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Development and validation of HPLC method to measure active amines in plant food supplements containing *Citrus aurantium* L

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ABSTRACT

Citrus aurantium L. (bitter orange) is frequently used as an ingredient of food supplements aimed to reduce body weight or improve general physical performances. The most active compounds of *C. aurantium* are amines having adrenergic activity: octopamine, synephrine, tyramine, N-methyl-tyramine and hordenine. The quantification of these amines is critical since their content in food supplements is regulated by national/international rules. Some methods for the quantification of *C. aurantium* amines have been published, including the official method developed by AOAC, but most of them are not totally satisfactory for the analysis of complex matrixes, such as extracts or food supplements.

A new HPLC–UV–fluorescence procedure has therefore been developed; the method is quick and simple, and allows the analysis of samples after a rapid extraction procedure without any further cleaning step. The assay, using one or two detectors, showed good results during the validation tests performed according to the FDA guidelines.

1. Introduction

Botanicals and botanical preparations are widely consumed in Western diets from various sources; among them plant food supplements (PFS) are the most important. Unlike drugs, these products are generally perceived positively by consumers, since "natural" is often considered synonymous with safety (Hung, Hillier, & Ernst, 2011).

The popularity and the ease of access in shops or via the Internet have made for rapid diffusion of these products, and there are concerns about their quality, composition, and safety. The problem is particularly significant, when they are obtained from unregulated markets where illicit activities are not infrequent (Angell & Kassirer, 1998; Gurley, Gardner, & Hubbard, 2000).

Labeling of PFS is a further problem, since the accuracy of the declared composition requires reliable analytical methods (Gurley et al., 2000). Cianchino, Acosta, Ortega, Martinez, and Gomez (2008) analyzed four herbal dietary supplements aimed at weight control and identified undeclared active compound such as ephedrine and norephedrine. The claimed properties of these food supplements derived from the illegal additions, not the declared ingredients (Gurley et al., 2000). In 2010, Vaysse et al. (2010) analyzed 20 herbal medicines and dietary supplements marketed as 'natural' slimming products; 14 of them were adulterated with sibutramine and synephrine.

Citrus aurantium L., also known as bitter orange, is a botanical ingredient frequently used in food supplements aimed at reducing body weight or improving general performance. The most important active ingredients of *C. aurantium* are amines having adrenergic activity: octopamine, synephrine, tyramine, N-methyl-tyramine and hordenine (Fig. 1). The *C. aurantium* extract is permitted in food supplements, but several countries have established limits for the content of active amines (among others Italian Ministry of Health, 2012).

The background is that the FDA's ban of ephedrine-containing supplements led to increased use of *C. aurantium* as an alternative to *Ephedra*, with possible risk for consumers (Haller & Benowitz, 2000; Stohs, Preuss, Keith, Keith, Miller, & Kaats, 2011). Quality control of PFS containing *C. aurantium* is therefore very important and a suitable analytical method is needed, to enable producers to check the raw material and extracts.

Several analytical approaches have been developed for the detection and quantification of the amines contained in *C. aurantium*. Pellati, Benvenuti, Melegari, and Firenzuoli (2002)

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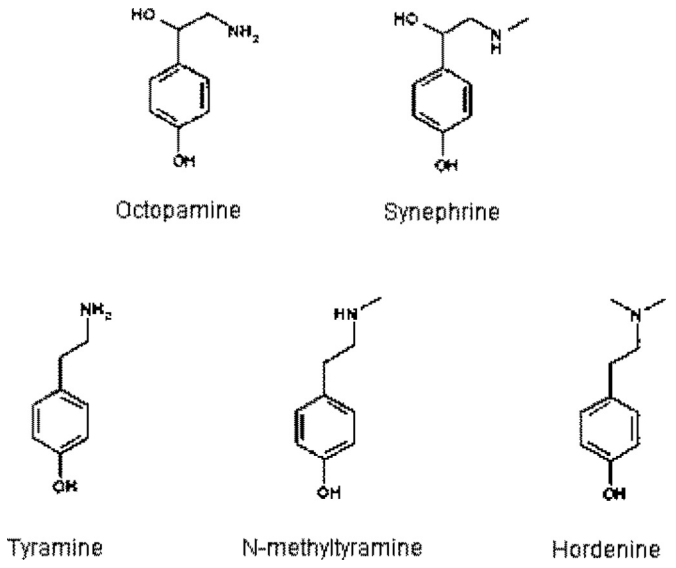

Fig. 1. Chemical structure of octopamine, synephrine, tyramine, N-methyl-tyramine, hordenine.

