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Betaines in Fruits of *Citrus* Genus Plants

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ABSTRACT: Numerous compounds, many of them osmolytes, were quantified in natural juices and in frozen concentrate juices from fruits of plants of the *Citrus* genus. L-Proline, *N*-methyl-L-proline (hygric acid), *N*,*N*-dimethyl-L-proline (stachydrine), 4-hydroxy-L-prolinebetaine (betonicine), 4-hydroxy-L-proline, γ-aminobutyric acid (Gaba), 3-carboxypropyltrimethylammonium (GabaBet), *N*-methylnicotinic acid (trigonelline), and choline in the fruit juices of yellow orange, blood orange, lemon, mandarin, bitter orange (*Citrus aurantium*), chinotto (*Citrus myrtifolia*), and grapefruit were analyzed by sensitive HPLC-ESI−tandem mass spectrometry procedure. It was found that the most represented osmolytes in the juices, that is, L-proline, stachydrine, and betonicine, can be quantified with minimal sample preparation and short analysis time (about 1 min) also by flow injection analysis (FIA) ESI-MS/MS with the same results as obtained by HPLC ESI-MS/MS. In all of the juices, discrete amounts of choline and trigonelline were present. Conversely, GabaBet was always below detection limits. Notably, *N*-methyl-L-proline and 4-hydroxy-L-prolinebetaine, which were discovered for the first time in the juice of bergamot (*Citrus bergamia* Risso et Poit), are also present in all of the citrus juices examined.

KEYWORDS: citrus fruits, *N*-methyl-L-proline, 4-hydroxy-L-prolinebetaine, γ-aminobutyric acid betaine, choline, trigonelline, betonicine, stachydrine

INTRODUCTION

Betaines are quaternary ammonium compounds, ubiquitous in the vegetal world, originating from amino and imino acids through specific biosynthetic pathways. Being compatible with cellular metabolism even at molar concentrations, they are generally referred to as compatible osmolytes.[1,2] These substances, like their amino or imino acid parents, tend to accumulate in the cytoplasm and intercellular fluids, where they exert protective functions for proteins, nucleic acids, and cell membranes[3,4] in response to abiotic stresses such as cold, freezing, presence of toxic metals, reduced availability of water, and/or "high salinity". Despite numerous studies on the resistance of plants to abiotic stress,[2,5,6] literature data on the *Citrus* genus are rather scarce.[7−9] More in detail, these studies have shown that unstressed citrus leaves accumulate high levels of free proline[7] and that leaves of several citrus species or hybrids in stress conditions accumulate *N*,*N*-dimethyl-L-proline (also known as stachydrine or proline betaine).[8] Nolte et al.[9] observed that betaines in plants of the *Citrus* genus were essentially those derived from proline. These authors reported the presence of stachydrine in leaves of the subfamily of Aurantioideae and other species of Rutaceae and that this osmoprotectant accumulates in the cytoplasm of leaf cells in stress conditions. Moreover, most of those plant species also accumulated discrete amounts of 4-hydroxy-L-prolinebetaine, also known as betonicine, which represented 3−15% of the stachydrine levels. As for juices, Rapp et al.[10] first revealed the presence of stachydrine in orange juice by a ^{13}C NMR approach. They found that stachydrine concentration in orange juice ranged between 240 and 700 mg/L, which makes it one of the major compounds in orange juice after sugars and organic acids. Successively, in an analytical study on choline-containing compounds and betaine in 145 common foods, the presence of glycine betaine at low levels (0.11−0.23 mg/100 g) in a sample of orange juice and in orange and grapefruit fruits was reported.[11] In another study on the content of betaines in 74 processed foods, the presence of stachydrine in orange juices and in a unique sample of mandarin fruit was shown.[12]

Recently, we determined the content of proline and some of its metabolites in bergamot (*Citrus bergamia* Risso et Poit) fruit, juice, and seeds with a HPLC ESI-MS/MS method, which allowed fast determination of those substances with minimum sample preparation and, importantly, for the first time we reported the presence of *N*-methylproline and 4-hydroxyprolinebetaine in the fruit juice from a plant of the *Citrus* genus.[13] Therefore, we considered it to be of interest to extend the investigation to more common and industrially important citrus species such as yellow and blood oranges, lemon, grapefruit, and mandarin. To this aim, citrus juices freshly prepared in the laboratory and juices of industrial origin were analyzed. Moreover, we optimized the analytical method to assess the possible presence of and quantify in the citrus juices some less represented betaines, such as Gaba-betaine and choline, deriving from amino acids other than proline, and trigonelline (Trg), deriving from nicotinic acid, which also acts as an osmolyte in higher plants and is a sensitive indicator of salt stress[14] (Figure 1).

