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Determination of octopamine and tyramine traces in dietary supplements and phytoextracts by high performance liquid chromatography after derivatization with 2,5-dimethyl-1H-pyrrole-3,4-dicarbaldehyde

Rita Gatti*, Cinzia Lotti, Rita Morigi, Aldo Andreani

Department of Pharmaceutical Sciences, Faculty of Pharmacy, Alma Mater Studiorum, University of Bologna, Via Belmeloro 6, 40126 Bologna, Italy

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ABSTRACT

The use of 2,5-dimethyl-1H-pyrrole-3,4-dicarbaldehyde (DPD) as a pre-column derivatization reagent for HPLC (high performance liquid chromatography) analysis of octopamine (oct) and tyramine (tyr) is proposed. The compound reacts under mild conditions (2 min at ambient temperature) with primary amino groups. The derivatization conditions were optimized by considering different parameters (temperature, time and reagent concentration). The synthesized oct and tyr adducts were characterized by ^{1}H NMR (nuclear magnetic resonance), ESI-MS (electrospray ionization mass spectrometry), IR (infrared) and UV (ultraviolet). Derivative chromatographic separations were performed on a Sinergy Hydro-RP column (150 mm × 4.6 mm i.d.) using a mobile phase consisting of methanol and triethylammonium phosphate buffer (pH 3; 10 mM) at varying composition gradient elution and at a flow rate of 0.8 mL/min. Detection was set at λ = 320 nm. The obtained results were compared with those achieved by a validated direct HPLC method with detection at λ = 275 nm using a Sinergy Polar-RP column (250 mm × 3 mm i.d.) by isocratic elution conditions with a mobile phase consisting of methanol/acetonitrile/sodium pentanesulphonate (SPS; pH 3; 10 mM), 7.5:7.5:85 (v/v/v) at a flow rate of 0.3 mL/min. Derivatization method sensitivity proved to be ten times higher than direct method. Limit of detection of oct and tyr was 0.010 and 0.008 μg/mL, respectively. The reliability of the pre-column method was satisfactory also in terms of linearity (from 0.028 to 1.255 and 0.024 to 1.244 μg/mL for oct and tyr, respectively), precision (relative standard deviation ≤2, without significant differences between intra-day and inter-day data) and recovery (from 98.9 to 101.2%). The proposed method showed to be suitable for a reliable determination of oct and tyr traces in commercially available phytoproducts using the instrumentation usually present in any analytical laboratory.

1. Introduction

Over the last few years, after the ban of *Ephedra*-containing dietary supplements, *Citrus aurantium*-containing formulations have rapidly replaced *Ephedra* products for the treatment of obesity in humans, though they might carry the same health risks as *Ephedra* [1].

C. aurantium (bitter orange) contains a variety of flavonoids [2,3] and a number of biogenic amines such as synephrine (syn), oct, tyr, N-methyltyramine (Nmtyr) and hordenine (hor). Syn is similar in structure to ephedrine, while oct differs structurally from norepinephrine in that it lacks one hydroxyl group on the aromatic ring. Oct is found in nervous tissue of both invertebrates and vertebrates. In invertebrates oct almost certainly functions as a neurotransmitter [4,5]. On the other hand, both the presence and effects of oct in mammals may be indicative of a "false neurotransmitter" action at noradrenergic terminals and receptors [4–6]. Syn has lipolytic effects in human fat cells only at high doses, and oct does not have lipolytic effects in human adipocytes [1]. Syn is the main biogenic amine present in *C. aurantium*, whereas the other phenethylamine alkaloids were found at significantly lower levels. One study compared syn, oct and tyr content in fruits, extracts and herbal products of *C. aurantium*. HPLC analysis was carried out on a LiChrospher RP-18 column (125 mm × 4.0 mm i.d., 5 μm), using a mobile phase of 0.02 M citric acid/0.02 M NaH_2PO_4 (7:3, v/v) (adjusted to pH 3.0) at a flow rate of 1.0 mL/min. Detection was set at 220 nm. Of the three examined alkaloids, syn was the main constituent with the lowest concentration occurring in fresh fruits. Oct and tyr were present in only very small amounts, ranging from below the limit of quantification to <0.03 and <0.06%, respectively. In only one sample (herbal product) a high content of oct (about 0.15%) was found [7].

The use of dietary supplements containing *C. aurantium* extracts may cause increase in blood pressure, heart rate and incidence of myocardial infarction. *C. aurantium* has been implicated in adverse

* Corresponding author. Tel.: +39 051 2099707; fax: +39 051 2099734.
E-mail address: rita.gatti2@unibo.it (R. Gatti).

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cardiovascular reactions, although currently data are insufficient to support any of these adverse events [8–10]. Oct and tyr are the phenethylamine alkaloids most suspected to cause toxicological effects. Consumption of high amounts of these amines can result in intoxication symptoms such as migraine, other types of headache and hypertension [11,12]. Consumers should be advised that *C. aurantium* products have adverse effects on hemodynamics and may interact with many drugs, owing to the inhibition of intestinal cytochrome P450 (CYP) 3A4, which is an enzyme responsible for the metabolism of a large variety of drugs [1]. Therefore, oct was included since 2006 in the "The Prohibited List International Standard" by WADA (World Anti-Doping Agency) among the drugs banned from competitions [13].

Oct and tyr quantification can be considered a significant indicator of weight-loss product quality and safety, as this is useful to evaluate whether manufactures are "spiking" products with higher levels than would normally be found in *C. aurantium*. Continued researches are steered towards the application of fully validated methods to monitor the quality of new *C. aurantium* dietary supplements and extracts brought onto the market. In particular, formulations containing Sinetrol® are commercially available, since it seems to contribute to the loss of the body fat weight without any secondary effect. Sinetrol® is a polyphenolic mixture of flavonoids and is composed of *Citrus*-based fruits extracted by physical treatment of specific varieties of red orange (*Citrus sinensis* L. *Osbeck*, *Blood group*), sweet orange (*C. aurantium* L. *var. sinensis*), bitter orange (*C. aurantium* L. *var. amara*), grapefruit (*Citrus paradise*) and guarana (*Paulinia cupanna*) [14]. However, as far as we know the determination of individual phenethylamines in Sinetrol® is not reported in literature.

Analytical determination of biogenic amines is not simple, because they have a polar structure and they are present at lower levels in a complex matrix. Several techniques have been reported for their quali-quantitative analysis, but chromatographic and electrophoretic methods are the preferred analytical techniques, because of their highly efficient separation before detection of individual components [15]. HPLC with UV detection at the wavelengths of 210, 225, 273, 275 and 280 nm [7,8,15–22] is the most used analytical technique for detection of *Citrus* alkaloids. Anyway, the minor compounds were frequently not found in the examined materials, owing to the poor absorptivity of the biogenic amines at the ultraviolet–visible (UV–vis) wavelengths.