analyzed by reversed-phase only synephrine, octopamine and tyramine. Ephedrine alkaloids and synephrine were assayed using column-switching technique coupled to a cation exchange column with scanning wavelength ultraviolet and fluorescence detector (Niemann & Gay, 2003). Penzak, Jann, Cold, Hon, Desai, Gurley, and Seville (2001) analyzed synephrine and octopamine using IPC (Ion Pair Chromatography). None of these methods, however, demonstrated the ability to analyze the compounds of interest with acceptable validation data. The AOAC official method (Roman et al., 2004) allows the separation of most *Citrus* amines, but there are several problems in applying this method to PFS: efficacy in resolution is incomplete, the analysis takes too long and is costly, and chromatographic performance quickly deteriorates. Among published methods, that by Putzbach, Rimmer, Sharpless, and Sander (2007) based on ion-pair HPLC and fluorimetric detection (FD) was considered the most promising, being more sensitive and specific than other methods using UV detection (Arbo et al., 2008; Gurley, Wang, & Gardner, 1998) However, problems appeared when it was applied to complex matrices and multi-ingredient samples, in particular PFS.

Our aim was to develop a simple, inexpensive HPLC method for quality control of products containing *C. aurantium,* including PFS.

2. Material and methods

2.1. Reagents and chemicals

Standards of synephrine, octopamine hydrochloride, hordenine, tyramine and N-methyl-tyramine were purchased from Sigma–Aldrich (St. Louis, MO, USA), with purity ≥95%. The standards were stored according to the supplier's instructions. LC-grade water, methanol and acetonitrile were purchased from Sigma–Aldrich (St. Louis, MO, USA). Other reagents including 85% ortho-phosphoric acid, 0.1 N hydrochloric acid and sodium dodecyl sulfate were from Merck KGaA (Darmstadt, Germany).

2.2. Instrumentation

The HPLC equipment was from Jasco (Tokyo, Japan) and consisted of two pumps (Intelligent HPLC Pump model PU-880), a fluorimetric detector (model FP-1520), a UV/Visible detector (model UV-875) and an injection valve (Rheodyne, Cotati, CA, USA) with a 100 μL loop. The ChromNAV software Jasco was used for data acquisition and processing.

2.3. Chromatographic conditions

The chromatographic separations were carried out on a reversed phase LiChrospher RP-18 column (250 × 4 mm ID, particle size 5 μm, Merck, Darmstadt, Germany) in association with a LiChrospher 100 RP-18, 5 μm guard column, both maintained at 24 °C. The fluorescence detector was set at 270/305 nm ($\lambda_{Ex}/\lambda_{Em}$), and UV–Vis detector at 224 nm.

Gradient elution at a flow rate of 1 mL/min used the following mobile phases: A, 2.9 g/L sodium dodecyl sulfate in water adjusted to pH 4.2 with 85% ortho-phosphoric acid; B, 2.9 g/L sodium dodecyl sulfate:acetonitrile (62:38, v/v) adjusted to pH 4.2 with 85% ortho-phosphoric acid. The gradient is illustrated in Table 1.

At the end of the gradient program, the column was maintained at the initial conditions for 10 min before the next injection. The total run time was 40 min.

2.4. Preparation of stock and working solutions

A stock solution of each amine was prepared at a final concentration of 1 mg/mL in 0.1 N HCl and further diluted with 0.1 N HCl to obtain working solutions in the range 0.1–10.0 μg/mL; for synephrine only the range was 5.0–125.0 μg/mL. All solutions were stored at −20 °C until use.

2.5. Sample preparation

About 100 mg of finely ground and homogenized samples were precisely weighed and added to 25 mL of 0.1 N HCl plus 75 mL of a water:methanol 75:25 solution (v/v). Extraction efficiency was assessed by preparing PFS samples with and without the addition of 2.5 μg/g of analytes and extracting for 5, 10, 15, 20, 25 and 30 min under stirring by a magnetic device: 20 min was sufficient for optimal extraction. After extraction, samples were filtered through a 0.45-μm filter and injected into the chromatographic equipment.

2.6. Validation

A full validation of the method with two detectors was performed according to the current FDA Guidelines on Bioanalytical Method Validation (FDA, 2013).

2.6.1. System suitability test

The following parameters were calculated using the ChromNAV software: retention factor (K), separation factor between two neighboring peaks (α), peaks' tailing factor and column efficiency (number of theoretical plates).

2.6.2. Linearity

For each run, five standard stock solutions were prepared and analyzed with three independent injections; the concentrations ranged between 5 and 125 μg/mL for synephrine and between 0.1

Table 1
Gradient elution used in HPLC separation.

