocarp.

The preparations started with water washing of the bergamot fruits followed by the manual scraping of the exocarp in order to remove the essential oils from the utricles on the fruit surface. Then, after washing again with water, the peel and seeds were separated manually.

Peel Extracts. The peel (flavedo and albedo) was homogenized in a mixer with water in a 1:1 (w/w) ratio. The homogenate was kept for 2 h under constant agitation and then centrifuged at 18000g for 30 min at 4 °C. The supernatant was finally frozen and kept at –20 °C until used for analytical determinations.

Endocarp Extracts. The endocarp (edible part of the fruit constituted by juice and pulp) deprived of seeds was homogenized in a mixer, centrifuged at 18000g for 30 min at 4 °C, and the supernatant frozen and kept at –20 °C until used for analytical determinations.

Seed Extracts. The seeds recovered from each lot were initially washed with water, drained and dried on filter paper. Successively, 2–4 g of the seeds were homogenized in a mixer with 20 mL of Milli-Q water. The homogenate was kept for 3 h under constant agitation, then centrifuged at 18000g for 30 min at 4 °C, and the supernatant frozen and kept at –20 °C until used for analytical determinations.

Juice. The bergamot juice was obtained by manually squeezing fruits with a squeezer. The juice obtained from about 2 kg of fruit of each lot was depulped by centrifugation at 18000g for 30 min at 4 °C and the supernatant frozen and kept at –20 °C until used for analytical determinations.

Instrumentation and Analytical Methods. Determinations on the extracts and juices by HPLC-ESI-MS/MS were performed in two different ways, i.e., without any further sample preparation except dilution 1:25 (v/v) of centrifuged samples (juice or extracts) with 0.1% formic acid in water or by subjecting the centrifuged samples on a column (5 × 1 cm) of Bio-Rad AG 50WX8-(H+) resin as described for the amino acid determinations and reconstituting the dried eluate with volumes of 0.1% formic acid in water which corresponded to an initial sample dilution of either 1:10 or 1:25.

HPLC-MS/MS Analysis. Chromatographic separations were performed with a 150 × 3.0 mm i.d., 5 μm, Supelco Discovery-C8 column at a flow rate of 100 μL/min. The chromatography was conducted isocratically with 0.1% formic acid in water. Samples of 20 μL of standard solutions or fruit extracts were injected. The HPLC analyses were performed on an Agilent 1100 series liquid chromatograph equipped with an LC-MSD SL quadrupole ion trap. The HPLC system had an on line degasser and an automatic injector. The conditions for ESI-MS/MS analyses, made in positive ion mode, utilizing nitrogen as the nebulizing and drying gas, were nebulizer pressure, 30 psi; drying temperature, 350 °C; and drying gas, 7 L/min. The ion charge control (ICC) was applied with the target set at 10000 and maximum accumulation time at 20 ms. In order to obtain efficient collision induced fragmentations of the positively charged parent ion, the ion trap, molecular weight cutoff, and amplitude potential and other instrumental parameters were previously optimized for each analyte. The retention time (in min) and peak areas of the monitored fragment ions were determined by the Agilent software Chemstation, version 4.2. The quantification was achieved by comparison with the calibration curve obtained with standard solutions.

Validation of Analyte Quantification. The stock standard solutions N,N-dimethylproline, 4-hydroxy-L-prolinebetaine, L-proline, N-methyl-L-proline, and 4-hydroxy-L-proline were prepared at 2000 ng/mL, and 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 curve was built using these standard solutions. The linear regression analysis was carried out by plotting the peak areas of the monitored fragment ion against the concentrations of the analyte standard solutions. The linearity of the instrumental response was demonstrated by a correlation coefficient (r^2) greater than 0.99.

HPLC Analysis of Amino Acids. Quantification of L-proline, 4-hydroxy-L-proline, and other free amino acids present in the samples was performed by reverse phase HPLC employing a Waters instrument, model 2690, equipped with the fluorescence detector, model 474. The amino acids were derivatized with Waters AccQ FLUOR Reagent (18). The juice or extract samples of about 10 mL were centrifuged at 12000g at 4 °C for 10 min. Then, 1 mL of supernatant, after filtration, was loaded on a column (5 × 1 cm) filled with Bio-Rad AG 50WX8-(H+) resin. After loading, the column was washed with five volumes of milli Q water, and then the amino acids were one step eluted with 10 mL of ammonia solution (1:1 v/v water) and 5 mL of water. The eluted solution was dried with a rotavapor, dissolved in 5 mL of 0.01 M HCl, and passed through 0.45 μm filter.