It is now well known that detection at short wavelengths (190–220 nm) increases the sensitivity combined with a loss in selectivity, whereas long wavelength detection, with its higher selectivity, usually requires higher concentration (less sensitivity) of the analyte due to the lower molar absorptivity (ε) at these wavelengths. To improve the detectability of compounds the HPLC derivatization is performed using various detection-sensitive properties such as electrochemical, absorbance and fluorescence [23–32]. The advantage of UV–vis detection over other methods, however, is its extended applicability combined with a good sensitivity. Moreover, contrarily to other detection systems, the UV–vis detectors are very reliable instruments. Derivatization reactions allow an increase of UV–vis detectability by pre- or post-chromatographic techniques offering the potential to optimize selectivity as well as the sensitivity of detection [23]. Frequently, the derivatization methods permit the determination of compound traces where otherwise it would have been impossible. In particular, pre-column derivatization presents the advantage to increase the hydrophobicity of the molecules so that they can be retained on the reversed-phase columns.

Commercially available derivatization reagents commonly used for derivatization of amino compounds are phenyl isothiocyanate (PITC), 4-nitrobenzoyl chloride, *p*-nitrobenzyl bromide, 2,4-dinitrofluorobenzene (DNFB), *o*-phthaldialdehyde (OPA), 9-fluorenylmethyl chloroformate (FMOC-Cl), dansyl chloride (Dns-Cl), 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F), naphthalene-2,3-dicarboxaldehyde [24,25]. However, these reagents can involve different drawbacks such as limited selectivity and sensitivity, toxicity, low stability of the derivatives, a time-consuming derivatization procedure or need of extraction method before the analysis.

Recently, we have proposed 2,5-dimethyl-1H-pyrrole-3,4-dicarbaldehyde (DPD) as a new pre-column derivatization reagent for HPLC analysis of amino acids [26]. DPD reacted in milder and faster reaction conditions than PITC and FMOC-Cl, whose derivatization procedure is lengthy, because the reagent excess has to be removed before analysis. The derivatization conditions resulted better also than those obtained using as reagents 2,7-dimethyl-3,8-dinitrodipyrazolo[1,5-a:1′,5′-d]pyrazine-4,9-dione (DDPP) and 4,7-phenanthroline-5,6-dione (panquinone), which were proposed in our previously studies on amino acid analysis [27–30]. In addition, DPD was selective towards the primary amino group in comparison with Dns-Cl, FMOC-Cl, NBD-F, fluorescein isothiocyanate, DDPP and DNFB, showing also to be devoid of toxic effects.

Derivatizing reagents recently used for the analysis of oct and tyr are the 2,6-dimethyl-4-quinolinecarboxylic acid N-hydroxysuccinimide ester (DMQC-OSu), Dns-Cl and OPA. In general, OPA is a compound employed as post-column derivatization reagent of primary phenethylamine alkaloids owing to the instability of its adducts [15,20]. However, pre-column derivatization procedure provides, with similar limits of sensitivity [4,11,31], the advantage of a simpler methodology with a less expensive and less complex equipment. DPD is a compound structurally analogue to OPA reagent and reacts quickly (1–2 min at ambient temperature) as OPA, but it has the convenience of having highly stable derivatives. In addition, the derivatization reaction goes on without a mercapto compound as co-reagent. On the other hand, the derivatization by using DMQC-OSu presents the disadvantage of reaction mixture incubation at 20 °C for 40 min leading to few by-products [33]. Dns-Cl needs a long time of reaction and a low concentration, otherwise the increase of the peak area background decreases the sensitivity of determination [34,35].

The objective of this work is to present a new UV–DAD HPLC method based on DPD derivatization for the sensitive detection of oct and tyr traces in dietary supplement, Sinetrol® and *C. aurantium* dry extract samples. The synthesized oct and tyr DPD derivatives were characterized by ^{1}H NMR, ESI-MS, IR and UV. The performance of the HPLC method was evaluated with respect to linearity, accuracy, precision, detection and quantification limits, specificity and solution stability. The obtained results were compared with those found by a direct method used as reference method.

2. Experimental

2.1. Materials

(±) Octopamine (oct) hydrochloride >95%, tyramine (tyr) hydrochloride 99%, (±) synephrine (syn) 98%, cinchonine 98% (used as internal standard, IS), phosphoric acid ≥85%, methanol Chromasolv®, acetonitrile Chromasolv®, SPS and 2,5-dimethylpyrrole used for the DPD synthesis were purchased from Sigma–Aldrich (Milan, Italy), whereas hordenine (hor) and N-methyltyramine (Nmtyr) from Carbone Scientific Co. Ltd. (London, United Kingdom). Phytoproducts were purchased from a local pharmacy; boric acid ≥99.5% and triethylammonium (TEA) ≥99.5% were obtained from Fluka (Buchs, Switzerland). DPD was synthesized and purified as previously described [36]. Purified water by a Milli-RX (Millipore, Milford, MA, USA) apparatus was used for the preparation of all solutions and mobile phases.

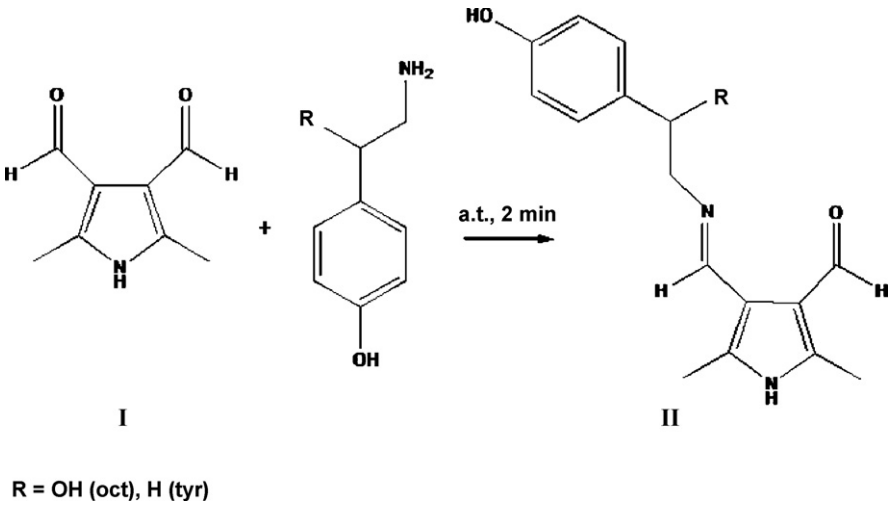

Fig. 1. Reaction scheme of phenethylamines with DPD (I).

2.2. Solutions

DPD solution (about 55 mM) was prepared daily in a mixture A:B (70:30, v/v), where A is methanol and B is borate buffer (pH 10; 0.4 M). Then, IS solution (0.4 mM) was prepared by dissolving cinchonine in methanol/water 25:75 (v/v). Phenethylamine solutions were prepared in water (concentration as calibration ranges). Borate buffer solution (pH 10; 0.4 M) was prepared by dissolving boric acid in water and adjusting to pH 10 with 0.1 M sodium hydroxide. TEA phosphate buffer (pH 3; 0.01 M) was prepared by dissolving TEA in water and adjusting to pH 3 with phosphoric acid. SPS (pH 3, 10 mM) was prepared by dissolving SPS in water and adjusting to pH 3 with phosphoric acid.