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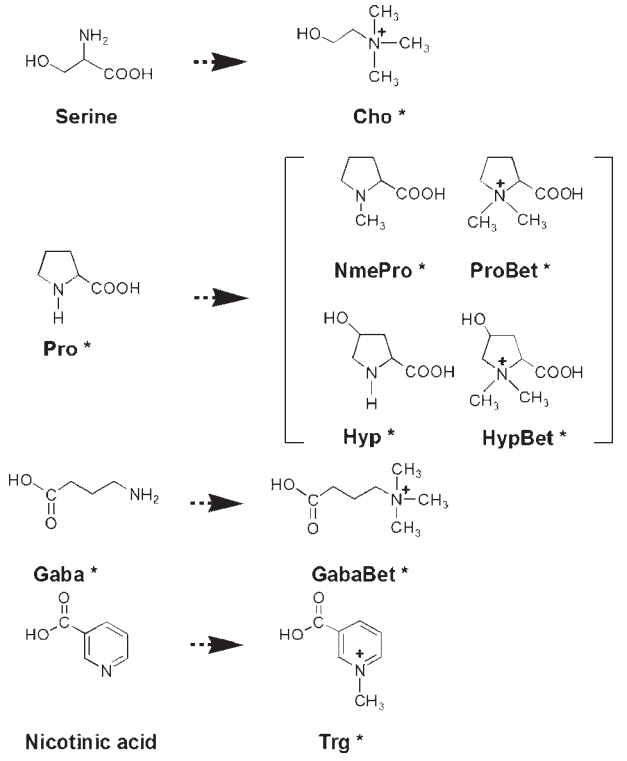

Figure 1. Chemical structures of substances deriving from serine, proline, γ-aminobutyric acid, and nicotinic acid. Asterisks indicate compounds analyzed in this study.

MATERIALS AND METHODS

Reagents. L-Proline (Pro), *N*-methyl-L-proline (NmePro), 4-hydroxy-L-proline (Hyp), γ-aminobutyric acid (Gaba), (3-carboxypropyl)trimethylammonium (GabaBet), choline (Cho), and *N*-methylnicotinic acid (trigonelline, Trg) were from Sigma-Aldrich (Milan, Italy). *N*,*N*-Dimethyl-L-proline (stachydrine, ProBet) and 4-hydroxy-L-prolinebetaine (betonicine, HypBet) 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.

Citrus Juice Samples. Forty samples of citrus juice (single-strength juices, frozen concentrated juices (FCJ), and juices prepared in the laboratory) were used: 33 juices were supplied by industries located in the Italian regions of Calabria and Sicily (southern Italy), and 7 were prepared in the laboratory.

Industrial Juices. The juices employed were three samples of frozen concentrated yellow orange juice (47 °Brix), four samples of yellow orange single-strength juice, six samples of frozen concentrated blood orange juice (54 °Brix), three samples of blood orange single-strength juice, three samples of lemon juice, six samples of frozen concentrated lemon juice (47.7 °Brix), three samples of mandarin juice, and five samples of frozen concentrated mandarin juice (63.5 °Brix).

The concentrated juices were treated, according to AIJN guidelines,[15] by diluting samples with Milli-Q water to a level of soluble solids (expressed as °Brix) equal to the corresponding natural juice, that is, 8 °Brix for reconstituted lemon juice, 11.2 °Brix for reconstituted orange juice, and 10.0 °Brix for reconstituted mandarin juice. After dilution, the juices were centrifuged and stored as described for the manually prepared juices.

Preparation of the Samples for HPLC-ESI-MS/MS Analyses. The determinations of analytes in the samples were performed by HPLC-ESI MS/MS in two different ways: (1) without any further sample preparation except dilution of the centrifuged juice with 0.1% formic acid in water in the ratios (v/v) 1:10, 1:25. or 1:50; (2) by subjecting the centrifuged samples to a passage on a column (5 × 1 cm) of Bio-Rad AG 50WX8-(H^+) resin. In brief, the column, after the load of 1 mL of centrifuged juice, 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 solution was dried in a rotavapor and reconstituted with 1 mL of 0.1% formic acid in water.

Laboratory Juices. The juices employed were three samples of chinotto (*Citrus myrtifolia*) juice, two samples of bitter orange (*Citrus aurantium*) juice, and two samples of grapefruit juice. The juices were prepared using a manual squeezer, filtered through a stainless steel filter with 1.18 mm mesh diameter, centrifuged at 18000*g* for 60 min at 4 °C, placed in 100 mL aliquots in plastic bags, and stored at −20 °C until used.