Total time	Phase A (%)	Phase B (%)
0 min	40	60
11 min	0	100
25 min	0	100
30 min	40	60

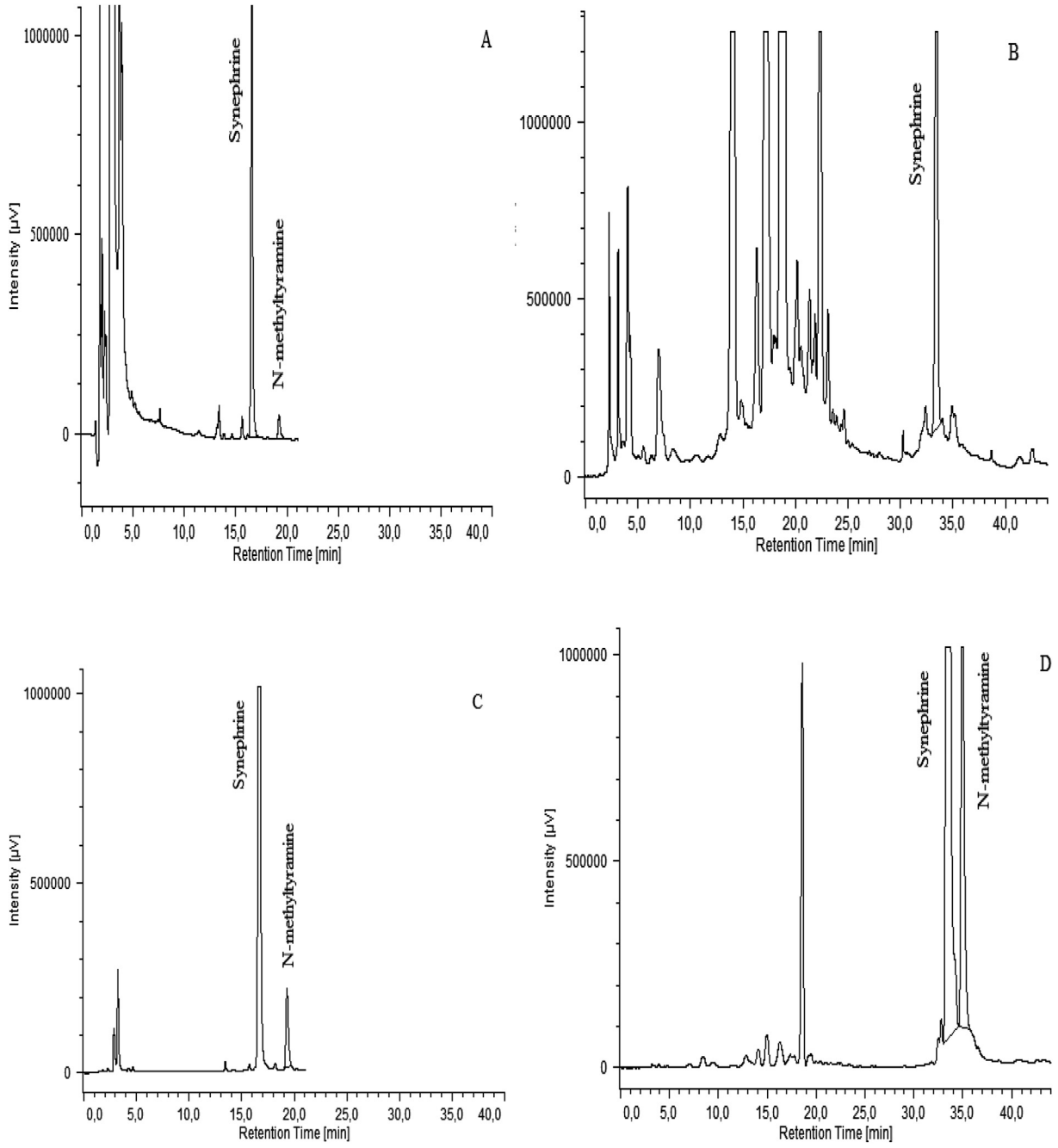

Fig. 2. Chromatograms of a PFS containing *Citrus aurantium* analyzed by the new method and Putzbach's protocol. Legend: Figure A: new method with UV detector; B: Putzbach's method with UV detector; C: new method with fluorimetric detector; D: Putzbach's method with fluorimetric detector.

Table 2
Results of system suitability tests for the five amines obtained during test analysis ($n = 3$).