The derivatization was conducted by adding in a 1 mL vial 10 μL of sample, 60 μL of AccQ Fluor borate buffer, and 20 μL of AccQ Fluor reagent (in 10 mM acetonitrile). The reaction mixture was allowed to react for 10 min at 55 °C in a Pierce preheated block. Ten microliter standard solutions of each amino acid at concentrations 2.5, 5, and 10 μM were subjected to the same derivatization procedure in order to build up the calibration curves. The chromatography was performed with a column C18, 3.9 × 150 mm (Waters), conditioned at 39 °C. The injection volume was 5 μL. The elution gradient and flow rate were those reported by van Wandelen and Cohen (18). Quantification was made using the peak area of

Table 1. Monitored Mass Transitions and Respective Sensitivities of the Analyzed Proline Derivatives

compound	mass transition (m/z)	sensitivity (peak area/ng)
Pro ^a	116 → 70	61680
HyPro ^b	132 → 68	1860
	132 → 86	27400
NmePro ^c	130 → 82	3730
	130 → 84	151400
ProBet ^d	144 → 84	37500
HypBet ^e	160 → 88	5120

^a Pro: L-proline. ^b HyPro: 4-hydroxy-L-proline. ^c NmePro: N-methyl-L-proline. ^d ProBet: N,N-dimethylproline. ^e HypBet: 4-hydroxy-L-prolinebetaine.

the fluorescence emission intensity by exciting at 350 nm and recording the fluorescence emission at 395 nm. Amino acids were identified on the basis of their retention times and quantified by comparison of the sample peak area with the respective calibration curve.

RESULTS AND DISCUSSION

Instrumental Conditions. The data were obtained by selected reaction monitoring (SRM) in positive ion mode using standard solutions of each analyte at various concentrations as described in the Materials and Method section. It is evident from **Table 1** that the relative intensity at the optimized instrumental settings, expressed as peak area per nanogram of injected analyte, is very different among the various substances. When recording the intensity of the most abundant fragment, N-methyl-L-proline is that showing the highest response, followed by L-proline, N,N-dimethylproline, 4-hydroxy-L-proline, and 4-hydroxy-L-prolinebetaine (**Table 1**).

Chromatographic Separation of the Proline Derived Osmolytes. As a first stage, we sought to develop a chromatographic method to analyze the compounds of interest in a short time and possibly without using a gradient. The use of a gradient, in fact, has adverse effects both on time, requiring column equilibration after each analysis, and on the detection response, the analyte ionization in the ion trap source being strongly dependent on the eluent composition. For example, ion-exchange chromatography requires the use of eluents containing high and variable concentrations of inorganic salts which may dampen electrospray ionization. Therefore, we tried a sorbent having the octyl phase (C8) as the matrix active group, which is normally used for the chromatographic separation of hydrophobic compounds in RP-HPLC analyses. With our quaternary ammonium compounds being scarcely hydrophobic, we expected that a certain retention by the C8 sorbent could be anyway effective in a purely aqueous eluent. As reported in **Figure 2**, a complete chromatographic separation of all the examined osmolytes was obtained using Supelco Discovery-C8 at a flow rate of 100 μ L/min in less than 10 min in isocratic conditions using 0.1% formic acid in water as the eluent (**Figure 2**). In particular, 4-hydroxy-L-prolinebetaine, with a retention time of 8.4 min is completely separated from N,N-dimethylproline, which is eluted at 9.5 min. This confirms that the retention is mainly due to the hydrophobic interaction of the compounds with the stationary phase, as 4-hydroxy-L-prolinebetaine differs from N,N-dimethylproline only by the presence of a hydrophilic hydroxyl group in position 4 of the pyrrolidine ring (**Figure 1**). The same consideration holds for 4-hydroxy-L-proline (r.t. 8.1 min) and L-proline (r.t. 9.0 min). The retention times were 8.10 min, 4-hydroxy-L-proline; 8.4 min, 4-hydroxy-L-prolinebetaine; 9.0 min, L-proline; 9.2 min, N-methyl-L-proline; 9.5 min, N,N-dimethylproline.

