Citrus Genus Plants Contain N-Methylated Tryptamine Derivatives and Their 5-Hydroxylated Forms

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ABSTRACT: The occurrence and distribution in *Citrus* genus plants of N-methylated derivatives of tryptamine and their 5-hydroxylated forms are reported. Tryptamine, *N*-methyltryptamine, *N,N*-dimethyltryptamine, *N,N,N*-trimethyltryptamine, 5-hydroxytryptamine (serotonin), 5-hydroxy-*N*-methyltryptamine, 5-hydroxy-*N,N*-dimethyltryptamine (bufotenine), and 5-hydroxy-*N,N,N*-trimethyltryptamine (bufotenidine) were quantitated by LC-ESI-MS/MS. Leaves of all citrus plants examined contained *N,N,N*-trimethyltryptamine, a compound that we first discovered in the bergamot plant. Interestingly, we also found out that all plants examined contained 5-hydroxy-*N,N*-dimethyltryptamine and 5-hydroxy-*N,N,N*-trimethyltryptamine, compounds never described so far in the *Citrus* genus. As *N,N,N*-trimethyltryptamine and 5-hydroxy-*N,N,N*-trimethyltryptamine possess nicotine-like activity by exerting their action on acetylcholine receptors, it is conceivable that both represent the arrival point of a biosynthetic pathway aimed to provide *Citrus* plants with chemical defense against aggressors. This hypothesis is supported by our finding that leaves and seeds, which are more frequently attacked by biotic agents, are the parts of the plant where the highest levels of those compounds were found.

KEYWORDS: *5-hydroxy-N,N,N-trimethyltryptamine, bufotenine, psilocin, tryptamine derivatives, hydroxylated tryptamine derivatives, citrus plants, biotic stress*

■ INTRODUCTION

Recently, we reported the occurrence in the bergamot[1] plant (*Citrus bergamia* Risso et Poit) of secondary metabolites derived from tryptamine, a substance with well-known repelling activity on insect herbivory,[2] which is biosynthesized starting from tryptophan by the action of the enzyme tryptophan decarboxylase.[3,4] As bergamot is an agriculturally important plant, the defense mechanisms against phytopathogenic organisms has attracted considerable attention especially regarding the economic impact related to the industrial production of juice and essential oil.[5−7] Our results[1] showed not only the presence of partially methylated tryptamine derivatives, such as *N*-methyltryptamine and *N,N*-dimethyltryptamine, which were distributed in all parts of the fruit (peel and edible part) and with higher levels in leaves and seeds, but also the occurrence of the trimethylated form of tryptamine, that is, *N,N,N*-trimethyltryptamine, a metabolite never found before in plants. The occurrence of all the N-methylated tryptamine derivatives in the bergamot plant led us to hypothesize the involvement of tryptophan decarboxylase in a new metabolic pathway different from the one widely studied, which leads to the formation of monoterpene indole alkaloids in plants.[8]

In the hypothesized new pathway, the formation of *N,N,N*-trimethyltryptamine from tryptamine, as a consequence of successive methylation reactions, catalyzed by the same or more *N*-methyltranferase(s), could represent the ultimate purpose of a plant defense mechanism against herbivory, as its accumulation mainly occurs in leaves. This possibility finds support in a pharmacological study[9] conducted on sections of frog and guinea pig intestines showing that *N,N,N*-trimethyltryptamine is a stimulant of parasympathetic ganglia and possesses nicotine-like activity by exerting its action on acetylcholine receptors. On the basis of those previous results and, above all, considering that the same pharmacological studies[9] reported that 5-hydroxy-*N,N,N*-trimethyltryptamine, also called bufotenidine or cinobufotenine, was an even more potent cholinergic substance (about 10-fold) than *N,N,N*-trimethyltryptamine, we sought to verify not only in bergamot but also in many other economically important plants of the *Citrus* genus plants, that is, orange, lemon, mandarin, chinotto (*Citrus myrtifolia*), and citron, the presence and the distribution in the various tissue parts of both N-methylated and 5-hydroxy-N-methylated derivatives of tryptamine. The chemical structures of the compounds discussed herein are shown in Table 1.

Table 1. Structural Representations of Tryptophan, N-Methylated Tryptamine Derivatives, and Their 5-Hydroxylated Forms Analyzed in This Study

Structural Rapresentation of Tryptamine Derivatives

Compound	R_1	R_2	R_3	R_4	R_5	R_α	Name
1	H	H	H	H	H	-COOH	tryptophan
2	H	H	H	H	-OH	-COOH	5-hydroxytryptophan
3	H	H	H	H	H	H	tryptamine
4	-CH₃	H	H	H	H	H	N-methyltryptamine
5	-CH₃	-CH₃	H	H	H	H	N,N-dimethyltryptamine
6	-CH₃	-CH₃	-CH₃	H	H	H	N,N,N-trimethyltryptamine
7	H	H	H	H	-OH	H	5-hydroxytryptamine (serotonin)
8	-CH₃	H	H	H	-OH	H	5-hydroxy-N-methyltryptamine
9	-CH₃	-CH₃	H	H	-OH	H	5-hydroxy-N,N-dimethyltryptamine (bufotenine)
10	-CH₃	-CH₃	-CH₃	H	-OH	H	5-hydroxy-N,N,N-trimethyltryptamine (bufotenidine)
11	-CH₃	-CH₃	H	-OH	H	H	4-hydroxy-N,N-dimethyltryptamine (psilocin)
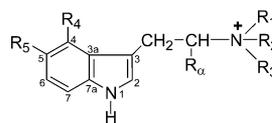
■ MATERIALS AND METHODS