2.3. Equipment

The liquid chromatograph consisted of a PU-1580 pump equipped with the LG-1580-02 ternary gradient unit and a diode array detector (DAD) model MD-910 (Jasco Corporation, Tokyo, Japan). Data were collected on a PC equipped with the integration program Borwin-PDA. The solvents were degassed on line with a degasser model DG 2080-53 (Jasco Corporation). Manual injections were carried out using a Rheodyne model 7725i injector with 20 μL sample loop. A column inlet filter (0.5 μm × 3 mm i.d.) model 7335 Rheodyne was used. Sonarex Super RK 102 (35 KMZ) Bandelin (Berlin, Germany) equipment with thermostatically controlled heating (30–80 °C) was used for ultrasonication. The ^{1}H NMR spectra were recorded in $(CD_3)_2SO$ on a Varian MR 400 MHz (ATB PFG probe); the chemical shift (referenced to solvent signal) is expressed in δ (ppm) and J in Hz; abbreviation: ph = phenyl. ESI-MS was performed using a Finningan LCQ Deca instrument from Thermo Electron (San Jose, CA) equipped with Xcalibur software. Full mass and MS/MS spectra were acquired in positive mode and in the MS1 scanning mode (m/z 100–300). The compounds were dissolved in methanol and infused in the ESI source by using a syringe pump; the flow rate was 5 μL/min. The IR spectra were recorded in KBr on a Nicolet Avatar 320 ESP spectrometer; ν_{max} is expressed in cm^{-1}. UV spectra were recorded on a Hewlett Packard 8453 spectrophotometer (Waldbronn, Germany).

2.4. Synthesis of phenethylamine adducts

The methanol solution of DPD (0.264 mmol) and oct or tyr hydrochloride (0.264 mmol) was refluxed for 2–4 h according to a TLC test. After addition of diethyl ether, the pure product was collected by filtration and characterized as follows.

2.4.1. Oct adduct: (±)-4-[(E){[2-hydroxy-2-(4-hydroxyphenyl)ethyl]imino}methyl]-2,5-dimethyl-1H-pyrrole-3-carbaldehyde hydrochloride

mp = 195 °C (dec.), uncorrected value; yield = 50%; IR: 3220, 1670, 1647, 1075, 955; ^{1}H NMR: 2.52 (3H, s, CH_3), 2.59 (3H, s, CH_3), 3.85 (1H, m, CH_2), 4.02 (1H, m, CH_2), 4.82 (1H, m, CHOH), 5.90 (1H, d, CHOH, J = 4.4), 6.75 (2H, d, ph, J = 8.6), 7.21 (2H, d, ph, J = 8.6), 8.81 (1H, d, CH=NH^+, J = 15.0), 9.44 (1H, s, phOH), 9.79 (1H, s, CHO), 12.50 (1H, dt, CH=NH^+, J = 15.0, J = 7.4), 13.31 (1H, s, NH). After D_2O addition, owing to proton exchange of OH and NH^+ groups, an evident simplification of CH_2, CHOH and CH=NH^+ signals was observed. The ^{1}H NMR spectra changes as follows: 2.49 (3H, s, CH_3), 2.57 (3H, s, CH_3), 3.83 (1H, dd, CH_2, J = 13.2, J = 8.0), 3.99 (1H,

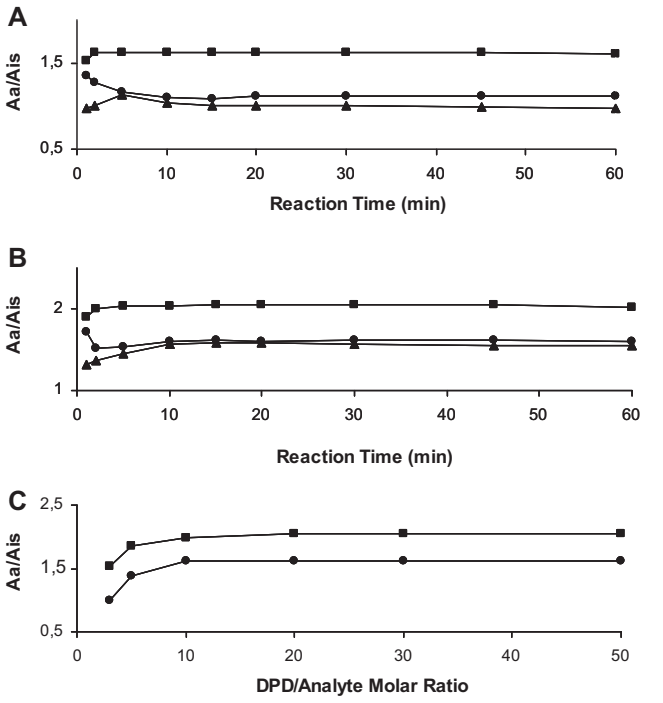

Fig. 2. Effect of the temperature on the derivatization reaction of oct (A) and tyr (B) with DPD. Reaction mixture at ambient temperature (■), 50 °C (●) and 70 °C (▲). (C) Influence of the reagent to phenethylamine molar ratio on the derivatization reaction of oct (●) and tyr (■) with DPD; A_a, analyte area and A_{IS}, IS area. Each point was obtained from the mean of three values (RDS <1.5%).

Table 1
Data for calibration graphs ($n=7$), limit of detection (LOD) and quantification (LOQ).

Compounds	Method	Slope[a]	y-Intercept[a]	Determination coefficient	Concentration range (μg/mL)	LOD (μg/mL)	LOQ (μg/mL)
Oct	A	0.1024 (±0.00060)	−0.00094 (±0.0001)	0.9999	0.028–1.255	0.010	0.033
	B	–	–	–	–	0.100	0.332
Tyr	A	0.1061 (±0.00056)	−0.00056 (±0.00007)	0.9999	0.024–1.244	0.008	0.027
	B	–	–	–	–	0.090	0.301
Syn	B	34708.6 (±103.4)	+40694.6 (±9357.4)	0.9999	12.99–182.0	0.173	0.577
Nmtyr	B	31933.6 (±178.2)	−2511.8 (±3133.1)	0.9998	2.230–35.71	0.124	0.413
Hord	B	–	–	–	–	0.129	0.430

[a] According to $y = ax + b$, where x is the analyte concentration expressed as μg/mL, y is the ratio of analyte peak-area to IS peak-area (method A) and analyte peak-area (method B).

dd, CH_2, $J=13.2$, $J=3.2$), 4.80 (1H, dd, CHOH, $J=8.0$, $J=3.2$), 6.74 (2H, d, ph, $J=8.4$), 7.20 (2H, d, ph, $J=8.4$), 8.72 (1H, s, CH=NH^+), 9.77 (1H, s, CHO). The geometrical configuration was determined by means of a NOE (nuclear overhauser effect) experiment: irradiation of CH=NH^+ (8.81 ppm), gave NOE at 2.52 ppm (CH_3), at 3.85 ppm (CH_2) and at 4.02 ppm (CH_2). These results are in agreement with the E configuration. ESI-MS (positive mode): m/z 287 $[M+H]^+$, MS/MS m/z 269, 152 and 136. UV (methanol) $\lambda = 218$ nm ($\varepsilon = 37{,}600$), $\lambda = 316$ nm ($\varepsilon = 17{,}200$).