HPLC-ESI-MS/MS and Flow Injection Analysis (FIA)-ESI-MS/MS Analyses. The optimization of the instrumental parameters for Gaba, GabaBet, Cho, and Trg was performed by continuous infusion of the standard solution of each analyte in 0.1% formic acid. The mass cutoff and the fragmentation amplitude were optimized to obtain for each analyte the most efficient MS/MS transition from the positively charged precursor ion $[M + H]^+$ to the fragment ions. Successively, the substances were analyzed by HPLC-MS/MS as described for the dosage of proline derivatives.[13] Briefly, chromatographic separations were performed with a Supelco Discovery-C8 column, 150 × 3.0 mm, and particle size = 5 μm, at flow rate of 100 μL/min. The chromatography was conducted isocratically with 0.1% formic acid in water. Volumes of 20 μL of standard solution or sample were injected. The HPLC analyses were performed on an Agilent 1100 series liquid chromatograph equipped with an online degasser and an automatic injector. The ESI-MS/MS analyses were performed, both for FIA and 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 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). In Table 1, the monitored transitions and the instrument settings are reported along with the instrumental response expressed as the peak area of the monitored ionic fragment per nanogram of analyte. The quantification was achieved by comparison with the calibration curves obtained with standard solutions. The retention time (in minutes) and peak areas of the monitored fragment ions were determined by the Agilent software Chemstation, version 4.2.

Preparations of Standards. Standard stock solutions of ProBet, HypBet, Pro, NmePro, Hyp, Gaba, GabaBet, Cho, and Trg were prepared at 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 of the analytes.

HPLC Analysis of Amino Acids. Quantification of Pro, Hyp, Gaba, and the other free amino acids present in the samples was performed by reverse phase (RP) HPLC employing a Waters instrument model 2690 equipped with fluorescence detector model 474. The amino acids were derivatized with Waters AccQ FLuor reagent according to the method of van Wandelen and Cohen.[16] Quantification was made using the peak area of the fluorescence emission intensity by excitation at

Table 1. Main Ion Trap Instrumental Conditions, MS/MS Transitions Monitored, Instrumental Response for the Analyzed Compounds, and Retention Times of the Compounds Analyzed by RP Chromatography on a C8 Column

		ion trap conditions			
compound	MS/MS transition monitored (m/z)	cutoff (m/z)	amplitude (V)	(peak area)/ng	retention time (min)
Gaba	104 → 87	40	0.90	5750	7.8
GabaBet	146 → 87	50	1.00	400700	7.0
Cho	104 → 60	40	1.00	203100	8.0
Trg	138 → 94	70	1.30	65600	9.2

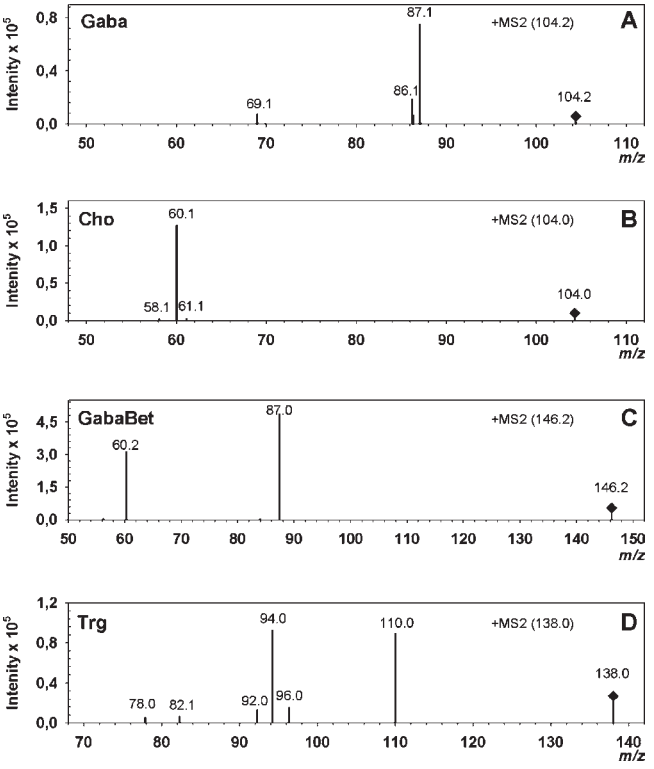

Figure 2. Fragmentation patterns of (A) Gaba, (B) Cho, (C) GabaBet, and (D) Trg.