Before chromatographic analyses, extracts and juices were previously subjected to passage on a column (5 × 1 cm) of Bio-Rad AG 50WX8-(H+) resin. This is a standard procedure generally used for the chromatographic analysis of amino acids

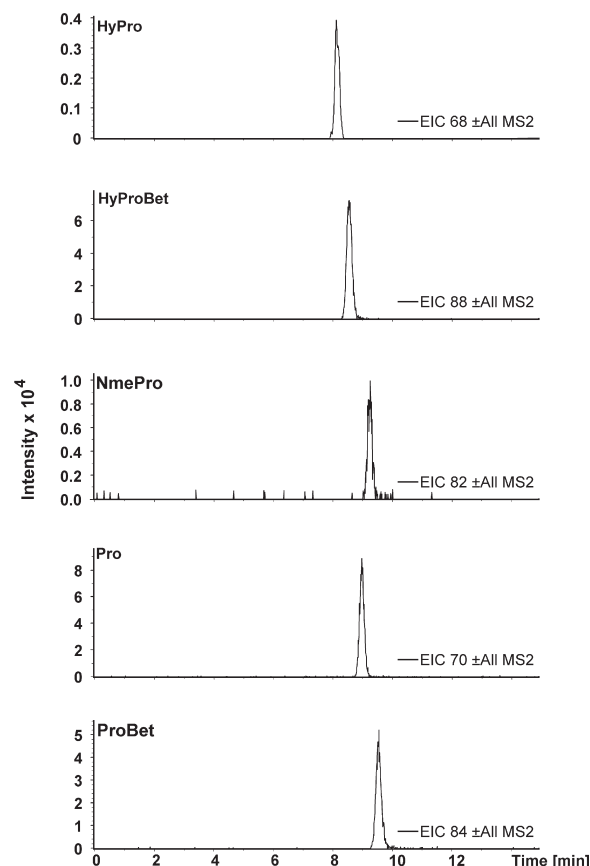


Figure 2. Chromatographic separation of proline derived compounds by HPLC-ESI-MS/MS. The extracted ion chromatogram (EIC) at the indicated mass (m/z) for each compound is reported. HyPro: 4-hydroxy-L-proline. HypBet: 4-hydroxy-L-prolinebetaine. NmePro: N-methyl-L-proline. Pro: L-proline. ProBet: N,N-dimethylproline.

in fruit juices. The dried residue was dissolved in water containing 0.1% formic acid and analyzed. As expected, N,N-dimethylproline and 4-hydroxy-L-prolinebetaine, being positively charged at acid pH, were strongly retained on the anionic exchange resin. However, at alkaline pH they become zwitter ions (**Figure 1**) and could be still retained on the AG 50WX8-(H+) resin also when eluting with ammonia solution, which is used to recover the amino acids retained at acid pH. However, we found that N,N-dimethylproline and 4-hydroxy-L-prolinebetaine were quantitatively recovered from the resin by elution with ammonia. In fact, further addition to the column of ammonia solution containing 2 M NaCl did not result in any further release of these compounds.

The various analytes were identified by comparison with authentic compounds on the basis of their chromatographic retention times and the MS/MS fragmentation patterns.

It is worth noting that, as far as the content of L-proline, N,N-dimethylproline, N-methyl-L-proline, and 4-hydroxy-L-prolinebetaine is concerned, the chromatographic analyses conducted on various samples of juice and extracts, which had been simply diluted 1:25 v/v with 0.1% formic acid in water, gave results comparable to those obtained with samples previously purified with passage on AG50WX8 resin (data not shown). Therefore, analyses of those compounds can be consistently accelerated avoiding that purification step.

Quantification of Proline Metabolites in Bergamot Extracts and Juices. The distribution of proline derived compounds determined by HPLC-ESI-MS/MS in the extracts and juice of the bergamot fruits is reported in **Table 2**. From a qualitative point of view, the most significant result emerging from **Table 2** is relative