2.4.2. Tyr adduct: 4-[(E){[2-(4-hydroxyphenyl)ethyl]imino}methyl]-2,5-dimethyl-1H-pyrrole-3-carbaldehyde hydrochloride.

mp = 215 °C (dec.), uncorrected value; yield 55%; IR: 3172, 1675, 1628, 838, 542; ^{1}H NMR: 2.48 (3H, s, CH_3), 2.58 (3H, s, CH_3), 2.93 (2H, t, CH_2, $J=7.0$), 4.02 (2H, m, CH_2), 6.71 (2H, d, ph, $J=8.4$), 7.06 (2H, d, ph, $J=8.4$), 8.76 (1H, d, CH=NH^+, $J=14.0$), 9.36 (1H, s, OH), 9.75 (1H, s, CHO), 12.34 (1H, s broad, CH=NH^+), 13.41 (1H, s broad, NH). After D_2O addition, ^{1}H NMR spectra changes as follows: 2.45 (3H, s, CH_3), 2.55 (3H, s, CH_3), 2.92 (2H, t, CH_2, $J=7.0$), 4.00 (2H, t, CH_2, $J=7.0$), 6.69 (2H, d, ph, $J=8.4$), 7.06 (2H, d, ph, $J=8.4$), 8.66 (1H, s, CH=NH^+), 9.72 (1H, s, CHO). In order to determine the geometrical configuration, a NOE experiment was performed: when the CH=NH^+ (8.76 ppm) was irradiated, NOE was observed at 2.48 ppm (CH_3) and at 4.02 ppm (CH_2) thus confirming the E configuration. ESI-MS (positive mode): m/z 271 $[M+H]^+$, MS/MS m/z 151, 136 and 121. UV (methanol) $\lambda = 218$ nm ($\varepsilon = 38{,}900$), $\lambda = 316$ nm ($\varepsilon = 17{,}500$).

2.5. Derivatization reaction

To 100 μL aliquot of phenethylamine solution in presence of IS (12.5 μg/mL) DPD solution was added (40 μL). The reaction was carried out in a micro-centrifuge tube (1.5 mL) for 2 min after 5 s of ultrasonication at room temperature; then, 100 μL of mobile phase (TEA phosphate buffer/water 30:70, v/v) was added. Finally, a 20 μL aliquot was injected into the chromatograph.

2.6. Chromatographic conditions

The HPLC separations were performed at 33 ± 2 °C on a Phenomenex Sinergy 4μ Hydro-RP 80A (150 mm × 4.6 mm i.d.) stainless steel column, with a guard column packed with the same stationary phase (method A). Gradient elution conditions were used with a mobile phase consisting of a mixture A:B, where A is methanol and B is TEA phosphate buffer (pH 3; 0.01 M) at a flow rate of 0.8 mL/min. The adopted gradient profile was $t=0$ min, 17% A; $t=6$ min, 17% A; $t=8$ min, 23% A; $t=13$ min, 23% A; $t=14$ min, 62% A; $t=15$ min, 62% A; $t=18$ min, 17% A. UV detection at $\lambda = 320$ nm was used. A Phenomenex Sinergy 4μ Polar RP 80A (250 mm × 3 mm i.d.) stainless steel column with a guard column packed with the same stationary phase was adopted at 33 ± 2 °C for the direct analysis of phenethylamines by isocratic elution conditions (method B) using a mixture A:B, where A is a binary mixture of methanol/acetonitrile 50:50 (v/v) and B is SPS (pH 3; 10 mM), 15/85 (v/v), at a flow rate of 0.3 mL/min. UV detection at $\lambda = 275$ nm was used.

2.7. Analysis of dietary supplements and phytoextracts

2.7.1. Sample preparation

2.7.1.1. Capsules. The content of 10 capsules was mixed and 0.5 g of powder was extracted three times with about 30 mL of water and transferred to a 100 mL volumetric flask, in presence of IS (12.5 μg/mL) for the method A.