Reagents. Tryptophan, tryptamine, N-methyltryptamine, 5-hydroxytryptophan, 5-hydroxytryptamine (serotonin), methyl iodide, and 0.1% solution of formic acid in water used for LC-ESI-MS analyses were obtained from Sigma-Aldrich (Milan, Italy). N,N-Dimethyltryptamine, 4-hydroxy-N,N-dimethyltryptamine (psilocin), and 5-hydroxy-N,N-dimethyltryptamine (bufotenine) were from LGC Standards (Milan, Italy). SPE-C18 columns for flash chromatography (particle size 33 μm) were obtained from Phenomenex (Anzola Emilia,Italy). All other solvents and reagents were of analytical grade.

Plant Materials. Fruits and leaves of bergamot were harvested in October and December, 2011 in the "Pellaro" area near Reggio Calabria (Italy). Leaves of lemon, orange, mandarin, chinotto, and citron were harvested in December, 2011 in the SSEA Arboretum of Reggio Calabria.

Bergamot Peel Extracts. 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 seed were separated manually. The peel (flavedo and albedo) was homogenized in a mixer with water in the 1:1 (w/w) ratio. The homogenate was allowed to stand for 2 h under constant agitation and then centrifuged at 18000g for 30 min at 4 °C. The supernatant was finally frozen at −20 °C until used for the successive determinations.

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

Bergamot 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 was 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 at −20 °C until used for the successive determinations.

Bergamot and Other Citrus Plant 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 0.1% formic acid in Milli Q grade water and then kept under stirring for 3 h. The homogenate was finally centrifuged at 18000g for 30 min, and the supernatant was stored in 20 mL vials at −20 °C.

Synthesis and Purification of 5-Hydroxy-N,N,N-trimethyltryptamine. For the conversion of 5-hydroxytryptamine into its quaternary ammonium compound, that is, 5-hydroxy-N,N,N-trimethyltryptamine, a modified procedure proposed by Chen and Benoiton[10,11] was used, which is based on a heterogeneous phase reaction employing methyl iodide as methylating agent in the presence of KHCO₃. Briefly, 200 mg of serotonin was dissolved in 20 mL of methanol with 1 g of KHCO₃, then 10 mL of methyl iodide (CH₃I) was added. The mixture was stirred for 12 h at room temperature. The addition of methyl iodide (10 mL) and KHCO₃ (1 g) was repeated twice more. Finally, the mixture was centrifuged and the supernatant collected and evaporated to dryness at 40 °C in a rotary evaporator. The residue, containing the 5-hydroxy-N,N,N-trimethyltryptamine, was dissolved in 10 mL of Milli Q grade water and purified by flash chromatography on a SepPac C₁₈ cartridge (Phenomenex, Anzola Emilia, Italy). The sample-loaded column was washed with 100 mL of Milli Q water and then the retained 5-hydroxy-N,N,N-trimethyltryptamine was eluted with a 50 mL solution of H₂O/acetonitrile (80:20). The eluate was evaporated to dryness under a stream of air and dried overnight under vacuum in the presence of P₂O₅. The yield was 55%.

Standard and Sample Preparations. The standard stock solutions of tryptophan (**1**), 5-hydroxytryptophan (**2**), tryptamine (**3**), N-methyltryptamine (**4**), N,N-dimethyltryptamine (**5**), N,N,N-trimethyltryptamine (**6**), 5-hydroxytryptamine (**7**), 5-hydroxy-N-methyltryptamine (**8**), 5-hydroxy-N,N-dimethyltryptamine (**9**), 5-hydroxy-N,N,N-trimethyltryptamine (**10**), and 4-hydroxy-N,N-dimethyltryptamine (**11**) were prepared at a concentration of 2000 ng/mL and stored at 4 °C. Prior to injection, stock solutions were appropriately diluted with water containing 0.1% formic acid before being used as working solutions.

Instrumental Conditions for HPLC-ESI-MS/MS Analyses. The optimization of the instrumental parameters for the analyses of the tryptamine and 5-hydroxytryptamine derivatives was performed by continuous infusion of 5 μM standard solution in 0.1% formic acid. The mass cutoff and the fragmentation amplitude were optimized in order to obtain the most efficient MS/MS transitions from the positively charged precursor ion [M+H⁺] to the fragment ions. The transitions utilized for quantification were 205.1→188 for tryptophan, 161.1→144 for tryptamine, 175.1→144 for N-methyltryptamine, 189.1→144 for N,N-dimethyltryptamine, 203.2→144 for N,N,N-trimethyltryptamine, 221.2→204 for 5-hydroxytryptophan, 177.1→160 for 5-hydroxytryptamine, 191.1→160 for 5-hydroxy-N-methyltryptamine, 205.1→160 for 5-hydroxy-N,N-dimethyltryptamine,

$205.1 \rightarrow 160$ for 4-hydroxy-*N,N*-dimethyltryptamine, and $219.1 \rightarrow 160$ for 5-hydroxy-*N,N,N*-trimethyltryptamine.