Table 2. Distribution of Proline Derived Compounds in Bergamot Extracts and Juice

compound (mg/kg)	statistical parameters	parts of the fruit			
		peel	edible part	seed	juice
Pro ^a	min-max	191–798	161–479	1247–3616	211–440
	mean \pm std dev	445 \pm 208	341 \pm 106	2149 \pm 698	325 \pm 79
Pro ^{a,f}	min-max	185–700	155–440	1200–3400	200–422
	mean \pm std dev	425 \pm 208	320 \pm 88	2046 \pm 557	311 \pm 44
HyPro ^b	min-max	1.4–3.3	1.2–3.1	107–290	1.0–2.9
	mean \pm std dev	2.2 \pm 0.5	2.4 \pm 0.5	204 \pm 55	2.3 \pm 0.5
HyPro ^{b,f}	min-max	1.2–4.0	1.0–4.2	100–330	1.0–4.0
	mean \pm std dev	2.0 \pm 1.0	2.2 \pm 0.8	170 \pm 85	2.5 \pm 1.3
NmePro ^c	min-max	22.7–44.9	20.2–35.7	120–400	20.1–33.6
	mean \pm std dev	31.6 \pm 6.0	28.0 \pm 6.0	243 \pm 110	27.0 \pm 5.6
ProBet ^d	min-max	189–844	336–482	47–71	336–507
	mean \pm std dev	461.8 \pm 223.2	410.6 \pm 36.2	58 \pm 9	419.9 \pm 48.9
HypBet ^e	min-max	94.4–341.4	139–281	17–23	139–281
	mean \pm std dev	205.7 \pm 76.2	289.6 \pm 40.3	19 \pm 2	204.3 \pm 49.0

^a Pro: L-proline. ^b HyPro: 4-hydroxy-L-proline. ^c NmePro: N-methyl-L-proline. ^d ProBet: N,N-dimethylproline. ^e HypBet: 4-hydroxy-L-prolinebetaine. ^f Analyses performed by RP-HPLC analysis with fluorescence detection.

to the presence of the proline metabolites in all parts of the fruit and, above all, of *N*-methyl-L-proline and 4-hydroxy-L-prolinebetaine, which, to our knowledge, had never been detected before in the fruits of plants of the *Citrus* genus. Data from **Table 2** show that *N,N*-dimethylproline and L-proline are the main compounds present in the peel of bergamot fruits with a mean value of 462 mg/kg and 445 mg/kg, respectively, followed by 4-hydroxy-L-prolinebetaine (mean value 206 mg/kg), *N*-methyl-L-proline (mean value 32 mg/kg), and 4-hydroxy-L-proline, which is present at very low concentration (mean value 2.2 mg/kg).

Also in the edible portion of the fruit (endocarp extract) and in the juice, obtained by squeezing the edible portion, the distribution ratio of these compounds was similar to that of the pericarp, although the concentrations were on average lower.

As for seeds, the high level of *N*-methyl-L-proline found in this parts of the fruit is of interest. The content of *N*-methyl-L-proline, in fact, was about ten times higher than that elsewhere. Also the levels of L-proline and 4-hydroxy-L-proline were found to be higher in seeds than in other tissue parts of the fruit. However, it is difficult to explain the reason for these compositional differences, also considering the lack of data in the literature.

N,N-Dimethylproline. As far as the presence of the proline derived osmolytes in the juice, Rapp et al. (19) first reported the presence in orange juice of *N,N*-dimethylproline in a ¹³C NMR study. They found that the *N,N*-dimethylproline concentration in orange juice was between 240 and 700 mg/L, which makes *N,N*-dimethylproline one of the major constituents in orange juice after sugars and organic acids. Le Gall et al. (20) pointed out that *N,N*-dimethylproline could be a marker for the detection of pulp wash addition to orange juice, thus easily revealing this kind of juice adulteration. In our study, the variability of *N,N*-dimethylproline found in bergamot juice appears close to that reported (240–700 mg/L) for orange juice (19) and lower than that reported for just one sample of mandarin juice (21).