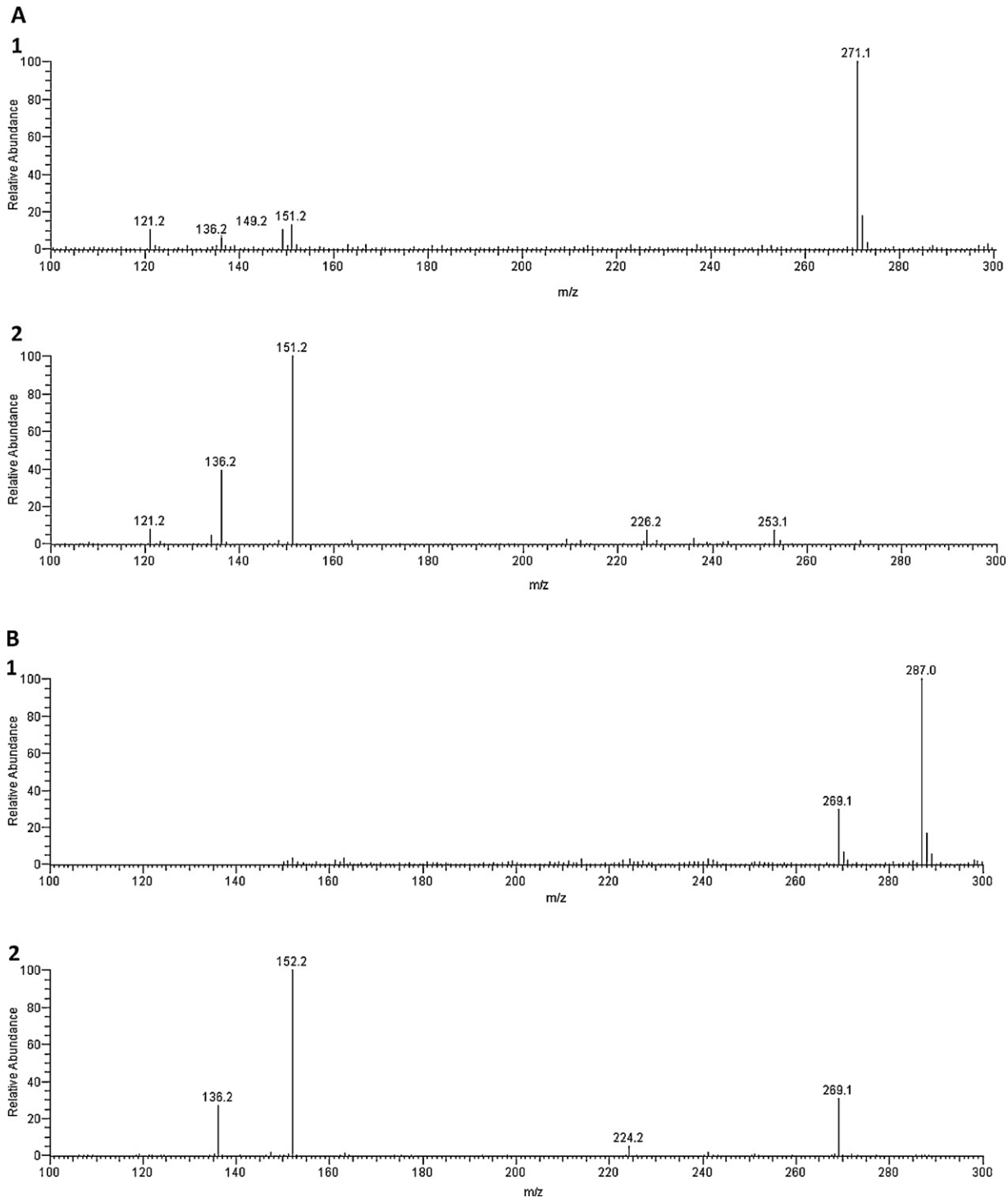

Fig. 3. (A) Mass spectra of tyr adduct: (1) Full (+)-ESI-MS spectrum and (2) MS/MS spectrum of *m/z* 271.1 (B) Mass spectra of oct adduct: (1) Full (+)-ESI-MS spectrum and (2) MS/MS spectrum of *m/z* 287.0.

2.7.1.2. Sinetrol® and C. aurantium dried extract (4% syn). A 0.5 g amount of each powder was dissolved with 100 mL of water, in presence of IS (12.5 μg/mL) for the method A.

2.7.1.3. Assay procedure. After appropriate extraction (capsules) or dissolution (extracts) of sample in 100 mL of water by ultrasonication for about 30 min and centrifugation for 15 min at 9000 rpm at ambient temperature, the supernatant or the resulting solution was filtered through a 0.22 μm membrane filter. Then, a 150 μL aliquot of the obtained solution (method B) and a 100 μL volume of the derivatized solution (method A) were diluted with 100 μL of the adequate mobile phase, respectively. Each final solution was injected in triplicate in the chromatograph. The phenethylamine content in each sample was determined by external standardization.

3. Results and discussion

3.1. Derivatization reaction

In order to apply DPD (Fig. 1) to the analysis of oct and tyr the optimization of derivatization system was investigated considering particularly the effect of temperature and reagent concentration on the course of the reaction of oct and tyr (Fig. 2). At ambient temperature, both derivatization reactions were found to be complete after about 1–2 min. As it can be seen in Fig. 2A and B, the response showed to be reproducible for all the time of the study indicating a good stability of the examined derivatives. At higher temperatures reduced responses were observed, probably owing to secondary reactions. In the chosen conditions (2 min at ambient temperature) the yields of phenethylamine adducts increased to reach a plateau at a molar ratio of reagent to phenethylamine of almost 10 and a further reagent excess did not interfere (Fig. 2C). According to our previous studies the derivatization reaction was carried out at mild conditions [26] with the advantage of an improvement both of reaction time and molar ratio of DPD to analyte. Under described conditions DPD did not react with secondary phenethylamines (syn, Nmtyr and hor) confirming to be selective towards the primary amino group. The derivatization reaction of oct and tyr was compared with an authentic specimen of appropriate adduct synthesized by preparative scale and the analytical data (IR, ^{1}H NMR, ESI-MS) were consistent with the expected structure according to previously synthesized derivatives [26]. The confirmation of the structure of the considered adducts was achieved by means of ESI-MS experiments; the obtained mass spectra (MS and MS/MS) are reported in Fig. 3.

3.2. Chromatography and detection

During the phase of optimization of the chromatographic system, different stationary phases and mobile phases were investigated to evaluate the effect of the solvent composition and pH on the compound separation. Since polar analytes do not are retained nor well separated on conventional C18 column, our attention was focused on Sinergy Hydro-RP C18 and Sinergy Polar-RP columns. Unlike conventional C18 column Hydro-RP C18 bonded phase is endcapped with a proprietary polar group to provide extreme retention of both hydrophobic as well as polar compounds via polar interactions, hydrogen bonding or electrostatic interactions. Sinergy Polar-RP column is an ether-linked phenyl phase which proprietary hydrophilic endcapping designed specifically to maximize retention and selectivity for polar and aromatic analytes. The ether-linkage increases the aromaticity of the phenyl group and also provides π–π interactions with conjugated compounds. It contributes to not only sharp peak shape for acidic and basic analytes, but also ensures stability under highly aqueous mobile phase conditions. Both Sinergy Hydro-RP C18 column under gradient elution conditions (method A) and Sinergy Polar-RP column under isocratic elution conditions (method B) allowed good separations of phenethylamine alkaloids in reasonably short times (Fig. 4). In particular, a selective separation of oct and tyr was obtained by the method A in absence of an ion-pair reagent, which, on the contrary, was necessary for the method B. The advantage obtained with the method A was due largely to the minor polarity of the derivatives in comparison with the original compounds. Moreover, the method A permitted the oct and tyr detection at the wavelength of 320 nm, where the interferences of the matrix are limited in comparison with those potentially present at lower wavelength.

3.3. Method validation

Both methods were accurately validated considering linearity, limits of detection (LOD) and quantification (LOQ), accuracy,

