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Development and validation of a pre-column reversed phase liquid chromatographic method with fluorescence detection for the determination of primary phenethylamines in dietary supplements and phytoextracts

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ABSTRACT

A sensitive and selective reversed-phase liquid chromatographic (RP-LC) method was developed and validated to determine octopamine, tyramine and Tyrosine (Tyr) in complex matrices as formulations and phytoextracts (*Citrus aurantium*), after pre-column derivatization with *o*-phthaldialdehyde (OPA) reagent. The chromatographic separations were performed at room temperature on a Phenomenex Luna C18 column using methanol and sodium acetate buffer (pH 5.5) by varying composition gradient elution as mobile phase and detected flurometrically at λ_{em} = 455 nm with λ_{ex} = 340 nm. The results obtained by the proposed method were compared with those achieved by a validated direct RP-LC method with fluorescence detection at λ_{em} = 310 nm with λ_{ex} = 275 nm, as reference method, using a Phenomenex Gemini C18 column under isocratic elution conditions with acetonitrile and sodium 1-heptanesulphonate (pH 3), as mobile phase. The higher sensitivity of the derivatization method (detection limit about 0.06 pmol) allowed the sure determination of octopamine present in traces in the examined samples. The repeatability of method (RSD) was ≤1.90% and there was no significant difference between repeatability and intermediate precision data. Recovery studies showed good results 99.5–101.3% with RSD ranging from 0.8 to 1.2%. All analyses were performed by mild conditions in absence of preliminary difficult extraction methodologies or laborious step of sample pre-treatment.

1. Introduction

Recently, dietary supplements formulated with fruits of *Citrus aurantium* have been marketed as an appetite suppressant to support weight loss. The unripe fruits of *C. aurantium* L. var. *amara* (Rutaceae; vernacular name: bitter orange) contain high levels of flavonoids (flavanones, flavones and flavonols) [1] and adrenergic amines which differ in the number and position of hydroxyl substituents and include synephrine, octopamine, tyramine, N-methyltyramine and hordenine. The most active components in *C. aurantium* are synephrine and octopamine. Synephrine is structurally similar to epinephrine, while octopamine is similar in structure to norepinephrine. Synephrine is the primary alkaloid found in the immature fruits, whereas the other alkaloids are present at significantly lower levels. *C. aurantium* alkaloids increase the metabolic rate and promote the oxidation of fat through increased thermogenesis. Synephrine and octopamine activate selectively the β-3 adrenoreceptors and seem to inhibit cAMP production [2–6]. After the ban in April 2004 of *Ephedra sinica* in the US many products for weight loss and bodybuilding now contain *C. aurantium* extracts instead. Although no direct adverse events have been associated with their ingestion thus far, they increase blood pressure and has the potential to rise the risk of cardiovascular events. In addition, *C. aurantium* should be used with caution because of suspected possible interferences with CYP 450 enzymes. Thus, it can cause an alarming increase in the blood levels of many drugs [4,7]. Octopamine is known as a "false neurotransmitter" in humans because it alters the normal function of the brain and is believed to stimulate the production of growth hormone [8]. Therefore, it was included since 2006 in the "The Prohibited List International Standard" by WADA (World Anti-Doping Agency) into stimulant category prohibited in sport competitions [9]. The seriousness of effects above given show the necessity of reliable analytical methods applicable to quality control of commercial dietary supplements, which are able to establish the octopamine content to avoid possible adulterations.

A variety of approaches have been used for the determination of phenethylamine alkaloids in plant materials and extracts, juice and in dietary supplements. Traditionally, the methods involve reversed-phase liquid chromatography (RP-LC) with UV, electrochemical and mass spectrometry detection or capillary

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electrophoresis (CE) [10]. RP-LC with UV detection is the preferred technique in phytochemical analysis for its versatility and the used mobile phases contain in several cases an ion-pair reagent to increase the peak resolution and symmetry [5,11–13]. On other hand, RP-LC with UV detection in absence of an ion-pair reagent was reported in other papers [14–17]. So far, the LC analysis of these compounds using fluorescence or chemiluminescence detection offers great sensitivity and selectivity [5,18,19]. In particular, LC with fluorescence detection was used for the determination of bioactive primary amines in orange juice and drinks by post-column derivatization with *o*-phthaldialdehyde (OPA) reagent [11,20,21].