350 nm and fluorescence emission recording 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.

RESULTS AND DISCUSSION

Instrumental Conditions and Quantification of Gaba, GabaBet, Cho, and Trg by LC-ESI MS/MS. The optimization of the MS/MS parameters for Gaba, GabaBet, Cho, and Trg was performed as described under Materials and Methods. In Table 1, the monitored transitions and the instrument settings are reported along with the instrumental responses expressed as the peak area of the monitored ionic fragment per nanogram of analyte. The retention times were obtained from chromatographic analyses performed with a Supelco Discovery-C8 column in isocratic conditions using 0.1% formic acid in water as eluent at flow rate of 100 μL/min, by injecting 5 μL of the standard solution of each analyte (Table 1). The data were obtained by selected reaction monitoring (SRM) in positive ion mode using standard solutions of each analyte at various concentrations, as described under Materials and Methods. It is evident that the relative intensities at the optimized instrumental settings are very different among the various substances (Table 1). When the intensity of the most abundant fragments is recorded, GabaBet is that showing the highest response, followed by Cho, Trg, and Gaba. The quantification of Gaba and Cho in citrus juices deserves some attention. In fact, these compounds are isobars (the m/z of the precursor ions are 104 for both), and both substances are naturally present in citrus juices.[11,12] Moreover, Gaba represents a potential heavy interference in the analysis of choline as it is generally present at much higher concentration. Gaba is fairly represented in all citrus juices[15] with a range of variability between 60 and 570 mg/L, whereas the average content of Cho is significantly lower.[11,12] Furthermore, these two compounds, in our chromatographic conditions, were not sufficiently separated to ensure an effective chromatographic separation (Table 1). Fortunately, Gaba and Cho show different fragmentation patterns (Figure 2A,B), which allowed a reliablly selective determination of both. In our instrumental settings the main Gaba fragment ion is at m/z 87 and other minor fragments are at m/z 86 and 69. Importantly, no fragment at m/z 60 was observed. In contrast, the Cho fragmentation pattern (Figure 2B) shows an intense specific transition at m/z 60 and no fragment ion at m/z 87. On this basis, Gaba was determined by monitoring the MS/MS transition 104 → 87 in accordance with that reported by Piraud et al.,[17] and choline was monitored through the MS/MS transition 104 → 60.

As for GabaBet analysis, the experiments on standard solutions showed a pattern of fragmentation (Figure 2C) consisting mainly of two fragments at m/z 87 and 60. For the quantitative measurement of the compound, we used the MS/MS transition 146 → 87, which was somewhat more sensitive than the other one.

Finally, as for Trg, the compound showed a fragmentation pattern characterized mainly by two ions at m/z 94 and 110 and few other minor fragments (Figure 2D). For the quantitative measurement, we observed, in agreement with that reported by Lang et al.,[18] a high specificity by monitoring the transition 138 → 94, which is also characterized by high instrumental response (Table 1).

Betaine in Citrus Juices. *GabaBet.* The determination of GabaBet was performed on samples obtained by subjecting the centrifuged juice to a passage on a column (5 × 1 cm) of Bio-Rad AG 50WX8-(H^+) resin. Then the dried eluate, which contains the amino acids and betaines present in the juice,[13] was reconstituted with a volume of 0.1% formic acid in water corresponding to initial sample volume. This procedure was necessary because of the low expected concentrations of the compound in citrus juices. For this reason, the analyses were performed on undiluted juices, treated as described with the aim to exclude most of interfering substances through the purification step on the AG 50WX8- (H^+)

Table 2. Citrus Juice Content (Milligrams per Liter) of Gaba, Gaba-Betaine, Choline, Trigonelline, and Proline Derivatives Determined by HPLC-ESI-MS/MS