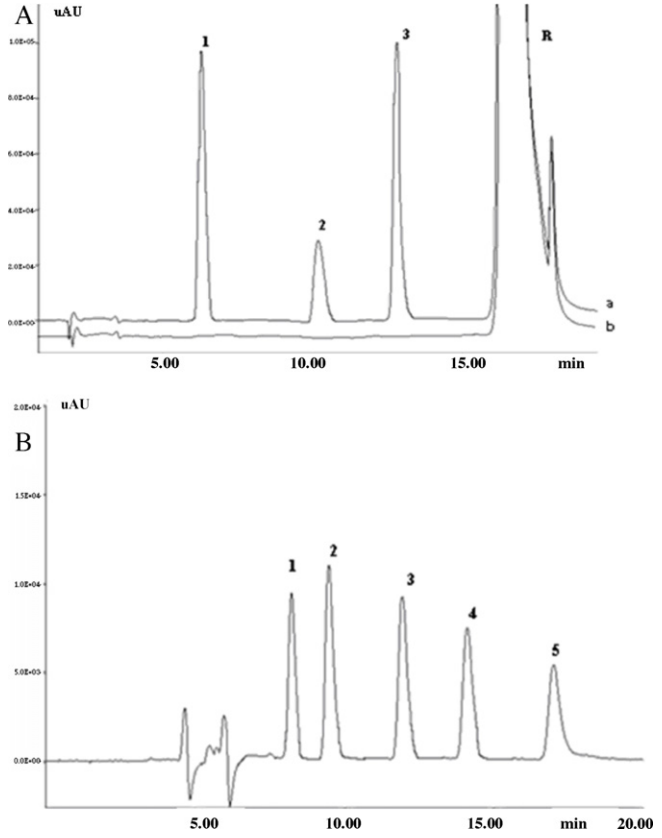

Fig. 4. Representative chromatograms of phenethylamines: (A) (a) phenethylamine separation after derivatization with DPD reagent (b) DPD under reaction conditions in absence of analytes (blank). Peaks: 1, oct (6.9 μg/mL); 2, IS (52 μg/mL); 3, tyr (5.8 μg/mL); R: reagent. UV detection at λ = 320 nm (method A). (B) Phenethylamine separation without derivatization reaction. Peaks: 1, oct (6.7 μg/mL); 2, syn (11 μg/mL); 3, tyr (6.2 μg/mL); 4, Nmtyr (7.4 μg/mL); 5, hor (6.0 μg/mL). UV detection at λ = 275 nm (method B). HPLC conditions as in Section 2.6.

precision, specificity and stability according to the 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 [37].

3.3.1. Linearity

The linearity was determined as linear regression with the least-square method on standard solutions. Concentrations (μg/mL) and calibration parameters are reported in Table 1. Phenethylamine solutions were injected after the described derivatization procedure for the method A, whereas they were injected directly for the method B. Triplicate injections for each final 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% [38].

3.3.2. Detection and quantification limits

LOD and LOQ have been established by the determination of the signal/noise ratio of 3:1 and 10:1, respectively. The data obtained by both methods were reported in Table 1. As it can be seen, LOD value of oct and tyr was found to be about 0.01 μg/mL (1 pmol) for the derivatization method. This procedure showed to be about

Table 2
Repeatability and intermediate precision data for retention time (t_R) and peak area of phenethylamine alkaloids.

Compounds	Method	Repeatability (n = 8, mean)			Intermediate precision (n = 24, mean)
		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%)	
Oct	A	5.9 (1.85)	5.9 (1.89)	6.1 (1.90)	6.0 (1.88)
	B	8.3 (1.20)	8.4 (1.22)	8.4 (1.15)	8.4 (1.19)
Tyr	A	12.7 (1.79)	12.8 (1.84)	12.6 (1.78)	12.6 (1.80)
	B	12.0 (1.18)	12.0 (1.14)	12.2 (1.15)	12.1 (1.16)
Syn	B	9.6 (1.22)	9.5 (1.26)	9.8 (1.28)	9.6 (1.25)
Nmtyr	B	14.6 (1.20)	14.4 (1.24)	14.6 (1.28)	14.5 (1.24)
Hord	B	17.5 (1.08)	17.7 (1.14)	17.6 (1.14)	17.6 (1.12)

Compounds	Method	A (mAU s)	$A_{analyte}/A_{SI}$ (RSD%)	A (mAU s)	$A_{analyte}/A_{SI}$ (RSD%)	A (mAU s)	$A_{analyte}/A_{SI}$ (RSD%)	A (mAU s)	$A_{analyte}/A_{SI}$ (RSD%)
Oct	A		1.55 (2.02)		1.54 (1.72)		1.56 (1.84)		1.55 (1.86)
	B	149732 (1.18)		149844 (1.20)		149746 (1.24)		149774 (1.21)	
Tyr	A		1.56 (1.80)		1.57 (1.75)		1.56 (1.88)		1.56 (1.81)
	B	164862 (1.15)		164943 (1.22)		164880 (1.20)		164895 (1.19)	
Syn	B	176521 (1.21)		176832 (1.29)		176625 (1.20)		176659 (1.23)	
Nmtyr	B	147990 (1.34)		148087 (1.41)		147854 (1.37)		147977 (1.37)	
Hord	B	122297 (1.19)		122354 (1.12)		121990 (1.11)		122214 (1.14)	

ten times more sensitive than the direct method, which presents a sensitivity of the same order of magnitude described in literature [19,22].

3.3.3. Precision

The precision of the chromatographic system of both methods, comprehensive of derivatization procedure for method A, was expressed as repeatability and intermediate precision. The repeatability was valued by injections of eight standard solutions containing oct and tyr for method A and oct, syn, tyr, Nmtyr and hor for method B (concentration for each amine as in Fig. 4). Moreover, the intermediate precision of the methods was determined with solutions prepared changing the parameters time-analyst: eight solutions were prepared by the analyst A in the day 1, eight solutions by the analyst B in the day 2 and eight additional solutions by the analyst C in the day 3. The repeatability of the methods was satisfactory for retention times as indicated by %RSD range 1.78–1.90 (method A) and 1.08–1.28 (method B) and also for peak areas as indicated by %RSD range 1.72–2.02 (method A) and 1.11–1.37 (method B) (Table 2). Furthermore, no statistically significant differences were found between inter-laboratory results.

3.3.4. Accuracy

The accuracy of both analytical methods was determined by analyzing commercial samples (capsules I and capsules II) fortified with known amounts of standard compounds corresponding to three levels of concentration (50, 100 and 200%) among found content for oct and tyr for method A and syn and Nmtyr for method B. The solutions were prepared in triplicate at each level of concentration. Quantitative recoveries were obtained in each instance: recovery = 98.9–101.2%, RSD = 0.9–1.3% for method A and recovery = 99.5–101.5%, RSD = 0.6–1.0% for method B (Table 3).

3.3.5. Stability

Stability preliminary studies of a reaction mixture showed that the DPD derivatives proved to be stable (peak area ratio variation within ± 2% of the initial value) for at least 12 h at ambient temperature and at least 3 days at 4 °C. Adduct solution stability, precision and applicability of the method in different laboratories provide an indication of the ruggedness and robustness of the methods.

3.3.6. Analysis of dietary supplements and extracts

Both methods were applied to the analysis of commercial formulations (capsules) used as dietary supplements, *C. aurantium*

Table 3
Accuracy data.

Formulations	Compounds	Method	Spiked amount (mg/g)	Determined amount (mg/g)	Mean recovery (%) (n = 3)	RSD (%)
Capsules I	Oct	A	0.03	0.09	101.2	1.1
			0.06	0.13	100.2	1.2
			0.13	0.19	99.1	1.3
	Tyr	A	0.02	0.08	98.9	0.9
			0.05	0.10	100.4	1.2
			0.10	0.15	100.0	1.0
	Syn	B	4.14	12.40	100.6	0.9
			8.26	16.32	99.7	0.6
			16.52	24.78	99.9	1.0
	Nmtyr	B	0.69	2.06	101.0	0.9
			1.37	2.74	100.1	1.0
			2.75	3.44	99.5	0.8
Capsules II	Oct	A	0.02	0.06	100.0	1.2
			0.04	0.08	100.7	0.9
			0.08	0.13	100.1	1.0
	Tyr	A	0.02	0.06	99.8	1.1
			0.04	0.08	99.6	1.0
			0.08	0.12	100.8	1.0
	Syn	B	2.67	8.01	101.5	0.7
			5.33	10.67	99.8	0.9
			10.67	16.01	101.2	0.9

Table 4
Results of HPLC analysis of phenethylamines in phytoproducts.