4-Hydroxy-L-prolinebetaine and N-Methyl-L-proline. The most interesting finding of our study regards the presence of 4-hydroxy-L-prolinebetaine and *N*-methyl-L-proline. The only data in the literature regarding 4-hydroxy-L-prolinebetaine were reported by Nolte et al. (13) who discovered the presence of a small amount of this substance in leaves of the Aurantioideae subfamily and other species of Rutaceae. The authors reported its tendency to accumulate in the cell cytoplasm of leaves and observed that 4-hydroxy-L-prolinebetaine was present in the examined species at levels which were 3–15% of that of *N,N*-dimethylproline. On the contrary, in all parts of the bergamot

fruit we examined (excepting seeds), 4-hydroxy-L-prolinebetaine is present at higher levels, between 94 and 341 mg/kg, representing about 50% of the *N,N*-dimethylproline concentration, which ranged between 189 and 844 mg/kg. 4-Hydroxy-L-prolinebetaine was identified in the samples by comparing with the authentic standard its retention time and fragmentation pattern in which the fragment ion at *m/z* 88 is peculiar for this compound (**Figure 3A and B**). 4-Hydroxy-L-prolinebetaine, with a retention time of 8.4 min, was completely separated from the other proline derived osmolytes (**Figure 2**). As for *N*-methyl-L-proline, also known as hygric acid, the concentration was essentially the same in the various fruit parts. To our knowledge, also the presence of *N*-methyl-L-proline was never reported before in *Citrus* genus plants. The highest *N*-methyl-L-proline content was observed in seeds (**Table 2**). The literature data regarding *N*-methyl-L-proline in plants are very scarce. Few papers report the *N*-methyl-L-proline presence in some species of Leguminosae and Caesalpi-noideae (22, 23). This substance is thought to confer resistance to abiotic stress (24) and to be a precursor in the biosynthetic pathway of *N,N*-dimethylproline through *N*-methylation of proline by a methyltransferase, via intermediate *N*-methylproline (14). However, the enzyme(s) involved in this pathway have never been characterized (14, 25).

From an analytical point of view, because of scarcity of literature data, we first investigated for the presence of possible interfering substances in MS analyses which could be mistaken for *N*-methyl-L-proline. For example, two of these substances are pyroglutamic and pipecolic acids, which are isobaric with *N*-methyl-L-proline and present in plant products. On the basis of this consideration, we started with the characterization of the MS/MS fragmentation pattern of *N*-methyl-L-proline and optimization of fragmentation conditions. The results, reported in **Table 1** and **Figure 4**, indicate that the main fragment is at *m/z* 84. Moreover, another fragment at *m/z* 82 is present, although with a relative intensity substantially lower than that at *m/z* 84 (ratio about 1:40) but sufficient for the *N*-methyl-L-proline dosage in our experimental conditions. We found that the fragment at *m/z* 82 is highly specific for *N*-methyl-L-proline. Neither pyroglutamic nor pipecolic acid fragmentation patterns show fragments at *m/z* 82. Moreover, also other possible interfering compounds are present in vegetal matrixes and in citrus juices (**Figure 5**), as glutamic acid, glutamine, and lysine. The guide by Association of the Industry of the Juices and Nectars from Fruits and Vegetables of the European Union (AIJN) (10) reports that in the main citrus juices (orange, lemon, grapefruit, and mandarin) these interfering