	Retention times (min) (mean ± SD)	K[a] (mean ± SD)	α[b] (mean ± SD)	Symmetry factor (mean ± SD)	Theoretical plates (N) (mean ± SD)
Octopamine	17.68 ± 0.36	8.66 ± 0.25	1.08 ± 0.01	0.870 ± 0.02	42,232 ± 9582
Synephrine	19.00 ± 0.54	9.37 ± 0.21	1.08 ± 0.01	0.903 ± 0.01	43,208 ± 8888
Tyramine	21.40 ± 1.00	10.68 ± 0.19	1.10 ± 0.01	0.932 ± 0.04	41,542 ± 12,798
N-methyl -tyramine	23.26 ± 1.28	11.69 ± 0.27	1.08 ± 0.01	1.129 ± 0.004	29,020 ± 4532
Hordenine	24.95 ± 1.53	12.61 ± 0.37	1.08 ± 0.01	1.150 ± 0.01	15,845 ± 3635

[a] K (Retention factor) $= (t_R - t_0)/t_0$, where t_R and t_0 are retention times of sample components and sample solvent, respectively.
[b] α (Separation factor) $= (t_{R2} - T_0)/(t_{R1} - t_0)$, where t_{R2} and t_{R1} are retention times of two neighboring peaks.

Table 3
Limit of Detection (LOD), Limit of Quantitation (LOQ), sensitivity and results of linear regression analysis of calibration curves ($n = 3$) determined for the five amines using the UV or FD.

Analyte	Linear range (μg/mL)	Linearity equation UV	Linearity equation FD	Correlation coefficient (R^2) UV	Correlation coefficient (R^2) FD	LOD UV (ng/mL)	LOQ UV		LOD FD (ng/mL)	LOQ FD		FD/UV Sensitivity
							ng/mL	Area (mean ± SD)		ng/mL	Area (mean ± SD)	
Octopamine	0.1–5	$m = 252{,}843 \pm 6977$ $q = -18904 \pm 7613$	$m = 1{,}676{,}305 \pm 69{,}517$ $q = -7790 \pm 85{,}015$	0.9987 ± 0.0013	0.9998 ± 0.0003	30.0	100.0	19,451 ± 1324	7.5	25.0	48,482 ± 2748	4.0
Synephrine	5.0–125	$m = 185{,}596 \pm 388$ $q = 1{,}681{,}704 \pm 101{,}893$	$m = 397{,}861 \pm 5707$ $q = 1{,}197{,}631 \pm 287{,}316$	0.9997 ± 0.0019	0.9964 ± 0.0032	30.0	100.0	64,007 ± 1360	1.9	6.2	5762± 178	16.1
Tyramine	0.2–5.0	$m = 285{,}408 \pm 216$ $q = -10{,}113 \pm 614$	$m = 2{,}722{,}364 \pm 50392$ $q = 60{,}438 \pm 27{,}098$	0.9999 ± 0.00001	0.9998 ± 0.0002	60.0	200.0	42,962 ± 3011	1.8	6.0	22,557± 2362	33.3
N-methyl-tyramine	0.5–5.0	$m = 275{,}075 \pm 17{,}782$ $q = -2746 \pm 34{,}305$	$m = 2{,}270{,}365 \pm 24{,}515$ $q = 805{,}378 \pm 133{,}821$	0.9991 ± 0.0004	0.99231 ± 0.0048	30.0	100.0	19,075 ± 280	1.9	6.2	14,466± 1077	16.1
Hordenine	0.5–5.0	$m = 249{,}605 \pm 10{,}318$ $q = -23{,}019 \pm 21{,}818$	$m = 2{,}246{,}598 \pm 102{,}821$ $q = 135{,}339 \pm 176{,}818$	0.9989 ± 0.0007	0.9989 ± 0.0012	150.0	500.0	51,388 ± 3881	4.5	15.0	29,178 ± 1974	33.3

and 5 μg/mL for the other amines. Linear regressions were obtained by plotting the areas of analyte peaks *vs* the nominal concentrations. The calibration curve equations and the corresponding correlation coefficients (R^2) were calculated using both detectors.

2.6.3. Limits of Detection and Quantitation

The Limit of Detection and Quantitation (LOD and LOQ, respectively) were determined from calibration curve: the LOQ was defined as the lowest concentration of the calibration curves and was estimated after 5 injections based on a signal-to-noise ratio of 10. A signal-to-noise ratio of 3 was considered acceptable for estimating the LOD.