Typical fluorogenic derivatization reagents for amino function reported in literature are dansyl chloride (Dns-Cl), 9-fluorenylmethyl chloroformate (FMOC), *o*-phthaldialdehyde (OPA), fluorescamine, nynhydrin, phenylisocyanate, phenylisothiocyanate (PITC), 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F), 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) [22,23], but some of them can give a variety of drawbacks. For example, PITC derivatization is long and involves several stages of drying under vacuum before the injection into chromatographic system; on the other hand, with FMOC, the excess of the derivatization reagent can give rise to problems if it is not removed by extraction with pentane or it is not derivatized with an amine whose derivative does not interfere. In addition, Dns-Cl requires a long time of reaction and a low concentration, otherwise the increase of the peak area background decrease the sensitivity of the determination [24,25]. In general, OPA is used as post-column fluorogenic reagent owing to the instability of its adducts, but several papers show that it can be employed in pre-column derivatization with reliable results [26–30]. In general, to improve the sensitivity pre-column derivatization procedures are preferred because they can be carried out without further instrumentation, in short time of derivatization reaction and without limitation of reaction solvent or derivatization reaction rate. Besides, the pre-column labeling offers the advantage to decrease the polarity of the compounds so that they can be retained on the reversed stationary phase. OPA is a compound devoid of significant native fluorescence which react in short times (1 min) and in mild conditions (room temperature) selectively with the primary amino function to give highly fluorescent adducts. OPA pre-column derivatization does not show the presence of excess reagent interfering with the analytes resolution. However, as far as we know OPA was not previously studied as pre-column reagent for phenethylamine alkaloid analysis in plant materials, extracts, juice and dietary supplements.

The aim of the present work is to verify the applicability of OPA as pre-column derivatization reagent for the RP-LC fluorescent determination (λ_{em} = 455 nm with λ_{ex} = 340 nm) of octopamine and other primary phenethylamines in dietary supplements and phytoextracts having a complex matrix. In addition, results obtained by a reference direct RP-LC method using fluorescence detection (λ_{em} = 310 nm with λ_{ex} = 275) were included and evaluated critically. The performance of both methods was investigated with respect to linearity, detection and quantification limits, precision and accuracy.

2. Experimental

2.1. Materials

(±) Octopamine hydrochloride >95%, tyramine hydrochloride 99% (±) synephrine 98%, quinine 98% (used as internal standard, IS), methanol and acetonitrile were purchased from Sigma–Aldrich (Milan, Italy), whereas hordenine and N-methyltyramine from Carbone Scientific Co. Ltd. (London, United Kingdom). *o*-Phthaldialdehyde (OPA) and L-Tyrosine (Tyr) 99% were obtained from Fluka (Buchs, Switzerland). Purified water by a Milli-RX (Millipore, Milford, MA, USA) apparatus was used for the preparation of all solutions and mobile phases. All the other chemicals were of analytical reagent grade.

2.2. Solutions

Standard solutions of Tyr, octopamine and tyramine were prepared in water and were subjected to derivatization reaction (method A), whereas the standard solutions of all phenethylamines were prepared in a mixture A:B (60:40, v/v), where A is water and B is the appropriate mobile phase for the direct method (method B). Phenethylamine concentrations are reported in Table 1. The mass of the standard compound hydrochloride was converted to the corresponding free base form using a molecular mass conversion. The reagent OPA solution was prepared by dissolving 100 mg in 1.5 mL of methanol; then, to the obtained solution 100 μL of 2-mercaptoethanol and 11.2 mL of sodium borate buffer (pH 9.5; 0.4 M) were added. The mixture was stored in the dark at 4 °C and was allowed to stand for 24 h before use. An aliquot of 10 μL of mercaptoethanol was added every 2 days to help to maintain the reagent strength. The solution remains stable for about 2 weeks [31]. IS solution corresponding to 250 and 6.25 μg/mL for the analysis of formulations (capsules) and phytoextracts was prepared in water, respectively. Borate buffer (pH 9.5, 0.4 M) solution was prepared by adjusting the pH of a boric acid solution to 9.5 with 1 M sodium hydroxide [32]. Sodium acetate buffer (pH 5.5, 0.05 M) was prepared by dissolving sodium acetate in water and adjusting to pH 5.5 with glacial acetic acid [33]. Sodium 1-heptanesulphonate (SHS) (pH 3, 12 mM) was prepared by dissolving SHS in water and adjusting to pH 3 with phosphoric acid.