Sample	Compounds	% Found[a] (RSD%)	
		Method A	Method B
Capsules I[b]	Oct	0.0063 (2.08)	<LOQ
	Tyr	0.0050 (1.46)	<LOQ
	Syn	–	0.826 (0.87)
	Nmtyr	–	0.137 (0.95)
Capsules II[c]	Oct	0.0041 (2.21)	<LOD
	Tyr	0.0039 (2.09)	<LOD
	Syn	–	0.533 (0.90)
	Nmtyr	–	<LOD
Sinetrol®[d]	Oct	0.0043 (2.06)	<LOD
	Tyr	0.0040 (1.88)	<LOD
	Syn	–	1.674 (0.89)
	Nmtyr	–	0.120 (1.01)
Dry extract[e]	Oct	0.0070 (1.80)	<LOQ
	Tyr	0.0052 (1.89)	<LOQ
	Syn	–	3.858 (0.87)
	Nmtyr	–	0.295 (0.98)

(–) Not detected.
[a]Mean of five determinations expressed as a percentage (p/p) of the weight. Other ingredients capsules: [b]Sinetrol®, *Paullinia sorbilis Mart* e.s., *Phaseoulus vugatis* L., *Rhododendron Caucasian* e.s., *Lagerstroemia speciosa* e.s., magnesium stearate, gelatine; [c]*Cynara scolymus* e.s., theobromine, green coffee e.s., *Undaria pinnatifida* e.s, cellulose, dioxide silicium, magnesium stearate, gelatine; [d]polyphenols, flavanones, anthocyanins, caffeine; [e]flavonoids, essential oils, coumarin compounds.

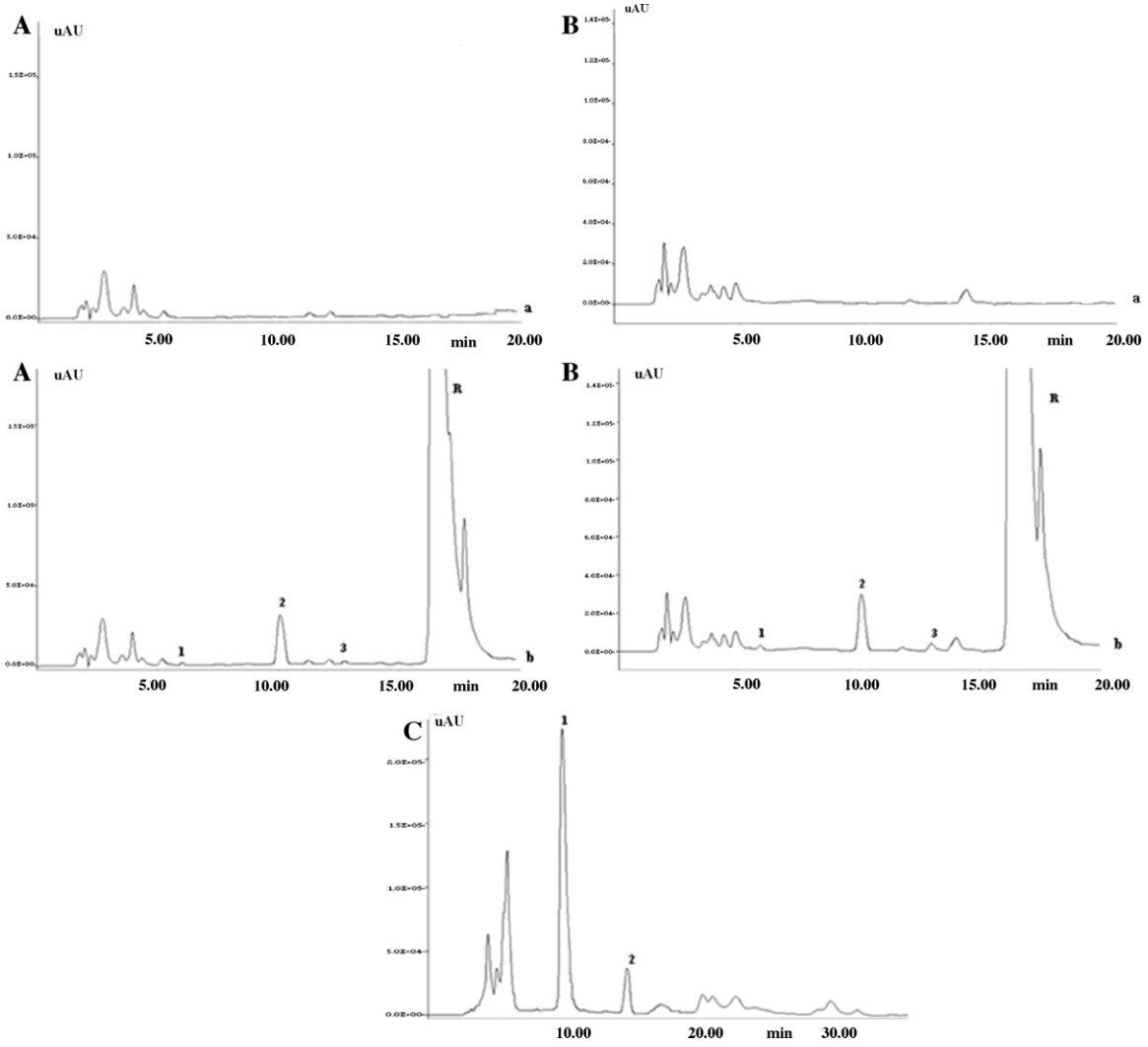

Fig. 5. HPLC separations of phenethylamine samples. (A) Dietary supplements (capsules I): phenethylamines before (a) and after (b) derivatization with DPD. Peaks: 1, oct; 2, IS; 3, tyr; R: reagent (method A). (B) Sinetrol®: phenethylamines before (a) and after (b) derivatization with DPD. Peaks: 1, oct; 2, IS; 3, tyr; R: reagent (method A). (C) *C. aurantium* dry extract. Peaks: 1, syn; 2, Nmtyr (method B). HPLC conditions and detection as in Fig. 4.

dried extract containing 4% of syn and Sinetrol®. The results were expressed as percentage of the weight (Table 4). As it can be seen, the method A based on the pre-column derivatization with DPD allowed the sure determination of oct and tyr traces, which were not detectable or not quantifiable by the reference method. A significant amount of syn and very low levels of primary phenethylamine alkaloids were found in Sinetrol®. Syn quantities in capsules and dry extract were in agreement with the claimed content, while Nmtyr values agreed with the data reported in literature [16,18,31]. Other components of the examined samples did not interfere with the analysis. Chromatographic separation examples are reported in Fig. 5. Peaks were identified on the basis both of the retention time (t_R) values and by spiking any sample with commercial standards. No interference relatively to blank (Fig. 4A) and matrix (Fig. 5A and B) was observed, showing a good specificity of the method A.