2.6.4. Precision

Intra-day precision was determined by preparing and analyzing, on the same day, five replicates of three different concentrations in the range 0.1–3.0 μg/mL for octopamine, tyramine, N-methyl-tyramine, hordenine and 10.0, 40.0, 80.0 μg/mL for synephrine.

Inter-day precision was evaluated by repeating the intra-day precision study on five different days. Precision was assessed by calculating the mean, standard deviation (SD), and the coefficient of variation (RSD%) of these values.

2.6.5. Accuracy

Accuracy was determined by evaluating spiked samples with two concentrations of octopamine, tyramine, N-methyl-tyramine, hordenine (1.0 and 3.0 μg/mL) and synephrine (8.0 and 12.0 μg/mL) and through the calculation of the RSD% between the calculated and the nominal values.

2.6.6. Extraction recovery

A PFS containing only *Hypericum perforatum* L. was selected as a blank matrix, since it does not contain the investigated amines. The content of ten capsules (0.5 g) was homogenized, spiked with the standard solutions and extracted as by the described procedure. The recovery for synephrine was carried out at low, medium and high concentrations (10.0, 50.0, 100.0 μg/mL); for octopamine, tyramine, N-methyl-tyramine, hordenine the concentrations were in the range of 0.1–5.0 μg/mL (three for each amine). Each concentration of both sets was analyzed in three replicates. The percentages of recovery were calculated by comparing the peak area ratio of the analytes measured in the samples to the peak areas of the corresponding standard solutions.

2.6.7. Stability

The stability of synephrine stock solutions (40.0 and 80.0 μg/mL) and of other amines (2.0 and 4.0 μg/mL) was determined on triplicate aliquots which were kept at room temperature for 8 h. Peak areas of these samples were compared with those of freshly prepared stock solutions. Similarly, the stability of the same standard solutions was assessed after storage for 20 days at −20 °C.

Freeze (−20 °C)-thaw stability of these solutions was assessed by comparing their concentrations after three freeze–thaw cycles with the concentrations determined at time zero.

2.6.8. Specificity

Potential interference between analytes and endogenous matrix components was investigated by analyzing six batches of *Hypericum perforatum* L. matrices. Peak areas of compounds co-eluting with the analytes should be less than 20% of the peak area of LOQ samples.

Table 4
Precision, accuracy and recovery of active amines determined with UV and FD ($n = 3$).

Analyte	Intra-day precision (RSD %)			Inter-day precision (RSD %)		Recovery (%) (mean ± SD)			Accuracy (%) (mean ± SD)		
	(μg/mL)	UV	FD	UV	FD	(μg/mL)	UV	FD	(μg/mL)	UV	FD
Octopamine	0.1	7.3	7.6	7.1	9.6	0.1	104.2 ± 12.8	107.8 ± 5.7	1.0	96.0 ± 0.1	98.5 ± 7.3
	2.0	3.4	5.8	3.8	5.6	1.25	89.5 ± 11.1	90.7 ± 12.8	3.0	103.9 ± 6.1	105.4 ± 6.2
	3.0	6.5	6.5	10.1	13.7	5.0	101.5 ± 7.8	102.8 ± 8.1			
Synephrine	10	1.3	1.2	3.3	1.3	10	85.9 ± 3.0	89.3 ± 2.6	8.0	95.8 ± 6.9	100.3 ± 10.8
	40	5.3	2.7	5.1	2.9	50	103.4 ± 14.3	90.3 ± 8.1	12.0	98.9 ± 5.2	108.2 ± 4.4
	80	4.8	4.3	3.5	3.6	100	96.2 ± 0.5	102.7 ± 2.2			
Tyramine	0.2	5.6	4.7	8.5	9.2	0.2	97.1 ± 7.7	103.9 ± 4.0	1.0	99.7 ± 31	110.3 ± 7.7
	2.0	5.0	4.6	4.0	3.8	1.25	81.4 ± 0.1	106.2 ± 2.8	3.0	102.5 ± 9.6	105.6 ± 5.2
	3.0	4.6	3.8	3.9	4.3	5	104.3 ± 5.5	106.6 ± 8.6			
N-methyl-tyramine	0.5	5.6	3.9	5.5	8.2	0.5	99.0 ± 1.3	102.1 ± 6.7	1.0	100.0 ± 4.4	103.7 ± 5.3
	2.0	5.0	6.8	5.8	6.2	1.25	95.8 ± 6.1	90.1 ± 6.9	3.0	96.9 ± 5.5	103.8 ± 5.9
	3.0	4.6	4.4	4.5	7.3	5.0	102.1 ± 4.5	105.7 ± 4.3			
Hordenine	0.5	2.8	3.2	4.8	10.7	0.5	108.4 ± 6.6	101.9 ± 11.3	1.0	90.2 ± 4.5	87.8 ± 8.1
	2.0	7.6	7.9	6.7	6.2	1.25	94.5 ± 3.2	109.5 ± 4.2	3.0	100.0 ± 0.9	103.2 ± 8.1
	3.0	7.8	2.8	7.0	6.4	5.0	90.5 ± 5.2	98.9 ± 4.8			