2.3. Equipment

The liquid chromatograph consisted of a Jasco Model LG-980-02S ternary gradient unit, a Jasco PU-1580 pump and a Jasco FP-920 fluorescence detector (Jasco Corporation, Tokyo, Japan). All separations were performed at ambient temperature (25 °C). The data were collected on a PC equipped with the integration program Borwin and ChromNav. The solvents were degassed on line with a degasser model Gastorr 153 S.A.S Corporation (Tokyo, Japan). Manual injections were carried out using a Rheodyne model 7125 injector with 20 μL sample loop. A column inlet filter (0.5 μm × 3 mm i.d.) model 7335 Rheodyne was used. The centrifugation was performed both by an ALC 4235A and an eppendorf Centric 150 Tehtnica (Opto-Lab, Concordia, Modena, Italy) centrifuge. Sonarex Super RK 102 (35 KMZ) Bandelin (Berlin, Germany) equipment with thermostatically controlled heating (30–80 °C) was used for ultrasonication.

2.4. Derivatization procedure

A 50 μL aliquot of the phenethylamines solution was treated with 50 μL of sodium borate buffer (pH 9.5, 0.4 M) and 40 μL of the reagent OPA solution were added. The reaction was carried out at ambient temperature in a tube (1.0 mL) for 1 min covering 15 s ultrasonication in presence of the appropriate IS solution. Then, 100 μL of a mixture A:B, where A is methanol and B is sodium acetate buffer (pH 5.5, 0.05 M) in the ratio 50:50, v/v, were added and 20 μL aliquot of the resulting clear solution was injected into the chromatograph.

Table 1
Data for calibration graphs ($n = 5$), limit of detection (LOD) and limit of quantification (LOQ).

Compounds	Method	Slope[a]	y-Intercept[a]	Correlation coefficient	Concentration range (nmol/mL)	LOD (pmol)	LOQ (pmol)
Tyr	A	0.104 (±0.0008)	+0.084 (±0.079)	0.9998	20.33–162.70	0.07[b]	0.23[b]
	B	34320.5 (±218.5)	−44871.5 (±81007.9)	0.9998	79.62–637.19	0.71[c]	2.37[c]
Octopamine	A	2.353 (±0.010)	−0.003 (±0.007)	0.9999	0.04–2.17	0.05[b]	0.17[b]
	B	–	–	–	–	1.04[c]	3.47[c]
Tyramine	A	2.675 (±0.007)	−0.004 (±0.008)	0.9999	0.03–1.35	0.07[b]	0.23[b]
	B	2527353.0 (±18957.0)	−418.5 (±9985.4)	0.9998	0.10–3.37	0.21[c]	0.70[c]
Synephrine	B	956543.7 (±8721.6)	+46558.1 (±138487.7)	0.9998	1.46–29.30	0.40[c]	1.34[c]
N-Methyl-tyramine	B	2096474.0 (±8856.1)	+19377.2 (±30105.9)	0.9999	0.22–7.21	0.32[c]	1.08[c]
Hordenine	B	–	–	–	–	0.50[c]	1.66[c]

[a] According to $y = ax + b$, where x = analyte concentration expressed as nmol/mL, y = ratio of analyte peak-area to IS peak-area (method A) and analyte peak-area (method B).
[b] Gain 1000.
[c] and gain 100.

2.5. Chromatographic conditions

The routine chromatographic separations were performed at ambient temperature on a Phenomenex Luna 5 μm C18 (250 mm × 4.60 mm i.d.) stainless steel column under gradient elution conditions (method A) and a Phenomenex Gemini 5 μm C18 (250 mm × 3 mm i.d.) stainless steel column (method B) under gradient elution conditions using different mobile phases according to the developed method.

