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Enantioselective LC analysis of synephrine in natural products on a protein-based chiral stationary phase☆

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Abstract

An enantioselective LC method with photodiode array detection (PAD) was developed for the enantioseparation of (±)-synephrine from *C. aurantium* L. var. *amara* fruits and phytotherapic derivatives by using a protein-based chiral stationary phase with cellobiohydrolase as the chiral selector (Chiral-CBH). Analyses were carried out on a Chiral-CBH column (100 × 4.0 mm i.d., 5 μm), with a mobile phase consisting of 2-propanol (5%, w/w) in sodium phosphate buffer (pH 6.0; 10 mM) and disodium EDTA (50 μM). The flow rate was 0.8 mL/min. Detection was set at 225 nm. To identify the order of elution, the racemate was resolved by the preparation of suitable diastereoisomeric salts with antipodes of appropriate organic acids.

Isolation of synephrine from *C. aurantium* fruits and phytoproducts was performed by solid-phase extraction (SPE) with a strong cation-exchange phase.

The method developed was validated and was found to be linear in the 0.40–40.14 μg/mL range ($r^2 = 1.000$, $P < 0.0001$) for both synephrine enantiomers. The limit of detection (LOD) for each enantiomer was 0.04 μg/mL. The limit of quantification (LOQ) for each enantiomer was 0.13 μg/mL. Intra-day precision (calculated as %R.S.D.) ranged from 0.03 to 0.24% for (−)-synephrine and from 0.03 to 0.35% for (+)-synephrine. Inter-day precision (calculated as %R.S.D.) ranged from 0.07 to 1.45% for (−)-synephrine and from 0.06 to 1.26% for (+)-synephrine. Intra- and inter-day accuracies (calculated as %recovery) were in the ranges of 97.4–100.6 and 98.0–101.6% for (−)-synephrine, and in the ranges 97.0–101.5 and 98.1–102.8% for (+)-synephrine.

The results of the application of the method to the analysis of *C. aurantium* samples showed that (−)-synephrine was the main component. (+)-Synephrine was not detected in *C. aurantium* fruits and was present in low concentration in the phytoproducts.

Keywords: *Citrus aurantium* L. var. *amara*; Adrenergic amines; Synephrine; Enantiomer separation; Protein-based chiral stationary phase; Chiral-CBH

1. Introduction

Citrus aurantium L. var. *amara* is a plant belonging to the Rutaceae family, whose fruit extracts have recently been used for the treatment of obesity.

The most important active constituents of *C. aurantium* fruits are flavonoids and adrenergic amines. Adrenergic amines are synephrine, octopamine and tyramine [1].

Synephrine is a primary synthesis drug developed as a sympathomimetic agent with pharmacological activities such as vasoconstriction, blood pressure elevation and bronchial muscle relaxation. Synephrine is also a known phenethylamine alkaloid present in the peel and the edible part of *Citrus* fruit [1,2]. Of the adrenergic amines of natural origin, synephrine has been found to be the main constituent of *C. aurantium* fruits and extracts; octopamine and tyramine are absent or present in low concentrations [3,4].

Synephrine is similar in structure to ephedrine (Fig. 1), the main active component of plants of the genus *Ephedra*. Only two substitutions are required to obtain synephrine from

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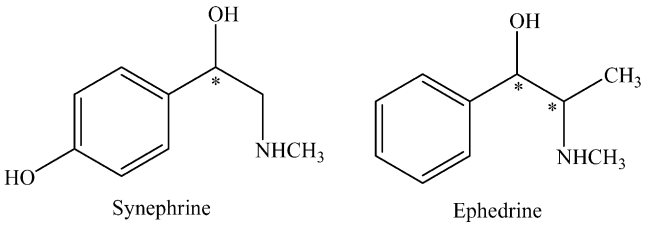

Fig. 1. Structure of synephrine and ephedrine.

ephedrine: one of the ring carbons is hydroxylated (OH replaces H), and a side chain methyl group (CH_3) is replaced by hydrogen.

Both ephedrine and synephrine are sympathomimetic compounds. The β3-adrenoreceptor appears to be responsible for the lipolytic and thermogenic effects of adrenergic agents [5,6].

It is known that synephrine and the other amines found in *C. aurantium* have adverse effects on the cardiovascular system owing to adrenergic stimulation [7,8]. Patients with severe hypertension, tachyarrhythmias, narrow-angle glaucoma and monoamine oxidase inhibitor recipients should avoid the use of *C. aurantium* extracts [9].