The substances were analyzed by HPLC-ESI/MS/MS, as described by Servillo et al.[1,12] for analysis of betaines and methylated tryptamine derivatives. 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 a flow rate of 100 μL/min. Volumes of 10–20 μL of standard solutions or samples were injected.

The HPLC-ESI-MS/MS analyses were performed with an HPLC Agilent 1100 series equipped with an on line degasser and automatic injector coupled online with an Agilent LC-MSD SL quadrupole ion trap. The MS acquisition was performed by using ESI in positive ion mode, with nitrogen as the nebulizing and drying gas under the following conditions: 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 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 the Agilent software Chemstation, version 4.2.

■ RESULTS AND DISCUSSION

Identification of Bufotenine in Citrus Plants. In our previous work,[1] aimed to verify the occurrence of tryptophan derived metabolites in the bergamot plant, we observed in the MS full scan chromatogram of leaf extracts the presence of two well resolved peaks both corresponding to m/z 205.1. The more retained peak, emerging at 14.2 min, was tryptophan, whereas the less retained one, emerging at 12.1 min, was not identified. We have herein focused our attention on this latter peak, performing the chromatographic analysis of bergamot leaf extracts by isolating at m/z 205.1 and subjecting ions to MS[2] fragmentation (Figure 1A and B). The fragmentation pattern of the peak at retention time 12.1 min is dominated by the fragment at m/z 160, which might indicate the presence of hydroxyvinylindole in the molecular structure of the unknown compound. This indication was confirmed by successive MS[3] analyses showing that the fragmentation of the MS[2] ions, isolated at m/z 160, gives a pattern dominated by a fragment at m/z 132 (Figure 1C). This fragment has been proposed to be produced from the hydroxyvinylindole ion by a neutral loss of CO from the benzene ring. The consequent benzene ring contraction generates two fused five-membered rings with the positive charge on the nitrogen atom of the pyrrole ring[13] (Figure 1C).

The same type of determination conducted on the leaf extracts of all *Citrus* genus plants examined showed the presence of the peak at m/z 205.1 with r.t. 12.1 min having the same MS[2] and MS[3] fragmentation patterns reported above.

The generation from the ion at m/z 205.1 of the MS[2] fragment at m/z 160 and the MS[3] fragment at m/z 132 from the MS[2] fragment at m/z 160 gives a strong indication of the occurrence of hydroxyvinylindole in the structure of the unknown compound. Moreover, the presence in its MS[2] fragmentation pattern of another fragment at m/z 58, which also occurs in the MS[2] spectrum of *N,N*-dimethyltryptamine,[1] led us to hypothesize that the unknown compound is a hydroxylated *N,N*-dimethyltryptamine. However, two compounds have been found in the natural world, both having m/z 205.1, which differ in the OH position in the indole structure, that is, psilocin (4-hydroxy-*N,N*-dimethyltryptamine) and bufotenine (5-hydroxy-*N,N*-dimethyltryptamine). Both sub-
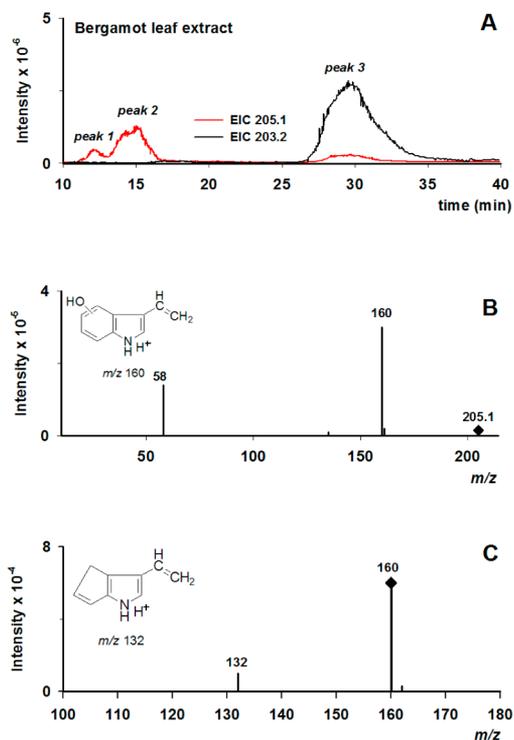

Figure 1. (A) LC-ESI-MS chromatogram conducted in zoom scan mode on a bergamot leaf extract in the m/z range 201–209 amu, extracted at m/z 205.1 (red line) and m/z 203.2 (black line). Peak 2 corresponds to tryptophan and peak 3 to *N,N,N*-trimethyltryptamine as reported in ref 1. (B) MS[2] fragmentation pattern of peak 1 in A. (C) MS[3] fragmentation pattern of the ion at m/z 160 in B. In B the proposed structure for the MS[2] ion at m/z 160 and in C that for the MS[3] ion at m/z 132 as reported in ref 13 are also indicated.