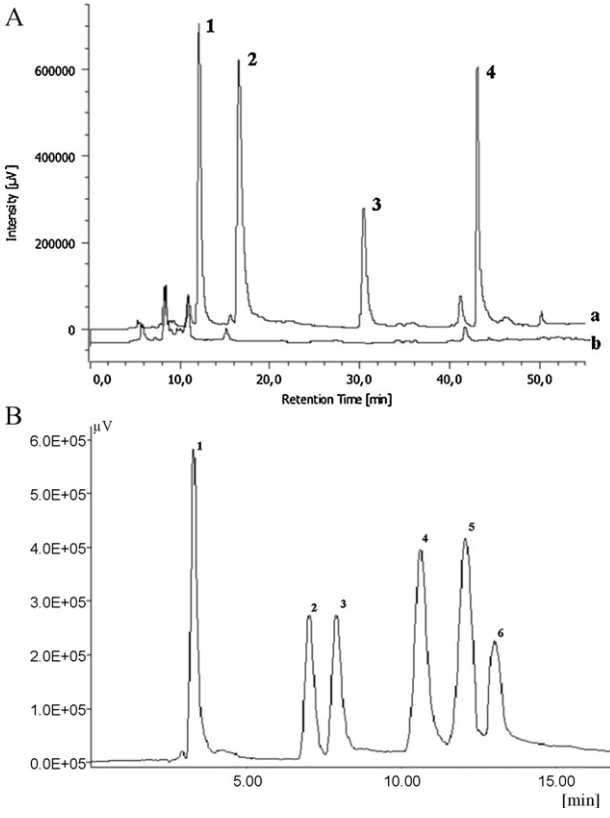

Fig. 1. Representative chromatograms of phenethylamines before the injection: (A) (a) phenetylamine separation after derivatization with OPA reagent; (b) OPA under reaction conditions in absence of analytes. Peaks: 1, Tyr (0.006 μmol/mL); 2, IS (0.20 μmol/mL); 3, octopamine (0.005 μmol/mL); 4, tyramine (0.003 μmol/mL); (B) phenetylamine separation by method B. Peaks: 1, Tyr (0.014 μmol/mL); 2, octopamine (0.010 μmol/mL); 3, synephrine (0.005 μmol/mL); 4, tyramine (0.005 μmol/mL); 5, N-methyltyramine (0.006 μmol/mL); 6, hordenine (0.006 μmol/mL). LC conditions and fluorescence detection as described in the text. Gain: 100.

A mixture A:B, where A is methanol and B acetate buffer (pH 5.5, 0.05 M) with the following gradient profile $t = 0$ min, 55% A; $t = 20$ min, 55% A; $t = 50$ min, 100% A; $t = 53$ min, 100% A and $t = 55$ min, 55% A at a flow-rate of 0.5 mL/min with fluorescence detection at $\lambda_{em} = 455$ nm with $\lambda_{ex} = 340$ nm was used for the method A; whereas a mixture A:B, where A is sodium SHS (pH 3, 12 mM) and B is acetonitrile, in the ratio 85:15, v/v, at a flow-rate of 0.5 mL/min with fluorescence detection at $\lambda_{em} = 310$ nm with $\lambda_{ex} = 275$ nm was used for the method B.

2.6. Analysis of dietary supplements and phytoextracts

2.6.1. Sample preparation

Capsules. The content of 20 capsules were powdered and an amount equivalent to about 100 mg of Tyr and 5 mg of synephrine was extracted twice with 24 mL of water by ultrasonication for about 15 min, followed by centrifugation for 15 min at 9000 rpm at ambient temperature. The supernatant was transferred to a 50 mL volumetric flask. After filtration through a 0.22 μm membrane filter a 0.15 mL supernatant aliquot was diluted to 5 mL with water (in presence of IS for the method A).

C. aurantium dried extracts. A weighted amount of two different extracts equivalent to 10 mg and 4 mg (dry extract I) and 15 mg and 6 mg (dry extract II) of synephrine for method A method B, respectively, was dissolved with 20 mL of water by ultrasonication for about 20 min, followed by centrifugation (9000 rpm) for 15 min at ambient temperature. After filtration through a 0.22 μm membrane filter a 1 mL aliquot was diluted to 2 mL with water in presence of IS for the method A, whereas a 0.10 mL supernatant aliquot was diluted to 5 mL for the method B.