Synephrine is a chiral compound (Fig. 2) and is clinically administered as the racemic mixture, although its enantiomers have been shown to exert different pharmacological activity on α- and β-adrenoreceptors [10,11]. In particular, (*R*)-(−)-synephrine is from 1 to 2 orders of magnitude more active than its (*S*)-(+)-counterpart.

The determination of the enantiomeric composition of pharmaceuticals and nutraceuticals is subject to severe attention from the pharmacological and toxicological point of view. This implies an increasing need for pertinent enantioselective analytical technologies, mainly based on liquid chromatography (LC), capillary electrophoresis (CE) or gas chromatography (GC).

In the literature, several methods for the enantioseparation of (±)-synephrine have been reported, including GC analysis with flame ionization detection of the diastereomers after pre-column derivatization [12]. LC has been applied both with an indirect method with UV detection [13] and with direct methods with electrochemical detection [14,15]. Furthermore, CE has been employed for the resolution of synephrine enantiomers [16].

However, the separation of synephrine enantiomers in *C. aurantium* complex matrices, such as crude drugs, extracts and dietary supplements, has not been investigated using the above-described LC techniques. Considering the great commercial proliferation of *C. aurantium* herbal medicines in recent years, the pharmacological activity and the possible toxicity of the plant extracts, the development of chromatographic methods for the phytochemical analysis of *C. aurantium* fruits and derivatives is very important. Since synephrine is the main compound responsible for the pharmacological and toxicological activities ascribed to *C. aurantium* extracts, a method able rapidly to separate and quantify synephrine enantiomers could represent a useful tool to define the identity and the quality of *C. aurantium* crude drugs and phytoproducts. Actually, the difference in the pharmacological effect between the two enantiomeric forms of synephrine has needed an efficient and reliable method for the enantioselective separation and determination in natural products.

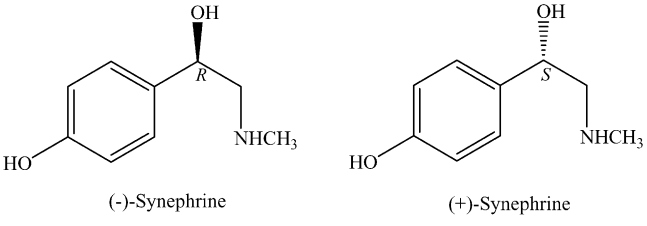

Fig. 2. Structure of synephrine enantiomers.

In a previous study [3], a partial enantioseparation of (±)-synephrine was achieved by using a β-cyclodextrin chiral stationary phase (CSP). In this study, the performance of several CSPs was evaluated. A sensitive and stereospecific assay for the direct separation of synephrine enantiomers was developed using a CSP with a chiral selector consisting of a stable enzyme, cellobiohydrolase (CBH), immobilized on silica particles. This column, used in reversed-phase mode, allowed a good enantioseparation of synephrine enantiomers. The analytical method developed was validated and successfully applied to determine the amount of synephrine enantiomers in *C. aurantium* fruits and derivatives.

2. Experimental

2.1. Chemicals and solvents

(±)-Synephrine, (±)-octopamine hydrochloride, tyramine and phosphoric acid were purchased from Sigma (Milan, Italy). [(1*S*)-(endo, anti)]-(−)-3-bromocamphor-8-sulfonic acid ammonium salt and [(1*R*)-(endo, anti)]-(+)-3-bromocamphor-8-sulfonic acid ammonium salt were from Aldrich (Milan, Italy). Hydrochloric acid (37%), Dowex 1X8, tetrabutylammonium hydrogen sulfate, copper(II) acetate, ammonium acetate, sodium dihydrogenphosphate, disodium hydrogenphosphate and ethylenediaminetetraacetic acid (EDTA) disodium salt were from Fluka (Milan, Italy). Copper(II) sulfate was from Carlo Erba (Milan, Italy). HPLC grade methanol, ethanol and acetonitrile were from J.T. Baker (Milan, Italy). HPLC grade 2-propanol was from Riedel-de-Haën. Water was purified using a Milli-Q PLUS 185 system from Millipore (Milford, MA, USA).

2.2. Plant material

Citrus aurantium L. var. *amara* fruits, kindly provided by Prof. Michele Melegari of the University of Modena and Reggio Emilia (Italy), were harvested from trees in January 2004. The whole fruits were immediately dried (at 40 °C in

a forced-air oven) to reach constant weight; some fruits were processed as peels, pulps and seeds and dried in the same way. The dried samples were stored at −20 °C, protected from light and humidity, until required for chemical analysis. The dried samples were ground to a fine powder on an IKA M20 grinder (Staufen, Germany) just before extraction.