stances are considered psychoactive. Psilocin is particularly present in some hallucinogenic species of mushrooms, bufotenine has been found in animals, mushrooms, and higher plants.[14,15] In order to determine which of the two substances corresponds to the peak at r.t. 12.1 min, standard solutions of both were analyzed by LC-ESI-MS/MS utilizing the same chromatographic conditions, which we previously used for the determinations of betaines in *Citrus* plants.[12,16] In these conditions, bufotenine and psilocin were separated; the first emerged at r.t. 10.5 min and the second at r.t. 12.1 min (Figure 2A). It is also interesting to note that the two compounds, in the same instrumental fragmentation conditions, behave differently. In fact, for psilocin, the two MS[2] fragments at m/z 160 and m/z 58 showed almost identical intensities (Figure 2C), whereas, for bufotenine, the fragment at m/z 160 showed an intensity about twice that of the fragment at m/z 58 (Figure 2B).

On the basis of these results, the extract of tissue parts of the various citrus plants were analyzed as described above. In all cases, we observed the peak at m/z 205.1, emerging at r.t. 10.5 min, which showed the same fragmentation pattern of bufotenine. As an example, the chromatogram of a bergamot leaf extract is reported in Figure 3A and B, where the bufotenine identification, besides the presence of the peak at r.t. 10.5 min, is confirmed by the higher intensity of the MS[2] fragment at m/z 160 than that of the fragment at m/z 58 (Figure 3C). It is noteworthy that an intense fragment at m/z 188 is also observed in the MS[2] fragmentation pattern of the peak at r.t 10.5 min, for leaf extracts. This is due to the presence
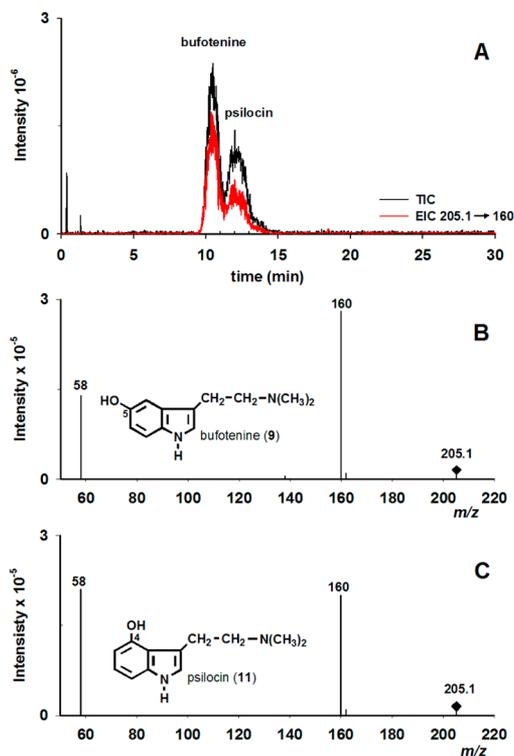
Figure 2. (A) Total ion current (TIC) chromatogram (black line) and MS^2 extracted ion chromatogram (EIC) at m/z 160 (red line) of a standard mixture of bufotenine and psilocin. The chromatographic conditions are reported in the text. (B) MS^2 fragmentation pattern of the bufotenine peak. (C) MS^2 fragmentation pattern of the psilocin peak.

of tryptophan in the extract. Actually, tryptophan (m/z 205.1) elutes at r.t. of 12.7 min, but it is much more abundant than bufotenine; therefore, the ascending part of the tryptophan peak partially coelutes with the descending part of the bufotenine peak (Figure 3A). However, in order to get further experimental evidence for bufotenine identification, we performed chromatographic analysis also with a longer C8 column (15 cm) in the same isocratic elution conditions. In this way, complete separation among bufotenine, psilocin, and tryptophan (Figure 3C and D) and the disappearance of the fragment at m/z 188 in the fragmentation pattern of the peak corresponding to bufotenine in the bergamot leaf extract analysis (Figure 3D, inset) were obtained.

Identification of 5-Hydroxytryptamine and Its *N*-Methyl Derivatives in Citrus Plants. The presence of bufotenine in all the examined citrus plants prompted us to look for the occurrence in the same sources of 5-hydroxytryptamine (serotonin) and possibly other compounds deriving from its N-methylation, that is, 5-hydroxy-*N*-methyltryptamine and 5-hydroxy-*N*,*N*,*N*-trimethyltryptamine. Moreover, 5-hydroxytryptophan, as a possible precursor of 5-hydroxytryptamine, was also searched for. In the MS^2 analyses, the monitored transitions were 177.1→160 for 5-hydroxytryptamine, 191.1→160 for 5-hydroxy-*N*-methyltryptamine, and 221.2→204 for 5-hydroxytryptophan.