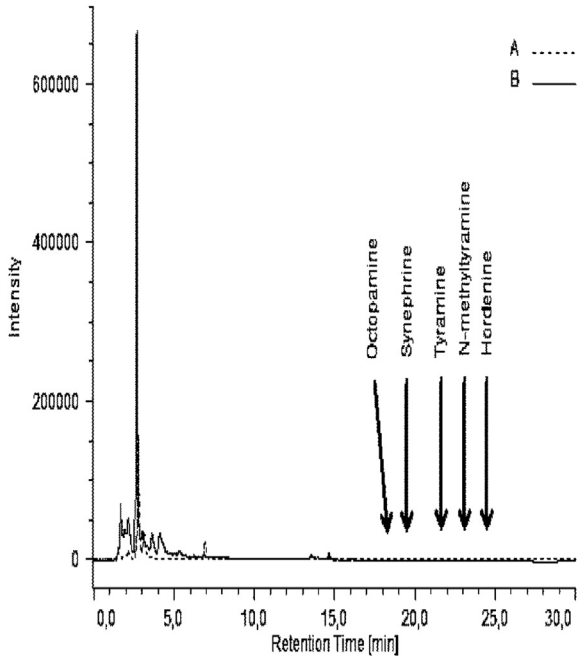

Fig. 3. Chromatogram of a blank extract (*Hypericum perforatum* L.) analyzed with fluorimetric (A) and UV (B) detector.

3. Results and discussion

3.1. Chromatography

The first step of the research was the application of known methods (Putzbach et al., 2007; Roman et al., 2004) for the detection of amines in *C. aurantium*, using both raw material and derivatives (extract and PFS). However, these approaches failed to provide an adequate chromatographic resolution probably because of the complexity of the extracted PFS matrices. After experimenting with different gradient conditions, a new ion-pair mobile phase was developed with a binary solvent system as described in Materials and Method. The characteristic analytes of *C. aurantium* (five amines) were well separated within 30 min at a flow rate of 1 mL/min at room temperature.

The chromatographic conditions used in the method here described were very different from those used in all other published methods: gradient elution, the ion-pair technique and the pH value (4.2). In particular, the pH 4.2 allowed the best separation of interfering peaks, when compared to other elution phases assayed. Fig. 2 shows a comparison between chromatographic separations of a PFS sample by the new developed method (left) and Puzbach's method (right). It is evident that the new method eliminates interferences due to complex matrix.

3.2. Identification of amines

The identity of the analytes was established by comparing the peak retention times and Relative Retention Times (RTT) with those of reference standards.

3.3. System Suitability Test (SST)

Table 2 shows data on suitability of the chromatographic system. Data obtained with UV and fluorimetric detectors were similar. The considered parameters show that the chromatographic system used in this assay is very efficient. Indeed, it yields retention factors (K) ranging between 8.7 and 12.6 (K acceptable values ≥ 2) (FDA, 2013) as well as symmetrical, sharp peaks and is therefore suitable for the quantification of the five analytes in complex matrices, such as PFS. Similar data on efficiency and selectivity were also obtained after about 200 injections of samples, showing the long life of the column and the consequent low cost of the assay.

Table 5
Extraction recovery (%) of active amines ($n = 3$).

Time of extraction (min)	Octopamine	Synephrine	Tyramine	N-methyl-tyramine	Hordenine
20	100.48 ± 2.69	101.88 ± 2.27	100.3 ± 2.73	99.79 ± 3.09	99.92 ± 3.14
15	99.97 ± 1.74	97.8 ± 3.01	99.81 ± 2.02	99.45 ± 2.86	98.9 ± 2.99
10	99.83 ± 3.22	99.85 ± 2.85	99.70 ± 1.98	99.34 ± 2.79	98.9 ± 3.29
5	92.49 ± 2.05	94.33 ± 1.10	94.75 ± 2.19	93.8 ± 2.67	95.02 ± 3.34