		industrial juices								laboratory juices		
compd	statistical parameter	FCJ orange[a]	orange juice[b]	FCJ blood orange[a]	blood orange juice[c]	FCJ lemon[d]	lemon juice[e]	FCJ mandarin[f]	mandarin juice[g]	chinotto juice[h]	grapefruit juice[i]	orange juice[j] (bitter)
Gaba	min−max	150−168	130−151	154−181	270−300	55−70	81−96	102−110	150−161	120−167	134−136	131−133
	mean ± SD	159 ± 9	139 ± 9	165 ± 9	285 ± 15	63 ± 5	87 ± 7	107 ± 4	155 ± 6	136 ± 21	135 ± 1	132 ± 2
GabaBet	min−max	nd[k]	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
	mean ± SD											
Cho	min−max	11−14	10−14	8−10	16−18	6−10	7.5−9.0	11−15	17−18	15−18	7−8	7.0−7.5
	mean ± SD	12 ± 2	12 ± 1.5	9.4 ± 0.4	17.1 ± 0.9	8 ± 2	8.3 ± 0.7	13.3 ± 1.6	17.5 ± 0.5	16 ± 2	7.9 ± 0.3	7.3 ± 0.2
Trg	min−max	6.5−8.0	8−10	3.0−4.5	4.5−5.5	6.0−7.2	9.0−10.2	11−14	7.0−9.2	4.2−5.4	1.0−2.4	5.2−6.3
	mean ± SD	7.6 ± 0.6	8.5 ± 1.0	3.8 ± 0.5	5.0 ± 0.4	6.8 ± 0.5	9.9 ± 0.4	12.6 ± 1.2	7.9 ± 1.1	4.8 ± 0.6	1.7 ± 1.0	5.8 ± 0.8
Pro	min−max	500−530	450−499	550−622	870−940	410−450	373−408	167−188	3−282	317−330	249−256	671−680
	mean ± SD	517 ± 15	467 ± 19	580 ± 25	597 ± 38	438 ± 15	395 ± 15	177 ± 10	279 ± 4	323 ± 6	252 ± 5	675 ± 6
Hyp	min-max	2.4−3.4	3.4−4.2	2.1−3.8	3.6−4.4	2.5−3.2	2.5−4.0	2.6−3.6	3.4−4.6	2.5−4.2	3.1−4.2	2.1−3.6
	mean ± SD	3.1 ± 0.7	3.9 ± 0.2	3.4 ± 0.3	4.0 ± 0.6	2.9 ± 0.4	3.6 ± 0.5	3.4 ± 0.4	3.8 ± 0.8	3.1 ± 0.6	3.6 ± 0.4	3.0 ± 0.3
NmePro	min−max	47−52	50−58	42−57	75−84	28−38	32−35	35−42	31−40	89−95	15−20	55−70
	mean ± SD	49 ± 3	54 ± 3	49 ± 6	79 ± 4	32 ± 4	34 ± 2	40 ± 2	33 ± 3	91 ± 3	16 ± 3	63 ± 3
ProBet	min−max	430−490	412−521	410−450	530−570	320−380	320−344	370−382	330−350	440−465	240−260	414−425
	mean ± SD	463 ± 30	486 ± 50	423 ± 15	548 ± 20	350 ± 25	332 ± 12	377 ± 6	342 ± 4	455 ± 13	246 ± 3	418 ± 6
HypBet	min−max	120−170	210−244	130−140	226−240	44−50	40−50	190−220	270−280	188−210	50−64	109−122
	mean ± SD	137 ± 28	228 ± 15	135 ± 4	235 ± 8	47 ± 2	47 ± 3	204 ± 11	274 ± 4	199 ± 11	57 ± 10	115 ± 5

[a] Frozen concentrated juice reconstituted to 11.2 °Brix. [b] Natural juice with soluble solid level ranging between 10.5 and 11.3 °Brix. [c] Natural juice with soluble solid level ranging between 12.4 and 13.9 °Brix. [d] Frozen concentrated juice reconstituted to 8.0 °Brix. [e] Natural juice with soluble solid level ranging between 7.6 and 7.9 °Brix. [f] Frozen concentrated juice reconstituted to 10.0 °Brix. [g] Hand-squeezed juice with soluble solid level ranging between 8.1 and 10.5 °Brix. [h] Hand-squeezed juice with soluble solid level ranging between 9.0 and 11.5 °Brix. [i] Hand-squeezed juice with soluble solid level of 10.5 and 10.9 °Brix. [j] Hand-squeezed juice with soluble solid level of 10.0 and 10.5 °Brix. [k] Not detected.

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resin. However, GabaBet was never detected in the juices examined (the detection limit in our conditions was 0.025 mg/L). In Table 2, which summarizes the analyses on citrus juices, the values of GabaBet concentration are reported as not detectable (nd).