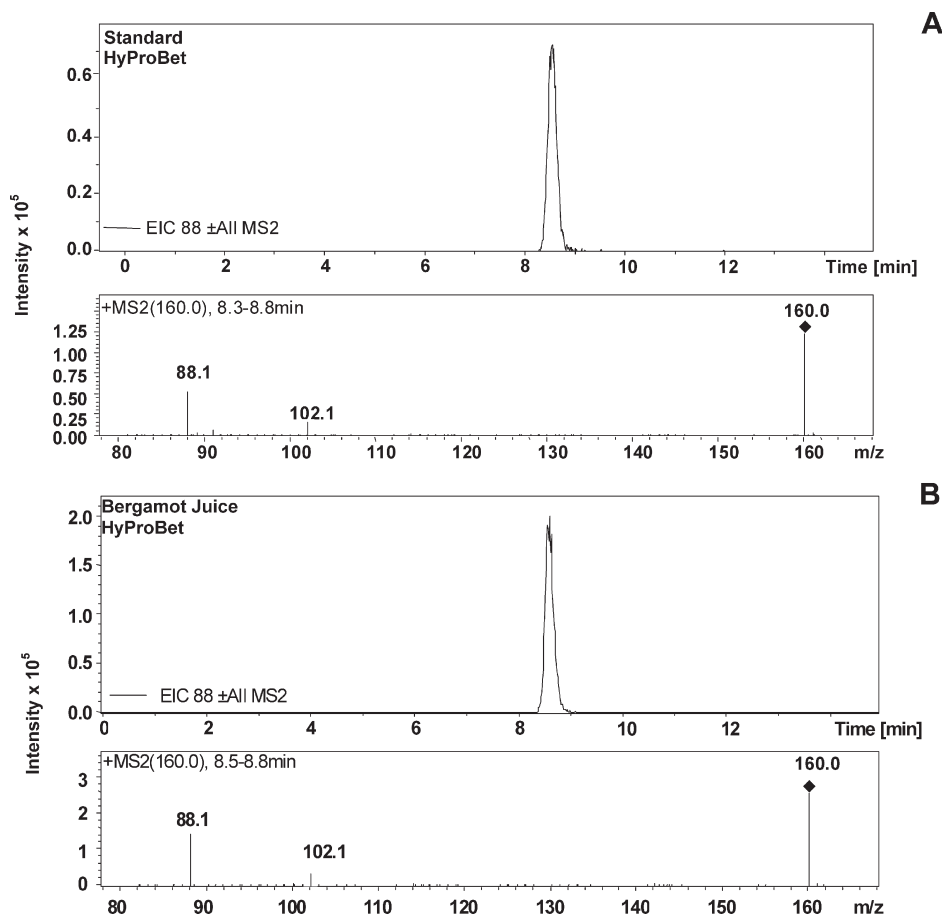


Figure 3. Extracted ion chromatograms of m/z 88 (MS^2) of bergamot juice (**B**) and standard 4-hydroxy-L-prolinebetaine solution (**A**). Below each chromatogram, the full scan MS^2 spectrum of the peak is reported. HypBet: 4-hydroxy-L-prolinebetaine.

compounds are widely expressed with a maximum value of 400 mg/L for glutamic acid, 75 mg/L glutamine, and 70 mg/L for lysine (10). Therefore, this must be taken into account in the quantitative determination of *N*-methyl-L-proline. Indeed, glutamic acid, glutamine and lysine have molecular masses higher than that of *N*-methyl-L-proline and should not interfere with its determination. However, interference can arise from in-source CID, a phenomenon in which fragments are produced before the parent ion enters into the ion trap (26, 27). Glutamic acid, glutamine and lysine easily undergo in-source CID and generate fragment ions at mass 130, the same mass of *N*-methyl-L-proline ($M + H^+$). Luckily, the fragmentation of the ions at mass 130, generated by in-source CID from this three compounds, show an intense fragmentation peak at m/z 84 but do not show any fragment at m/z 82. Anyway, in the case of bergamot juice, the chromatographic conditions we used seemed suitable to completely separate *N*-methyl-L-proline from other interfering compounds. In fact, it can be observed in **Figure 4**, which reports a typical analysis of a sample of bergamot juice diluted 1:25, that the EIC chromatogram at MS^2 84 shows three well resolved peaks, the last at the same retention time (9.2 min) as that of authentic *N*-methyl-L-proline (**Figure 4A**), whereas the EIC chromatogram at MS^2 82 shows only one peak at 9.2 min. Moreover, the ratio of the areas of the two peaks at MS^2 84 and 82 was the same as that of the two peaks originating from authentic *N*-methyl-L-proline, which is highly indicative of the absence of interfering substances. However, in order to have a safer determination of *N*-methyl-L-proline in the various fruit tissue, quantifications were conducted by monitoring the $130 \rightarrow 82$ transition, which is much more specific and shows an

instrumental response sufficient to quantify the analyte concentrations in our samples.

4-Hydroxy-L-proline and L-Proline. Also data regarding 4-hydroxy-L-proline in *Citrus* juices are rather limited in the literature. For this amino acid, the unique role, which has been hypothesized (14), is to be the primary source of the biosynthesis of 4-hydroxy-L-prolinebetaine, accomplished through its double *N*-methylation.

Determination of 4-hydroxy-L-proline in citrus juices is crucial for detecting possible frauds derived from adding amino acids, produced from the hydrolysis of proteins of animal origin, to the juice. The only data we found were from Wucherpennig and Millies (28), who reported the physiological presence of 4-hydroxy-L-proline in orange juice at a level lower than 10 mg/L, i.e., about 10 times lower than that of the least represented amino acids isoleucine and leucine, which are commonly determined in citrus juices. RP-HPLC analysis, coupled to fluorescence detection, of free amino acids essentially confirmed the low 4-hydroxy-L-proline level (mean value of about 3 mg/L) in bergamot juice (**Figure 5** and **Table 2**), in full agreement with values reported in orange juices (28).