2.6.2. Assay procedure

50 μL aliquot of the sample solution after derivatization procedure (method A) and a 150 μL volume of the sample solution (method B) were diluted with 100 μL of the appropriate mobile phase and injected in the chromatograph, respectively. The amine content in the samples was determined by comparison with an appropriate standard solution. To the end, each solution was injected in triplicate in the chromatograph.

3. Results and discussion

In general, the determination of biogenic amines in a complex matrix as dietary supplements and phytoextracts for weight loss is difficult and sophisticated or time-consuming sample preparation procedures were developed to resolve the problem [10]. In addition, the analysis of the dietary supplement examined in present work has got complicated owing to the remarkable difference of

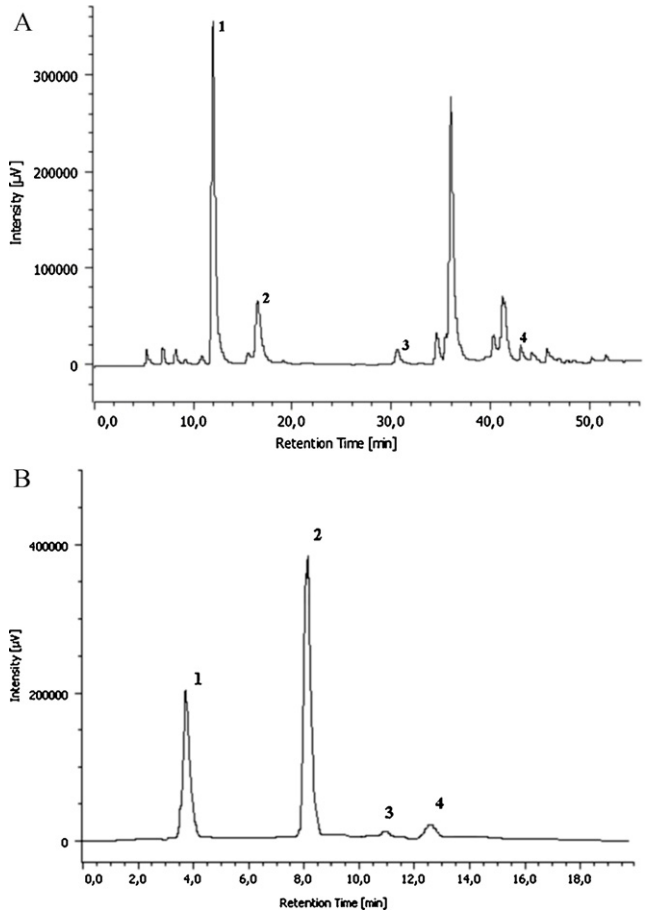

Fig. 2. LC separations of a phenethylamine sample (capsules): (A) phenethylamines after derivatization with OPA. Peaks: 1, Tyr; 2, IS; 3, octopamine; 4, tyramine. (B) Phenethylamines. Peaks: 1, Tyr; 2, synephrine; 3, tyramine; 4, N-methyltyramine. LC conditions and detection as in Fig. 1. Gain: $t = 0$, gain 10; $t = 25$, gain 100 for the method A; $t = 0$, gain 10; $t = 5$, gain 100 for the method B.