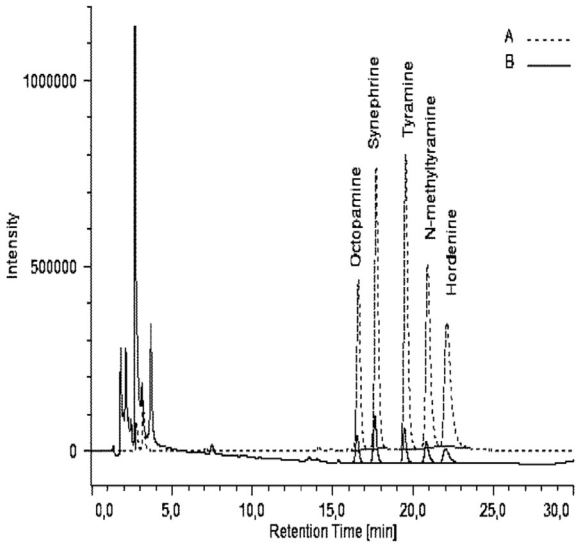

Fig. 4. Chromatogram of a blank extract (*Hypericum perforatum* L.) spiked with a standard mixture (10 μg/mL) analyzed with fluorimetric (A) and UV (B) detector.

3.4. Validation

3.4.1. Linearity, LOD and LOQ

The method was linear between 5 and 125 μg/mL for synephrine and between 0.1 and 5 μg/mL for other amines, corresponding to the tested concentrations, as shown by the correlation coefficients (R^2) being always greater than 0.99. Table 3 shows the linearity results (no significant variation of the slope between calibration curves, RSD being between 0.08 and 6.5%, as well as the Limit of Detection (LOD) and Limit of Quantitation (LOQ) values: LOD obtained by fluorescence detector was between 1.8 and 7.5 ng/mL and LOQ was between 6.0 and 25.0 ng/mL for tyramine and octopamine respectively. The fluorimetric detection was 4–30 times as sensitive as UV detection. These ranges of sensitivity were in every case suitable for accurate determination of the amines in *C. aurantium* samples.

3.4.2. Precision, accuracy, specificity and recovery

Table 4 shows the results of precision, accuracy and recovery experiments. Intra- and inter-day precision data showed RSD% values always <15% so that the method is precise, and as the