From literature data (26, 27, 29), it emerges that the isobaric amino acids 4-hydroxy-L-proline, Leu and Ile, show very similar fragmentation patterns with the most represented positive fragment at m/z 86. In other words, Ile and Leu, present in plant products at low level but higher than 4-hydroxy-L-proline, have fragmentation patterns very similar to that of 4-hydroxy-L-proline. It follows that a reliable determination of this compound would be impossible without a complete chromatographic separation. Luckily, 4-hydroxy-L-proline presents a highly specific fragment ion at m/z 68 (26) which can be utilized for a

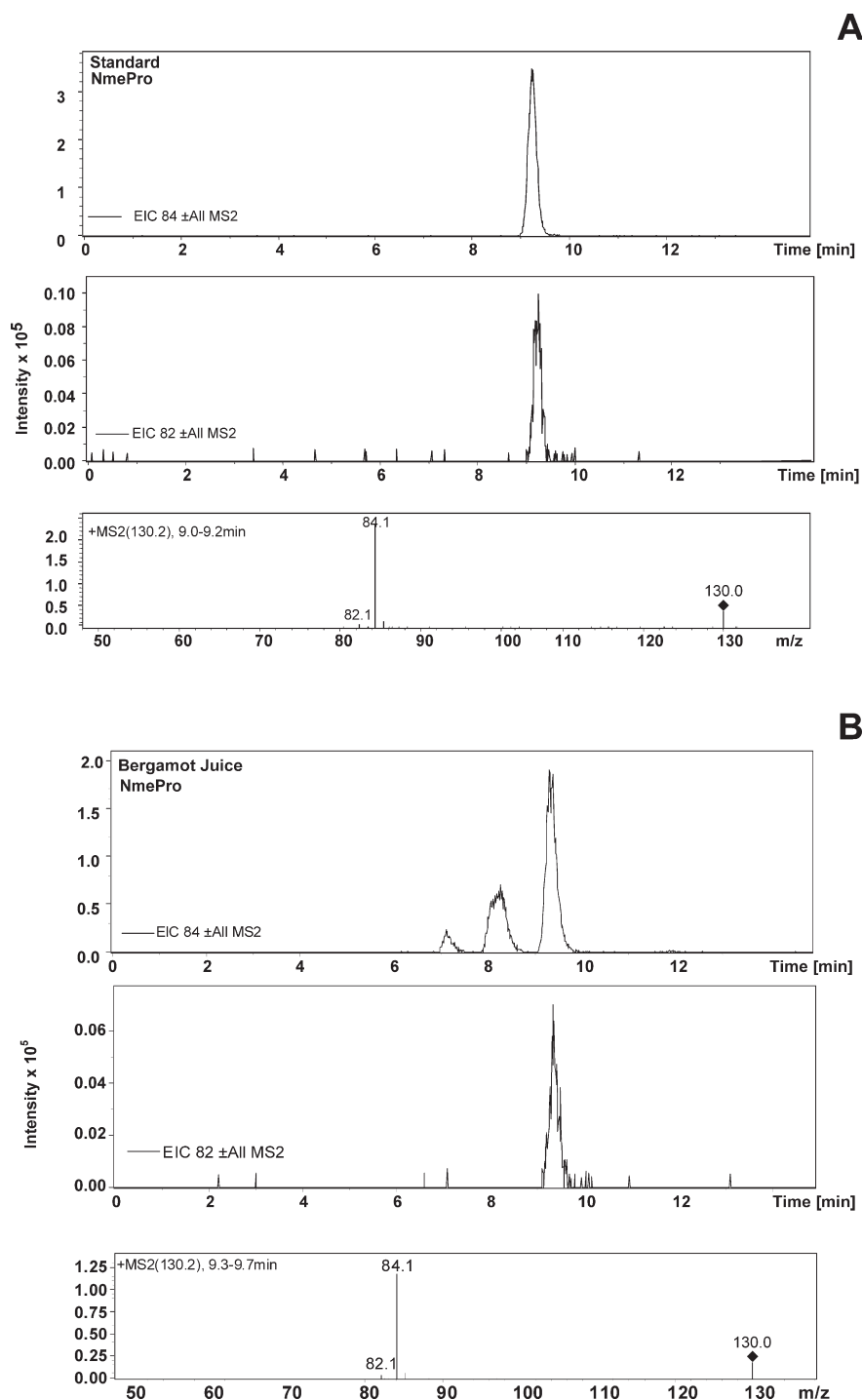


Figure 4. Extracted ion chromatograms of m/z 84 and 82 (MS²) of bergamot juice (**B**) and standard *N*-methyl-L-proline solution (**A**). Below each chromatogram, the full scan MS² spectrum of the peak at the retention time of 9.5 min is reported. NmePro: *N*-methyl-L-proline.