A complete chromatographic separation of all examined metabolites was obtained using the 10 cm Supelco Discovery-C8 column in a time less than 16 min and in isocratic condition with 0.1% formic acid in water as eluent. In particular, 5-hydroxytryptophan elutes first at r.t. 6.2 min, followed by 5-
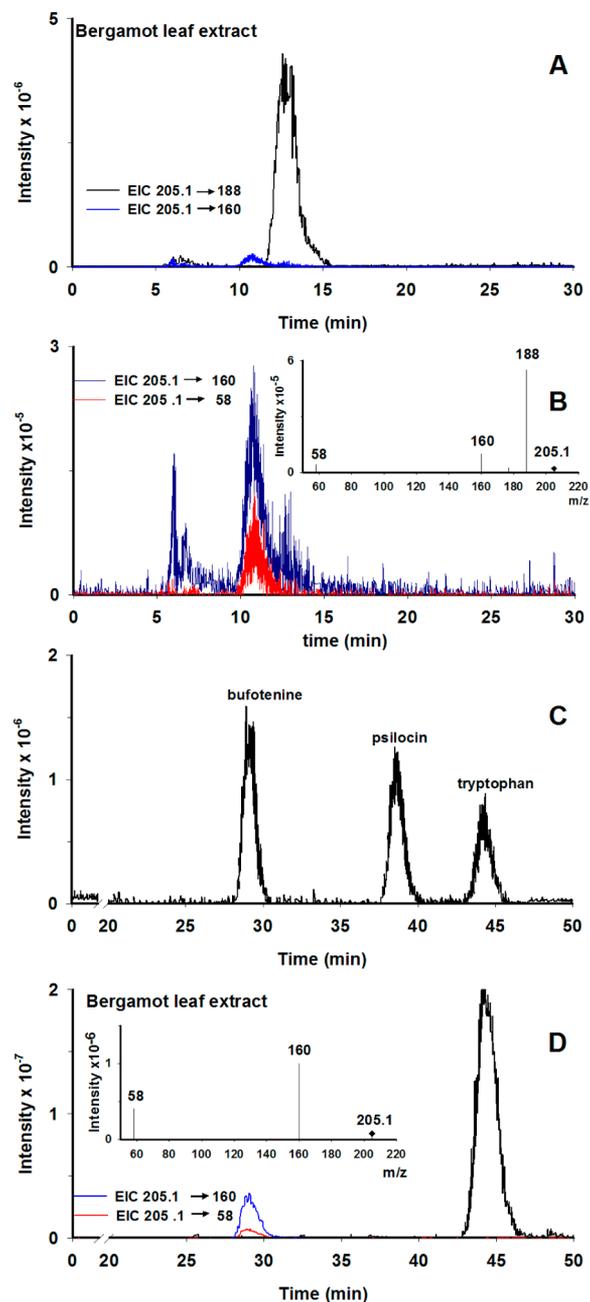

Figure 3. (A) MS^2 EIC chromatograms at m/z 188 (black line) and at m/z 160 (blue line) of bergamot leaf extract. The chromatography was performed with a C8 column (10 cm) eluted as reported in the text. (B) MS^2 EIC chromatogram of the peak at r.t. 10.4 min. In the inset, the MS^2 fragmentation pattern of the peak at r.t. 10.4 min is reported. The intense fragment at m/z 188, typical of the MS^2 tryptophan fragmentation, is due to the partial overlap of the tryptophan peak at r.t. 12.4 min, as is seen in A. (C) Standard mixture of bufotenine, psilocin, and tryptophan chromatographed on a longer C8 column (15 cm). (D) MS^2 EIC chromatograms at m/z 188 (black line) and at m/z 160 (blue line) of the same bergamot leaf extract as that in A but chromatographed on a longer C8 column (15 cm). In the inset, the fragmentation pattern of the peak corresponding to bufotenine is reported, in which the fragment at m/z 188 is no longer present.

hydroxytryptamine at r.t. 8.1 min, and 5-hydroxy-*N*-methyltryptamine at r.t. 10 min (Figure 4A). The MS^2 fragmentation patterns obtained from standard solutions of the metabolites are reported in Figure 4C−E. The chromatographic analysis
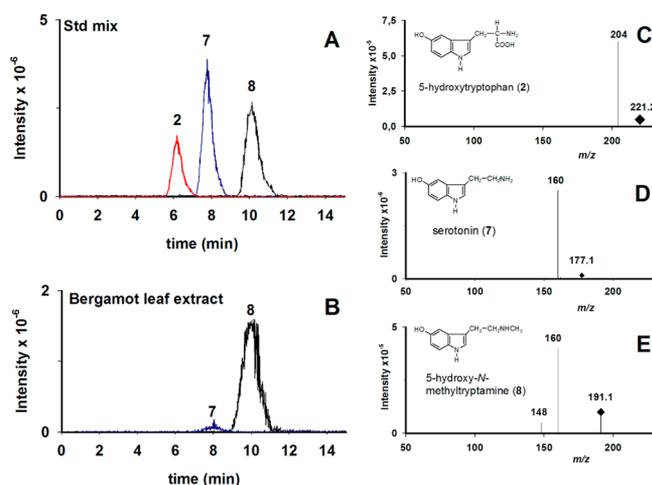
Figure 4. (A) MS² EIC of a standard mixture of 5-hydroxytryptophan (**2**), 5-hydroxytryptamine (**7**), and 5-hydroxy-*N*-methyltryptamine (**8**). The MS² transitions followed were 221.2→204 for (**2**) (red line), 177.1→160 for (**7**) (blue line), and 191.1→160 for (**8**) (black line). (B) MS² EIC of a bergamot leaf extract, where no signal from **2** is observed. (C, D, and E) MS² fragmentation patterns of the peaks depicted in A. The MS² fragmentation patterns of the peaks corresponding to **7** and **8** from the bergamot leaf extract being identical to those of the standard compounds were not reported. The chromatographic conditions are described in the text.