Gaba. This amino acid is present in citrus fruits at fair levels;[15] therefore, the analyses were conducted on juices diluted 10 times. The data obtained by HPLC-ESI-MS/MS (Table 2) are fully comparable with those obtained by HPLC with fluorescence detection (data not shown). In agreement with literature data,[15] Gaba was present in noticeable concentrations in all citrus juices examined, and yet it does not seem to function as a precursor of GabaBet, which, as seen before, was not detected in any juice. Crawford et al.[19] hypothesized that Gaba accumulation in vegetal matrices may represent the product of a regulation mechanism aimed to control pH in cytoplasm. In fact, glutamate conversion into Gaba and CO_2 by the glutamate decarboxylase action entails the pH increase due to proton consumption in the course of the reaction. In addition, it was also shown that cytoplasm acidification induced Gaba accumulation.[19]

Choline. As for Gaba, also the HPLC-ESI-MS/MS analyses of Cho were conducted on juices diluted 10 times. The highest free Cho content was found for mandarin and chinotto juices, with values in the range of 11−18 mg/L, followed by blood orange (8−18 mg/L), yellow orange (10−14 mg/L), and, finally, lemon, grapefruit, and bitter orange juices with values between 6.0 and 10.0 mg/L (Table 2). These data confirm the low content of free Cho in the citrus juices. However, the values reported by Zeisel et al.[11] for samples of orange and grapefruit juices, which were analyzed according to the Koc method,[20] are substantially lower than those detected in this study. The values of free Cho content we found for grapefruit juice were about 2 times higher and those of yellow orange juice about 3 times higher. This could be ascribed to two opposite factors. First, the method used in ref 11 for sample preparation entails many purification steps of the sample (such as extraction with solvent mixtures, phase separation, and removal of the protein fraction present in the samples), which could result in a lower recovery. The second factor could be ascribed, in the case of our samples of industrial origin, to the action of phospholipases, normally present in vegetal matrices, which, by hydrolyzing choline phospholipids, may increase over time the free choline content. In fact, some steps of the industrial juice production technology, such as the initial juice extraction and the subsequent finishing operations, are the slowest stages of the whole industrial preparation process of these products. Indeed, only after these operations are juices are heat-treated to inactivate the enzymes and microbial components. Therefore, the higher Cho levels we found in those two citrus juices of industrial origin could be due to this reason.

Trigonelline. The literature data on Trg content in citrus products are rather limited. The only data we found were from Slow et al.,[12] who reported the presence of Trg in mandarin fruit and in orange juice at levels lower than 20 μg/g. Analytically, it is possible to reliably quantify Trg with RP-HPLC-MS/MS by diluting the juice 10 times and monitoring the transition 138 → 94, which is highly specific and intense. Results show that Trg is present in natural mandarin juice at a mean level of 7.9 mg/L and in the reconstituted mandarin juice at 10 °Brix at mean level of 12.6 mg/L (Table 2). The mean Trg content for natural lemon juice was close to 10 mg/L, which was higher than that of reconstituted lemon juice at 8 °Brix (6.8 mg/L). The mean Trg content of the natural yellow orange juice was 8.5 mg/L and 7.6 mg/L for the juice reconstituted to 11.2 °Brix. Trig levels were

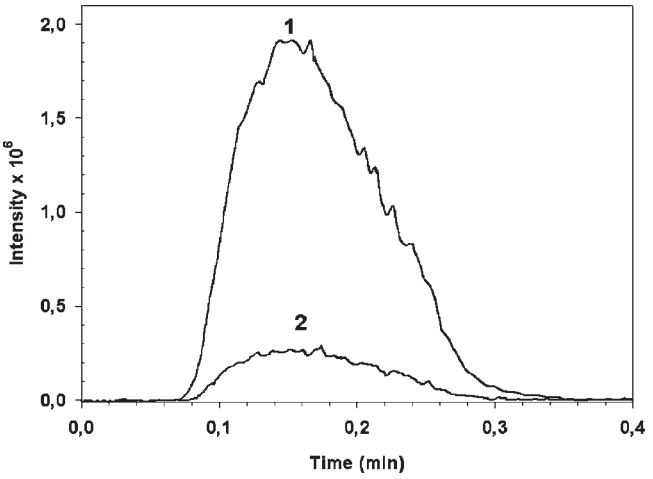

Figure 3. Typical FIA of the ProBet content in a sample of lemon juice. The juice was diluted 50 times with 0.1% formic acid, and 5 μL was injected at an eluent flow rate of 100 μL/min. Curves: 1, total ion chromatogram; 2, EIC at the MS/MS transition 144 → 84.

lower in all other citrus juices with mean values of <6.0 mg/L. The lowest content was found for grapefruit juice, with a mean value below 2.0 mg/L.