selective determination also in the presence of these interfering compounds. However, the relative intensity of the fragment ion at m/z 68 is consistently lower than that at m/z 86, which makes 4-hydroxy-L-proline detection through the fragment at m/z 68 less sensitive than at m/z 86. Despite the low instrumental response, our results indicated that it is possible to reliably quantify 4-hydroxy-L-proline with RP-HPLC-MS/MS, by diluting bergamot juice 10 times instead of 25 times. The data obtained by analyzing 4-hydroxy-L-proline with RP-HPLC-MS/MS (**Figure 2**) chromatography show that the compound eluted at 8.1 min, well resolved from Leu and Ile, which, being more hydrophobic, are eluted later.

Unlike for 4-hydroxyproline, the literature data for proline are particularly abundant. L-Proline is the main amino acid in citrus fruits, except grapefruit where aspartic acid predominates. Proline increases gradually with fruit ripening, and its concentration is also highly dependent on the origin of the fruit. For example, in the case of lemon juice, the fruits of South America show in some cases L-proline levels even lower than 100 mg/L. However, the L-proline levels in lemon juice from Mediterranean regions are on average greater than 350 mg/L and sometimes even reach 800 mg/L. In grapefruit juice of South American origin, L-proline levels are less than 200 mg/L, whereas they are much higher for juice of Israeli origin (10). In samples of bergamot that we analyzed, the

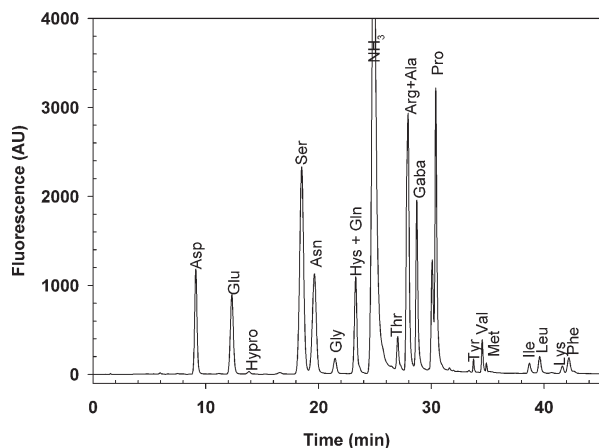


Figure 5. Typical RP-HPLC chromatogram with fluorescence detection of the free amino acid content in a sample of bergamot juice.

most interesting aspect concerns the high content of L-proline in seeds, with an average value of about 2200 mg/kg. From an analytical point of view, the L-proline determinations by HPLC-ESI-MS/MS were totally comparable to those obtained by HPLC with fluorescence detection (Table 2).

In conclusion, proline and its derived metabolites appear to be of great physiological importance in *Citrus* genus plants. The determination of their concentrations can be useful in the assessment of plant stress. Some proline derived compounds are detected and quantified with difficulty due to the lack of easily derivatizable groups. The method we developed for the analysis of proline derived osmolytes show several advantages in terms of rapidity and selectivity. No method was reported before for the analysis of proline derived compounds by isocratic chromatography which does not exceed 10 min. The procedure of sample preparation is particularly fast requiring only dilution and centrifugation. Moreover, the method can also be useful for the determination of those compounds in biochemical studies of proline metabolism in a wide range of organisms. As an example, *N,N*-dimethylproline was found in human urine, and a role in osmotic protection of human kidneys was invoked (30). The role in human nutrition of *N*-methyl-L-proline and 4-hydroxy-L-prolinebetaine, which are present in bergamot juice and in other citrus juices (unpublished results) at significant levels, deserves further studies.

Supporting Information Available: Main ion trap instrumental conditions for the analysis of proline derivatives by ESI-MS/MS in positive ionization mode. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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