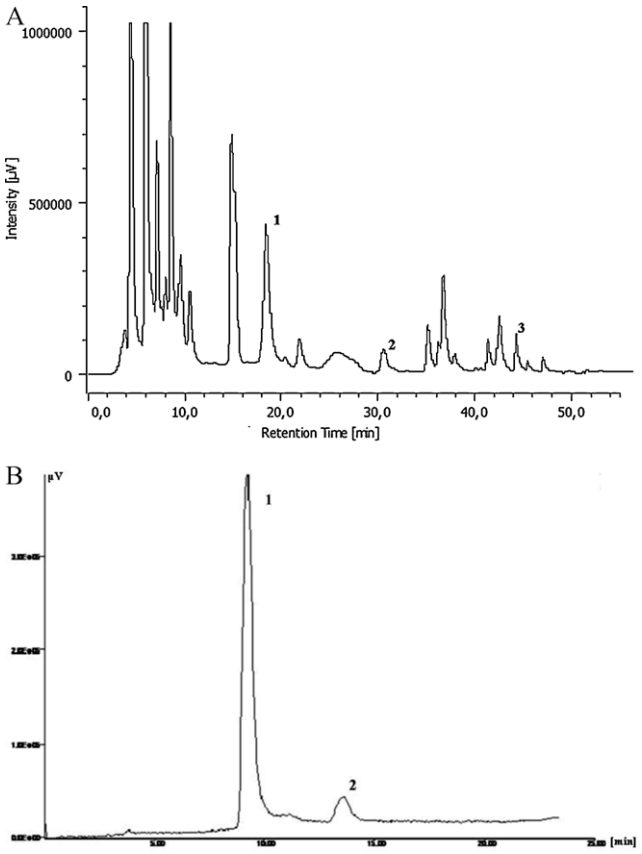

Fig. 3. LC separations of phenethylamine alkaloid samples (phytoextracts): (A) phenethylamines in dry extract II sample after derivatization with OPA. Peaks: 1, IS; 2, octopamine; 3, tyramine. (B) phenethylamines in dry extract I sample. Peaks: 1, synephrine; 2, N-methyltyramine. LC conditions, detection and gain as in Fig. 1.

concentration between the very high levels of the amino acid Tyr and the phenethylamine alkaloids at small signal (octopamine, tyramine and N-methyltyramine).

3.1. Chromatography and detection

To assure an adequate separation of the analytes the chromatographic conditions were investigated considering different RP-LC columns and mobile phases according to the adopted method. As a result of that, Luna ODS column was used for the separation of Tyr, octopamine and tyramine after derivatization reaction with OPA reagent (method A). Satisfactory results were obtained by a mobile phase constituted of a mixture of acetate buffer (pH 5.5) [16] and methanol without the necessity of use the more expensive acetonitrile. Moreover, an ion-pairing reagent was not necessary thanks to the lower polarity of the derivatives in comparison with those of natural phenethylamines. The profile of LC separations in presence of IS is reported in Fig. 1A. The long time of chromatographic course was chosen so that other components of the formulation or extracts did not interfere with the analysis assuring an unambiguous identification of octopamine present in traces. On the other hand, a Gemini ODS column was used and the addition of an ion-pairing reagent (SHS) to the mobile phase was required (method B), otherwise the analytes are not resolved as they are poorly retained on the stationary phase owing to their polarity [34]. When a mobile phase consisting of a solution of SHS (pH 3) and acetonitrile was used, three SHS concentrations (10, 12 and 15 mM) were evaluated and the separation results were compared. Baseline resolution of all phenethylamines was achieved only with 10 or 12 mM concentrations, however at a concentration of 12 mM of SHS the retention time of the compounds was shorter, resulting in a more rapid separation with a good compromise of resolution (Fig. 1B). SHS speed the chromatographic run respect to the use of sodium dodecyl sulfate [5]. The active components of *C. aurantium* were well separated within 15 min similarly to the use of the Discovery HS F5 column by UV detection at wavelength of 225 nm [15], but obtaining a good separation also for the Tyr, N-methyltyramine and hordenine peaks. In addition, Tyr was resolved from octopamine without the necessity of a ternary mixture as mobile phase applying a pH-gradient for elution [7].

3.2. Method validation

The developed methods were validated to show compliance with international requirements for analytical methods in the quality control of pharmaceuticals. For validation of the analytical methods, the guidelines of the International Conference on Harmonization of Technical Requirements for the Registration of Pharmaceuticals for Human Use were followed [35].

3.2.1. Linearity

Under the described conditions linear calibration graphs were obtained and the concentration (nmol/mL) and calibration parameters are shown in Table 1. Amine standard solutions were injected after the described derivatization procedure for the method A, whereas they were injected directly

Table 2
Repeatability and intermediate precision data for retention time (t_R).