performed in the same conditions of a bergamot leaf extract is reported in Figure 4B. The occurrence in the extract of 5-hydroxytryptamine and 5-hydroxy-*N*-methyltryptamine was confirmed by the identity of the retention times and MS² fragmentation patterns with the respective standards. 5-Hydroxytryptamine and 5-hydroxy-*N*-methyltryptamine were found in the leaf extracts of all citrus plants examined. However, 5-hydroxytryptophan was not detected in leaf extracts (Figure 4B).

Identification 5-Hydroxy-*N,N,N*-trimethyltriptamine (Bufotenidine) in Citrus Plants. The absence of 5-hydroxytryptophan in all of the citrus plant leaf extracts examined led us to hypothesize that 5-hydroxytryptamine and its *N*-methyl derivatives do not stem from decarboxylation of 5-hydroxytryptophan but rather that they come from tryptamine and/or its *N*-methyl derivatives by successive hydroxylation. As we recently discovered the occurrence in bergamot of noticeable levels of *N,N,N*-trimethyltryptamine,[1] we examined the possibility that the hydroxylation product of *N,N,N*-trimethyltryptamine was also present in this plant source. To this end, a bergamot leaf extract was analyzed by LC-ESI-MS/MS by isolating at *m/z* 219.1. As shown in Figure 5A, an intense total ion current signal is seen in the chromatogram region between 11 and 15 min, and the extracted ion chromatogram (Figure 5C) shows the presence of two fragments: the first at *m/z* 160 and the second, less intense, at *m/z* 60, indicative of the probable presence of the trimethylammonium group in the structure of the substance.[1,12] Moreover, the unknown compound also shows a retention time higher than that of 5-hydroxy-*N,N*-dimethyltryptamine, providing further support to the hypothesis that it was the hydroxylated form of *N,N,N*-trimethyltryptamine. This is analogous to our finding, previously reported for N-methylated tryptamines, that the retention time increases when methylation on amino nitrogen increases.[1] To confirm that hypothesis, as 5-hydroxy-*N,N,N*-trimethyltriptamine was not commercially
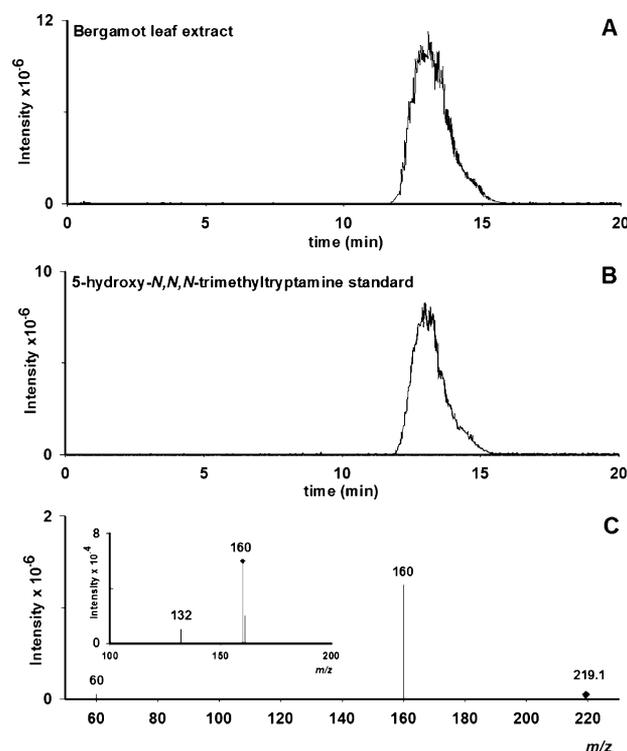

Figure 5. (A) Representative chromatogram of a bergamot leaf extract following the MS² transitions 219.1→160. (B) Chromatography of the synthesized 5-hydroxy-*N,N,N*-trimethyltryptamine (**10**) standard solution performed in the same experimental conditions. (C) MS² fragmentation pattern of the peak reported in A. Inset of C: MS³ fragmentation pattern of the peak in A by isolating at *m/z* 160. The MS² and MS³ fragmentation patterns of the peak in B (standard 5-hydroxy-*N,N,N*-trimethyltryptamine) being identical to those of the peak in A were omitted.