Proline Derivatives. The distribution of proline derivatives determined by HPLC-ESI-MS/MS in the *Citrus* species was also examined (Table 2). Pro, ProBet, and HypBet, as we already observed for the bergamot[13] on both the juice and other parts of the fruit tissues (albedo and seeds), are the osmolytes mainly expressed in citrus juices and, therefore, appear to be genus-specific. Similar results were previously obtained by Nolte et al.[9] on leaves of plants of the subfamily of Aurantioideae and other species of Rutaceae. Without considerations about the physiological role of proline and its methylated derivatives, which remains unclear despite numerous studies on abiotic stress resistance,[21,22] it appears, at least in terms of biochemical composition (Table 2), that the methylation of part of the free proline pool constitutes the main strategy adopted by all *Citrus* species to counteract abiotic stress.[9,13] This mechanism is expressed in a generalized way in each part of the plant tissues.

In terms of composition, results show that the highest ProBet contents are found in the juices of the red and yellow oranges, with average values of 548 and 486 mg/L, respectively, followed by chinotto juice (average value of 455 mg/L), bitter orange juice (average value of 418 mg/L), mandarin juice (average value of 342 mg/L), lemon juice (average value of 332 mg/L), and finally grapefruit juice, with the lowest content (average value of 246 mg/L) (Table 2).

The data on HypBet are also similar in terms of abundance (Table 2). This substance, except for the bergamot juice,[13] had never been identified before in other citrus juices. Results showed that HypBet is more expressed in the juices of yellow orange, blood orange, and mandarin, with substantially similar average values of about 240 mg/L, followed by chinotto and bitter orange juices (average values of 199 and 115 mg/L, respectively) (Table 2) and, finally, by lemon and grapefruit juices with average values of 47 and 57 mg/L, respectively.

The data for Pro, ProBet, and HypBet contents were obtained by HPLC-ESI-MS/MS as described under Materials and Methods on samples of juices diluted 25 or 50 times with 0.1% formic

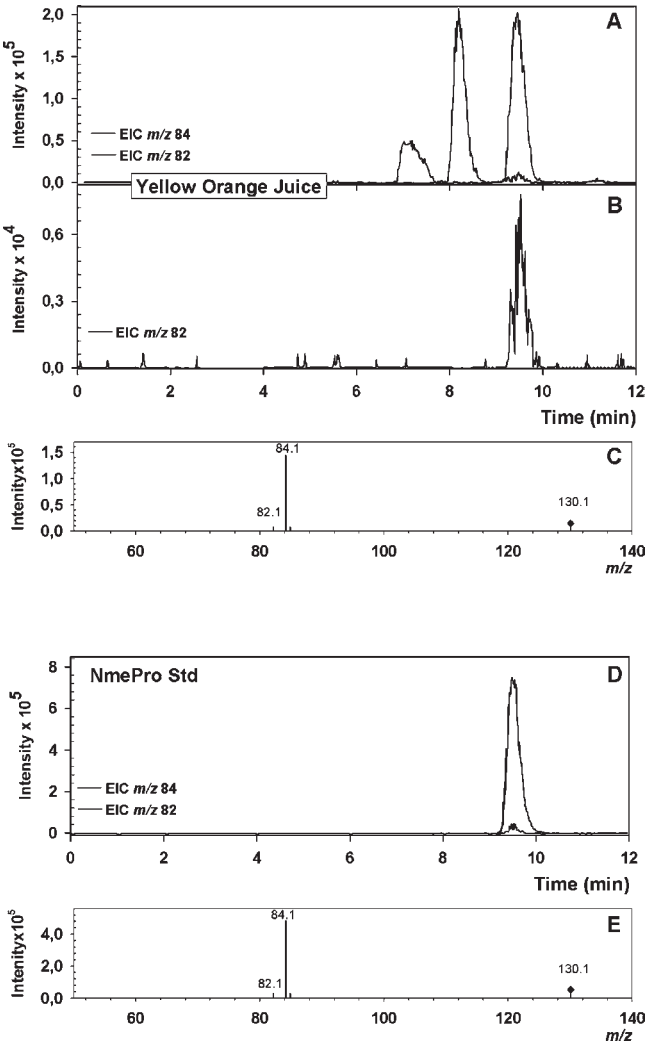

Figure 4. (A) EICs of yellow orange juice (EIC at MS/MS transition 130 → 84 (thin line), EIC at MS/MS transition 130 → 82 (thick line)). (B) Amplified scale of EIC of yellow orange juice at MS/MS transition 130 → 82. (C) Fragmentation pattern of the peak at retention time 9.2 min. (D) NmePro standard solution (EIC for MS/MS transition 130 → 84 (thin line), EIC for MS/MS transition 130 → 82 (thick line)). (E) Fragmentation pattern of the peak at retention time 9.2 min of NmePro standard solution chromatogram.