Compounds	Method	Repeatability						Intermediate precision	
		Day 1/Analyst A		Day 2/Analyst B		Day 3/Analyst C			
		t_R (min)	RSD (%)	t_R (min)	RSD (%)	t_R (min)	RSD (%)	t_R (min)	RSD (%)
Tyr	A	11.8	1.90	12.0	1.89	11.9	1.87	11.9	1.89
	B	3.5	1.28	3.8	1.35	3.6	1.26	3.6	1.31
Octopamine	A	30.6	1.40	30.4	1.36	30.5	1.44	30.5	1.42
	B	7.1	1.19	7.4	1.13	6.8	1.21	7.1	1.19
Tyramine	A	42.8	1.65	42.9	1.62	42.9	1.64	42.9	1.66
	B	10.8	1.14	10.9	1.13	10.7	1.19	10.8	1.18
Synephrine	B	8.4	1.36	8.9	1.40	9.1	1.29	8.9	1.39
N-Methyl-tyramine	B	12.3	1.65	12.7	1.60	13.3	1.58	12.8	1.64
Hordenine	B	13.4	1.05	13.5	1.10	13.7	1.08	13.5	1.12

Table 3
Accuracy data (capsules).

Compounds	Method	Spiked amount (mg/g)	Determined amount (mg/g)	Mean recovery[a] (%)	RSD (%)
Octopamine	A	0.10	0.31	99.9	0.9
		0.21	0.43	100.8	0.8
		0.42	0.63	99.8	0.9
Tyramine	A	0.06	0.18	100.0	1.2
		0.12	0.24	99.5	0.9
	B	0.25	0.37	101.3	1.0
		0.06	0.19	99.6	2.3
		0.13	0.26	98.8	1.9
		0.26	0.39	101.0	1.6
Synephrine	B	4.28	12.8	102.6	1.4
		8.55	17.1	99.9	1.2
		17.0	25.6	101.1	1.1
N-Methyl-tyramine	B	0.54	1.61	98.7	1.6
		1.07	2.14	100.0	1.5
		2.14	3.20	99.5	1.6

[a] $n = 2$ (method A) and $n = 3$ (method B).

Table 4
Results of LC analysis of phenethylamines in dietary supplements and extracts.

	Compounds	%Found[a] (RSD %)	
		Method A	Method B
Capsules[b]	Tyr	16.51 (0.87)	16.36 (1.02)
	Octopamine	0.021 (2.12)	<LOD
	Synephrine	–	0.855 (0.95)
	Tyramine	0.012 (1.91)	0.013 (1.85)
	N-Methyltyramine	–	0.107 (1.37)
Dry extract I[c]	Octopamine	0.007 (2.80)	<LOD
	Synephrine	–	4.15 (1.31)
	Tyramine	0.003 (2.05)	<LOD
	N-Methyltyramine	–	0.230 (1.13)
Dry extract II[c]	Octopamine	0.009 (2.05)	<LOD
	Synephrine	–	6.25 (1.19)
	Tyramine	0.005 (2.59)	<LOD
	N-Methyltyramine	–	0.48 (0.98)

(–) Not detected.
[a] Mean of five determinations expressed as a percentage (p/p) of the weight. Other ingredients:
[b] Guaranà, Sinetrol®, carnitine, magnesium stearate, chromium picolinate, silica.
[c] Flavonoids, essential oils, coumarin compounds.

for the method B. Duplicate (method A) or triplicate (method B) injections for each standard solution were made and the peak-area ratio of analyte to IS (method A) or the peak-area (method B) was plotted against the corresponding amine concentration to obtain the calibration graphs. Good linear relationship was found for each compound and for both methods, as indicated by the coefficient of determination $\geq$0.9998. Moreover, the linearity of calibration curves was confirmed using the quality coefficient (QC) as statistical test which was <5% [36].