available, it was synthesized according to the Chen and Benoiton method.[10,11] The synthesized 5-hydroxy-*N,N,N*-trimethyltryptamine was purified and subjected to mass spectrometric analysis in order to find the optimal instrumental conditions for detection and quantitation. The MS² fragmentation pattern of 5-hydroxy-*N,N,N*-trimethyltryptamine standard solution is reported in Figure 5A. The MS³ fragmentation pattern was obtained isolating the MS² fragment at *m/z* 160. This pattern was dominated by the product ion at *m/z* 132 (Figure 5C). Then, the chromatographic behavior and MS² fragmentation pattern of the synthesized 5-hydroxy-*N,N,N*-trimethyltryptamine were compared with those of the unknown compound from the leaf extract (Figure 5). Both compounds showed the same mass at *m/z* 219.1 of the parent ions, the same MS² fragmentation patterns, and the same intensity ratio between the two main fragments at *m/z* 160 and 60. Moreover, they eluted at the same retention time (Figure 5A and B). These observations sufficiently prove that the unknown compound is 5-hydroxy-*N,N,N*-trimethyltryptamine. Anyway, to obtain further evidence of the identity of the compound, 5-hydroxy-*N,N,N*-trimethyltryptamine standard solutions were added to bergamot leaf extracts and subjected to chromatography by utilizing C8 columns of 10 and 15 cm and two different isocratic elution conditions, that is, 100% water with 0.1% formic acid and a mixture of 75% water with 0.1% formic acid containing 25% methanol. In all cases, only one symmetrical peak was seen in the EIC chromatograms obtained by isolating the ion at *m/z* 219.1, thus confirming the identity

of the unknown compound from the leaf as 5-hydroxy-N,N,N-trimethyltriptamine.

Distribution of Tryptamine Derivatives in Various Parts of the Bergamot Plant and in Leaves of Other Citrus Plants. The quantitative distribution of the hydroxylated tryptamine derivatives in fruits and leaves of the bergamot plant is reported in Table 2.

Table 2. Distribution of the 5-Hydroxylated Forms of Methylated Tryptamine Derivatives in Various Parts of the Bergamot Plant[a]

| | parts of the bergamot fruit | | | |
compd	albedo	edible part	seed	leaf
2	absent/trace	absent/trace	absent/trace	absent/trace
7	trace	trace	0.5–6.0	0.1–1.0
8	trace	trace-0.05	5.0–25.0	1.0–3.0
9	0.03–0.05	1.0–2.0	5.0–30.0	1.0–3.5
10	0.4–0.8	trace-0.1	2.0–6.0	6.0–12

[a](**2**) 5-hydroxytryptophan, (**7**) 5-hydroxytryptamine, (**8**) 5-hydroxy-N-methyltryptamine, (**9**) 5-hydroxy-N,N-dimethyltryptamine, and (**10**) 5-hydroxy-N,N,N-trimethyltryptamine. Ranges are expressed as mg/kg. Trace = values less than 0.02 mg/kg.

It appears from Table 2 that 5-hydroxytryptophan is absent in all tissue parts of the fruit and leaves. Conversely, 5-hydroxytryptamine was relatively abundant in bergamot seeds and leaves but only present at trace level in the edible part of the fruit (fruit juice). As for 5-hydroxy-N-methyltryptamine, the highest levels were found in seeds (up to 25 ppm), while it is only at trace levels in the albedo and the edible part of the fruit, and intermediate amounts were found in leaves. Also, 5-hydroxy-N,N-dimethyltryptamine shows a distribution similar to that of 5-hydroxy-N-methyltryptamine, with the highest levels in seeds (up to 30 ppm) and the lowest in the fruit juice (up to 2 ppm) and intermediate levels in leaves. The highest amounts of 5-hydroxy-N,N,N-trimethyltryptamine were found in leaves (up to 12 ppm) and the lowest in albedo and fruit juice, while intermediate levels were present in seeds (up to 6 ppm).

Table 3 reports the ranges of tryptophan, tryptamine, and its N-methylated derivatives and those of 5-hydroxytryptamine and its N-methylated derivatives in leaves of the various citrus plants. For the sake of clarity, the last column of Table 3 lists the amounts of these compounds previously reported in bergamot leaves.[1] The data in Table 3 show the common presence of these indole metabolites in all the Citrus species examined. However, their levels significantly vary among the species. In particular, leaves of bergamot, lemon, and citron contain levels of N,N,N-trimethyltryptamine and 5-hydroxy-N,N,N-trimethyltryptamine consistently higher than those of orange, mandarin, and chinotto.

Tryptamine Metabolism in Citrus Plants. In plant metabolism, tryptamine represents an intermediate of the metabolic flow that leads from tryptophan to compounds important for plant physiology and defense. Tryptamine derives from tryptophan decarboxylation by the activity of the tryptophan decarboxylase enzyme. Once formed, it is converted into a variety of metabolites such as the phytohormone indole-3-acetic acid[17] and the monoterpene indole alkaloids such as the well-known anticancer agents vinblastine and vincristine.[8] However, the role of tryptamine as a precursor of simpler secondary metabolites involved in plant defense against biotic agents has not been well defined. Although there are no reports in the literature on the presence in Citrus genus plants of the enzyme tryptophan decarboxylase, the presence at noticeable levels of tryptamine and its N-methylated derivatives makes it reasonable to hypothesize the presence in this plant genus of tryptophan decarboxylase. Moreover, the occurrence of 5-hydroxytryptamine and its N-methylated derivatives in all the citrus plants we examined and the absence of 5-hydroxytryptophan also support the likelihood of tryptamine 5-hydroxylase activity. In fact, the absence of 5-hydroxytryptophan suggests that 5-hydroxytryptamine in Citrus plants is generated by the hydroxylation of tryptamine by tryptamine 5-hydroxylase, as it also occurs in the majority of plants.[20] Instead, in mammals and some plants such as Hypericum perforatum[18,19] (St. John's wort), 5-hydroxytryptamine is formed by the decarboxylation of 5-hydroxytryptophan. Therefore, the presence of the N-methylated derivatives of serotonin that we have found in the Citrus plants (Table 2 and 3) suggests that they may arise either from the methylation of serotonin or from hydroxylation of the N-methylated derivatives of tryptamine, which are also present at noticeable levels in this plant genus. However, the presence of both pathways is a possibility. Figure 6 summarizes the hypotheses for the formation of all of these tryptamine derivatives. Of course, further studies are required to support such hypothetical pathways.