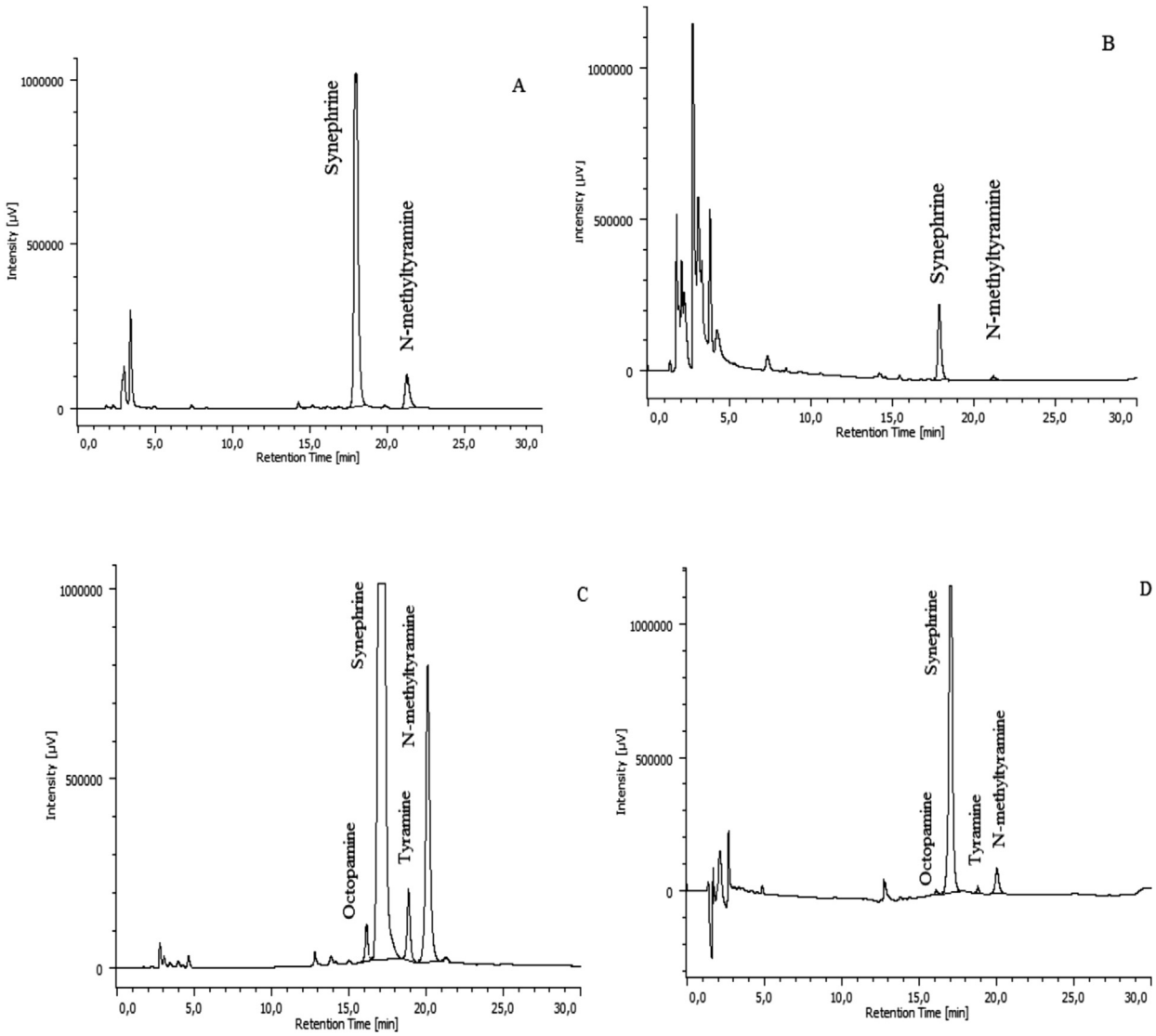

Fig. 5. Chromatograms of a plant food supplement (tablets, titrated at 5% synephrine) containing *Citrus aurantium* analyzed with fluorimetric (A) and UV (B) detector and of a *C. aurantium* extract revealed with fluorimetric (C) and UV (D) detector.

calculated accuracy was always within 15% of the nominal concentration, the method can be considered accurate. A blank sample containing only *H. perforatum* L. was tested to confirm the absence of peaks having retention times similar to those of *C. aurantium* amines (Fig. 3). No peak at the retention times of the amines had an area exceeding 20% of LOQ values. The recovery with both detection systems ranged between 80 and 110% for all amines.

3.4.3. Stability

Stock solutions of synephrine, octopamine, tyramine, N-methyltyramine and hordenine were stable for at least 24 h at room temperature: the peak areas in the stock standard solution compared with those for freshly prepared solution ranged between 99.50% ± 3.6 and 91.0% ± 3.2 for UV detector and between 90.5% ± 3.8 and 104.2% ± 0.5 for the fluorimetric detector. The same stock solutions were stable for at least 1 month at −20 °C: the long-term stability ranged between 91.4% ± 5.9 and 102.2% ± 3.7 for the UV detector and between 90.8% ± 5.9 and 108.9% ± 5.6 for the fluorimetric detector.

Freeze/thaw stability experiments showed that the analytes were stable in 0.1 N HCl for at least three freeze–thaw cycles at −20 °C: the stability ranged between 91.9% ± 1.3 and 101.9% ± 2.5 for the UV detector and between 91.9% ± 1.1 and 107.8% ± 2.2 for the fluorimetric detector.

Stability of the 5 amines in the final extract was at least 48 h at 4 °C.

3.5. Application of the method

3.5.1. Optimization of time extraction

Spiked blank samples analyzed after different extraction times showed that the recovery of the analytes reached a percentage close to 100% after 20 min of magnetic stirring (Table 5). Therefore, all analyzes were performed using that extraction-time.

3.5.2. Applicability

The applicability of the method was finally verified by using both a blank matrix of *H. perforatum* L. and two real samples (a *C. aurantium* extract and a commercial PFS). Standard stock solutions (10 μg/mL) of the amines were added to the blank matrix in order to evaluate peak separation and method suitability. Figs. 4 and 5 show the resulting chromatograms, where separation was highly satisfactory in all the samples.

4. Conclusion

This paper describes the development, validation and application of a LC/UV/Fluorescence assay for quantitative analysis of five active amines in *C. aurantium* raw material and derivatives. The method has been fully validated according to FDA Guidelines for Bioanalytical Method Validation (FDA, 2013) showing accurate, precise, selective, fast and relatively inexpensive analyzes. The quantification of these amines is particularly important since their concentrations in PFS are regulated by national and international laws and for post-market control of undeclared illegal addition of synephrine and octopamine. The improved chromatographic system and the use of a fluorescence detector provide a validated tool to analyze the five active amines in complex matrices, including plant food supplements.

Acknowledgments

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