3.2.2. Limit of detection and quantification

The limit of detection (LOD), evaluated as the injected amount of the compound that resulted in a peak-height of three times the noise level ($S/N = 3$), was in the range about of 0.05–0.07 (gain 1000) and 0.21–1.04 (gain 100) pmol for the methods A and B, respectively. On the other hand, the limit of quantification (LOQ), evaluated as the injected amount of the compound that resulted in a peak-height of ten times the noise level ($S/N = 10$), was in the range about of 0.17–0.23 and 0.70–3.47 pmol for the methods A and B, respectively. The LOD and LOQ values are reported in Table 1. The method A showed a higher sensitivity for the phenethylamine with primary amino group than these of the method B owing to easiness to work to gain 1000. That was not realizable by the method B, probably for the proximity of the excitation and emission wavelengths. In particular, the sensitivity increased 20, 10 and 3 times for octopamine, Tyr and tyramine, respectively.

3.2.3. Precision

Chromatographic separation precision of both methods, comprehensive of derivatization procedure for method A, was tested as repeatability and intermediate precision. The repeatability was valued by injections of five standard solutions containing Tyr, octopamine and tyramine for method A and ten standard solutions containing Tyr, octopamine, tyramine, synephrine, *N*-methyltyramine and hordenine for method B (concentration for each amine as in Fig. 1). Besides, the intermediate precision of the methods was determined with solutions prepared changing the parameters time-analyst: five solutions were prepared by the analyst A in the day 1, other five solutions by the analyst B in the day 2 and other five solutions by the analyst C in the day 3 (method A); ten solutions were prepared by the analyst A in the day 1, other

ten solutions by the analyst B in the day 2 and other ten solutions by the analyst C in the day 3 (method B). The repeatability of the methods was satisfactory as indicated by %RSD range 1.36–1.90 for the method A and 1.05–1.65 for the method B (Table 2). Furthermore, no statistically significant differences were found between inter-laboratory results.

3.2.4. Accuracy

The accuracy of both analytical methods was verified by analysing a commercial sample (capsules) fortified with known amounts of standard compounds corresponding to three levels of concentration among claimed content for octopamine, tyramine, synephrine and *N*-methyltyramine (approximately 50, 100 and 200%) and one level for Tyr (approximately 25%) of the nominal value of the formulation. The solutions were prepared in duplicate and in triplicate at each level for method A and method B, respectively. Quantitative recoveries were obtained in each instance: recovery = 99.5–101.3%; RSD = 0.8–1.2% for method A and recovery = 98.7–102.6%; RSD = 1.1–2.3% for method B as shown in Table 3.

3.3. Analysis of dietary supplements and phytoextracts

Both optimized methods were applied to the quali-quantitative analysis of phenethylamines in a commercial formulation (capsules) and phytoextracts (*C. aurantium*). The obtained results are reported in Table 4 and the data were expressed as percentage of the weight. Other formulation ingredients did not interfere with the analysis. Tyr and synephrine levels in the commercial formulation such as synephrine levels in the extracts were in agreement with the claimed content by manufacturers. Octopamine, tyramine and N-methyltyramine values agreed with the literature data [5,10,15], whereas hordenine was not detected in the examined samples. The values of Tyr and tyramine in capsules were comparable between methods A and B, whereas the methods showed to be in part complementary between them for the other amines: octopamine was quantified by the method A and the secondary amines by the method B. Chromatogram examples obtained from commercial dietary supplements and extracts are illustrated in Figs. 2 and 3, respectively. Peaks were identified on the basis both of the retention time (t_R) values and by spiking any sample with commercial standards. As it can be seen in Fig. 2A and B different gain (10 and 100) was used both for method A and method B in the same chromatographic course to obtain the simultaneous analysis of Tyr and phenethylamine alkaloids present at a notable different concentration in capsules.

4. Conclusion

OPA showed to be a fluorogenic pre-column reagent suitable for the simultaneous determination of Tyr, octopamine and tyramine present at very different amounts in a examined dietary supplement. The proposed derivatization method demonstrated a higher sensitivity for primary phenethylamines (about twenty-fold for octopamine) in comparison with the developed direct method. In addition, the derivatization method did not needs of an ion pair reagent to retain the primary phenethylamines and allowed a sure determination of octopamine in commercial products excluding all possibility of adulterations. Both described procedures are simple and the analyses were performed by mild conditions in absence of preliminary difficult extraction methodologies or laborious step of sample pre-treatment and without the necessity of an expensive instrumentation.

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