Table 3. Distribution of Tryptophan and Tryptamine Derivatives in Leaves of Citrus Plants[a]

compd	lemon	orange	mandarin	chinotto	citron	bergamot
1	8.0–24.0	15.0–44.0	27.0–77.0	27.0–80.0	31.0–92.0	21.0–60.0
2	absent/trace	absent/trace	absent/trace	absent/trace	absent/trace	absent/trace
3	0.7–3.0	trace –0.3	0.3–1.5	0.2–1.0	0.4–2.0	0.5–2.0
4	trace	trace	trace	trace	trace	0.2–0.5
5	0.1–0.2	trace –0.1	trace –0.1	0.2–0.5	0.10–0.20	0.2–0.4
6	2.0–4.5	0.6–1.5	trace –0.1	0.4–1.0	1.2–2.8	6.0–12.0
7	0.1–0.5	trace –0.1	trace –0.1	0.1–0.6	0.1–1.0	0.1–1.0
8	0.2–0.8	0.1–0.3	trace –0.1	0.5–3.0	0.2–0.9	1.0–3.0
9	0.1–0.2	0.4–2.0	trace	1.5–5.5	1.2–4.6	1.0 –3.5
10	3.0–7.0	trace –0.1	trace –0.1	trace –0.1	3.5–7.5	6.0 –12.0

[a](**1**) tryptophan, (**2**) 5-hydroxytryptophan, (**3**) tryptamine, (**4**) N-methyltryptamine, (**5**) N,N-dimethyltryptamine, (**6**) N,N,N-trimethyltryptamine, (**7**) 5-hydroxytryptamine, (**8**) 5-hydroxy-N-dimethyltryptamine, (**9**) 5-hydroxy-N,N-dimethyltryptamine, and (**10**) 5-hydroxy-N,N,N-trimethyltryptamine. Ranges are expressed as mg/kg. Trace = values less than 0.02 mg/kg.
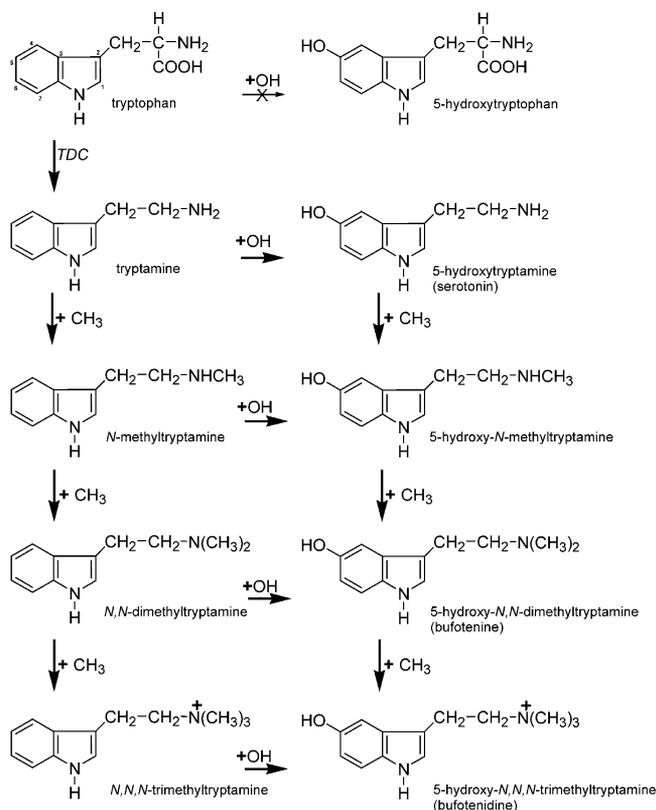
Figure 6. Hypothesized pathways for the biosynthesis of the N-methylated and 5-hydroxy-N-methylated tryptamine derivatives. The first step, in which tryptamine is produced, is catalyzed by the tryptophan decarboxylase (TDC) enzyme. The absence of 5-hydroxytryptophan in the citrus plants examined makes us hypothesize that the tryptamine derived hydroxylated forms do not stem from this amino acid. Once produced, tryptamine may be N-methylated by the action of the same or different methyltransferase enzyme(s) acting in a consecutive manner. Then, each methylated intermediate could be transformed in the corresponding hydroxylated form. Alternatively, the tryptamine could be first hydroxylated to serotonin, which, successively, is N-methylated in a consecutive manner by the same or different N-methyltransferase enzyme(s).

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Notes

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