acid in water (Table 2). However, we observed that FIA ESI-MS/MS, which avoids the chromatographic separation, could achieve the quantitative determination of these osmolytes in a rapid and accurate way also. The results by FIA differed by <7% (data not shown) from those obtained by HPLC ESI-MS/MS. As we pointed out previously,[13] the high concentrations in the juice and instrumental response for these substances, expressed as peak area per nanogram of injected analyte, allow high dilution of the samples, thus minimizing matrix effects. Proline had the highest instrumental response followed by ProBet and HypBet. Actually, the determination of these substances can be rapidly and reliably accomplished by FIA in analysis time of <1 min. As an example, Figure 3 reports a typical FIA of ProBet in a sample of lemon juice diluted 50 times. The possibility to easily analyze these compatible solutes may be of great interest, especially considering that the current strategies aimed to obtain salt- and drought-tolerant crops by genetically engineering biosynthesis of proline and its derivatives need the dosage of these osmolytes in a specific and rapid manner. Instead, in almost all studies concerning the biochemical and physiological role in transgenic plants of the overaccumulation of osmolytes in response to abiotic stress, the dosage of proline is still conducted with obsolete and poor specific methods.[23] Furthermore, the dosages of the other compounds from proline metabolism (Hyp, NmePro, ProBet, and HypBet)[24,25] are rarely accomplished due to the analytical complexity.

However, FIA cannot be applied to Hyp and NmePro determinations. These substances, in fact, are present in the juices at lower concentrations; furthermore, due to interferences represented by some amino acids generally present in the vegetal matrices (Leu, Ileu, Lys, Glu, Gln, and PyroGlu), one cannot use the most sensitive transitions (132 → 86 for Hyp and 130 → 84 for NmePro), which would allow high sample dilution but are in common with the interfering substances.[17,26] For these reasons, the use of the more specific, although much less sensitive, transitions (130 → 82 for NmePro and 132 → 68 for Hyp) is mandatory and, consequently, chromatographic separation on less diluted juice is required less diluted juice is required.

As for Hyp, which is normally present at low concentration in citrus juices,[13,27] all analytical tests were carried out on centrifuged samples subjected to passage on a Bio-Rad AG 50WX8-(H+) column and reconstitution of the dried eluate with volumes of 0.1% formic acid in water equal to the initial volume of the sample. The chromatographic analysis was performed by monitoring the fragment ion at m/z 68.[17,26] The results (Table 2) were substantially in good agreement with those obtained when the free amino acid content in citrus juices was analyzed by RP-HPLC with fluorescence detection (data not shown).

Another significant result concerns the presence, never reported before, of NmePro in all citrus juices examined. To have safer determinations of this substance in the juices, quantifications were conducted by monitoring the MS/MS transition 130 → 82, which is much less intense than the MS/MS transition 130 → 84 but highly specific for NmePro and sensitive enough to quantify the analyte concentrations in our samples.[13] The analyses were performed without subjecting samples to the purification step on AG 50WX8-(H^+) resin, but simply using the juices diluted 10 times with 0.1% formic acid in water before chromatographic separation. The presence of NmePro in all citrus juices analyzed was confirmed on the basis of comparison of retention time and fragmentation pattern with the authentic standard. As an example, in Figure 4, the EICs for yellow orange juice and NmePro standard solution are given. It is worth noting the similarity of the reported EIC of the yellow orange sample with that previously reported for bergamot juice.[13] Results show that the juice extracted from the fruit of chinotto is that with the highest NmePro content (mean values close to 91 mg/L), followed by the juices from blood orange, bitter orange, and yellow orange (mean values between 49 and 79 mg/L), Table 2. Lower contents were found in lemon and mandarin juices with average values ranging between 32 and 40 mg/L and, finally, in grapefruit juice, which is characterized by the lowest content, with an average value of 16 mg/L. The presence at not negligible concentrations of NmePro in all citrus juices appears to support the hypothesis that this substance may represent a precursor of ProBet through the biosynthetic pathway of the enzymatic proline N-methylation via the intermediate *N*-methylproline, as proposed by Rhodes et al.,[2,28] although the enzymes involved in this pathway have never been characterized so far.

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ABBREVIATIONS USED

Pro, L-proline; NmePro, *N*-methyl-L-proline (hygric acid); ProBet, *N*,*N*-dimethyl-L-proline (stachydrine); HypBet, 4-hydroxy-L-prolinebetaine (betonicine); Hyp, 4-hydroxy-L-proline; Trig, *N*-methylnicotinic acid (trigonelline); Cho, 2-hydroxyethyltrimethylammonium (choline); GabaBet, 3-carboxypropyltrimethylammonium (γ-aminobutyric acid betaine); Gaba, γ-aminobutyric acid.

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