4. Conclusions

DPD has been confirmed as a versatile and selective pre-column labelling reagent for primary amino group. It showed to be suitable to RP-HPLC analysis of primary phenethylamines, giving stable derivatives with UV absorbance at 320 nm. The method sensitivity allowed the determination of oct and tyr in complex samples without any matrix interference at levels not reachable by the direct method. Efficient chromatographic separations were performed in absence of an ion-pair reagent in the mobile phase. The validation procedure permits its application to the quality control of *C. aurantium*-containing products. The unambiguous determination of oct and tyr levels can be an indirect marker of safety, excluding possible adulterations. The proposed method is applicable in common analytical laboratories, not requiring an expensive and sophisticated instrumentation. Derivatization reaction rapidity (1–2 min at ambient temperature) makes DPD potentially useful as post-column reagent.

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References

[1] A. Fugh-Berman, A. Myers, Exp. Biol. Med. 229 (2004) 698.
[2] S. Nagy, P.E. Shaw, M.K. Veidhais, Citrus Science and Technology, vol. 1, The AVI Publishing Co., Bridgeport, CT, 1977.
[3] X. He, L. Lian, L. Lin, M.W. Bernart, J. Chromatogr. A 791 (1997) 127.
[4] L.D. Mell Jr., D.O. Carpenter, Neurochem. Res. 5 (1980) 1089.
[5] J.M. Saaverdra, J. Axelrod, Adv. Biochem. Psychopharmacol. 15 (1976) 95.
[6] I.J. Kopin, J.E. Fischer, J.M. Masocrhio, W.D. Horst, V.K. Weise, J. Pharmacol. Exp. Ther. 147 (1965) 186.
[7] F. Pellati, S. Benvenuti, M. Melegari, F. Firenzuoli, Phytochem. Anal. 29 (2002) 1113.
[8] M.C. Roman, J.M. Betz, J. Hildreth, J. AOAC Int. 90 (2007) 68.
[9] L.T. Bui, D. Nguyen, P.J. Ambrose, Ann. Pharmacother. 40 (2006) 53.
[10] S. Jordan, M. Murty, K. Pilon, Can. Med. Assoc. J. 171 (2004) 993.
[11] V. Pereira, M. Pontes, J.S. Câmara, J.C. Marques, J. Chromatogr. A 1189 (2008) 435.
[12] M.H. Silla-Santos, Int. J. Food Microbiol. 29 (1996) 213.
[13] The World Anti-Doping Code, The 2006 Prohibited List International Standard, http://www.wadaama.org/en/WorldAntiDopingProgram/SportsandAntiDopingOrganizations/Intenational-Standards/Prohibited-List/.
[14] C. Dallas, A. Gerbi, G. Tenca, F. Juchaux, Phytomedicine 15 (2008) 783.
[15] F. Pellati, S. Benvenuti, J. Chromatogr. A 1161 (2007) 71.
[16] F. Pellati, S. Benvenuti, J. Chromatogr. A 1165 (2007) 58.
[17] M. Ganzera, C. Lanser, H. Stuppner, Talanta 66 (2005) 889.
[18] K. Putzbach, C.A. Rimmer, K.E. Sharpless, L.C. Sander, J. Chromatogr. A 1156 (2007) 304.
[19] F. Tang, L. Tao, X. Luo, L. Ding, M. Guo, L. Nie, S. Yao, J. Chromatogr. A 1125 (2006) 182.
[20] S.M. Vieira, K.H. Theodoro, M.B.A. Gloria, Food Chem. 100 (2007) 895.
[21] B. Avula, S.K. Upparapalli, A. Navarrete, I.A. Khan, J. AOAC Int. 88 (2005) 1593.
[22] D. He, Y. Shan, Y. Wu, G. Liu, B. Chen, S. Yao, Food Chem. 127 (2011) 880.
[23] J.A.P. Meulendijk, W.J.M. Underberg, in: H. Lingeman, W.J.M. Underberg (Eds.), Detection-oriented Derivatization Techniques in Liquid Chromatography, Marcel Dekker, New York, 1990, p. 247.
[24] N. Seiler, in: K. Blau, J.M. Halket (Eds.), Handbook of Derivatives for Chromatography, John Wiley & Sons, New York, 1993 (Chapter IX).
[25] G. Lunn, L.C. Hellwig, Handbook of Derivatization Reactions for HPLC, John Wiley and Sons, New York, 1998, p. 253.
[26] R. Gatti, M.G. Gioia, A. Leoni, A. Andreani, J. Pharm. Biomed. Anal. 53 (2010) 207.
[27] M.G. Gioia, B. Cacciari, A. Leoni, R. Gatti, Anal. Chim. Acta 579 (2006) 152.
[28] R. Gatti, M.G. Gioia, A.M. Di Pietra, Anal. Chim. Acta 474 (2002) 11.
[29] M.G. Gioia, R. Gatti, M. Vannini, M. Hudaib, Chromatographia 56 (2002) 289.
[30] R. Gatti, M.G. Gioia, Biomed. Chromatogr. 22 (2008) 207.
[31] R. Gatti, C. Lotti, J. Chromatogr. A 1218 (2011) 4468.
[32] S. Lamba, A. Pandit, S.K. Sanghi, V.S. Gowri, A. Tiwari, V.K. Baderia, D.K. Singh, P. Nigam, Anal. Chim. Acta 614 (2008) 190.
[33] K.J. Huang, C.X. Jin, S.-L. Song, C.Y.##Y.M.L. Wei, J. Li, J. Chromatogr. B 879 (2011) 579.
[34] H.-M. Mao, B.-G. Chen, X.-M. Qian, Z. Liu, Microchem. J. 91 (2009) 176.
[35] G.H. Ruan, L.X. Zheng, Q.E. Wu, Z.J. Zhou, J. Hyg. Res. 35 (2006) 146.
[36] A. Andreani, A. Cavalli, M. Granaiola, M. Guardigli, A. Leoni, A. Locatelli, R. Morigi, M. Rambaldi, M. Recanatini, A. Roda, J. Med. Chem. 44 (2001) 4011.
[37] International Conference on Harmonization of Technical Requirements for the Registration of Pharmaceuticals for Human Use (ICH), Guideline Q2 (R1)-Validation of Analytical Procedures: Text and Methodology, ICH Secretariat, c/o IFPMA, Geneva, Switzerland, 2005.
[38] J. Van Loco, M. Elskens, C. Croux, H. Beernaert, Accredit. Qual. Assur. 7 (2002) 281.