C. aurantium hydroalcoholic dry extracts (indicated as dry extracts no. 1 and no. 2, respectively) were purchased from Polichimica (Bologna, Italy). The %level of synephrine claimed by the manufacturer of the commercially available hydroalcoholic dry extracts was 6.00% for dry extract no. 1 and 4.00% for dry extract no. 2.

C. aurantium phytoproducts (tablets and capsules) under investigation were purchased in local shops in March 2004 and are representative of the Italian market. These products are classified as dietary supplements and are indicated in the text as *C. aurantium* dietary supplement no. 1, no. 2, no. 3, no. 4 and no. 5.

2.3. Apparatus

Chromatography was performed on an Agilent Technologies (Waldbronn, Germany) modular model 1100 system consisting of a vacuum degasser, a quaternary pump, an autosampler, a thermostatted column compartment and a photodiode array detector (PAD). The chromatograms were recorded with Agilent ChemStation for LC and LC/MS system (Rev. A.08.03) on a Pentium III personal computer.

The polarimetric measurements were carried out using a Perkin-Elmer 241 Polarimeter (Norwalk, CT, USA).

^{1}H nuclear magnetic resonance (NMR) spectra were obtained with a Bruker DPX200 FT-NMR spectrometer (Rheinstetten, Germany). Infrared spectra were obtained with a Perkin-Elmer 1600 Series Fourier transform FT-IR equipment.

2.4. Preparation of synephrine enantiomers

In order to obtain (−)-synephrine hydrochloride [17], a solution of [(1*S*)-(endo, anti)]-(−)-3-bromocamphor-8-sulfonic acid ammonium salt (5.91 g) in water (21 mL) was added slowly to a solution of racemic-free base (3 g) in 35.4% hydrochloric acid (1.60 mL) and water (7.40 mL). The resultant solution was allowed to stand overnight at 4 °C to give (−)-synephrine (−)-3-bromocamphorsulfonate, which was recrystallized from water (3×). This colourless crystalline salt (yield 0.49 g (11.4%)), dissolved in water (30 mL), was converted into the corresponding (−)-hydrochloride salt using Dowex 1X8 (18 g), which was eluted with water. Lyophilization of the eluate afforded (−)-synephrine hydrochloride (yield 0.12 g (59.5%); mp 176 °C dec).

This procedure was repeated with the racemic free base (2 g) and [(1*R*)-(endo, anti)]-(+)-3-bromocamphor-8-sulfonic acid ammonium salt (3.94 g) to give the (+)-synephrine (+)-3-bromocamphorsulfonate (yield 0.27 g (9.4%)), which, after recrystallization from water (3×), was converted into (+)-synephrine hydrochloride (yield 0.07 g (60.9%); mp 178 °C dec) with Dowex 1X8 (12 g), as previously described.

2.5. Chromatographic conditions

In this study, a LiChroCART Chiradex column (250 mm × 4 mm i.d., 5 μm, Merck, Darmstadt, Germany), a Sumichiral OA-6000 column (150 mm × 4.6 mm i.d., 5 μm, Sumika Chemical Analysis Service, Osaka, Japan) and a Chiral-CBH column (100 mm × 4.0 mm i.d., 5 μm, ChromTech, Congleton, UK) coupled with a guard column (10 mm × 4.0 mm, 5 μm), were evaluated for the separation of synephrine enantiomers. All chromatographic experiments were performed in the isocratic mode.

With regard to the LiChroCART Chiradex column, the mobile phase consisted of methanol–NaH_2PO_4 (pH 3.5; 25 mM) (2:98, v/v) and tetrabutylammonium hydrogen sulfate (10 mM). The flow rate was 0.4 mL/min. The column was thermostatted at 2 °C. Detection was performed at 225 nm. The sample injection volume was 5 μL.

With regard to the Sumichiral OA-6000 column, the mobile phase was an aqueous solution of copper(II) acetate (1 mM) and ammonium acetate (10 mM) (pH* 6.4). The flow rate was 1.7 mL/min. The column was thermostatted at 26 °C. Detection was set at 225 nm. The sample injection volume was 5 μL.

With regard to the Chiral-CBH column, analyses were carried out with a mobile phase consisting of 2-propanol (5%, w/w) in sodium phosphate buffer (pH 6.0; 10 mM) and disodium EDTA (50 μM). The flow rate was 0.8 mL/min. The column was thermostatted at 20 °C. Detection was set at 225 nm. The sample injection volume was 20 μL.

2.6. Sample preparation

The sample preparation from *C. aurantium* fruits (whole fruits, peels, pulps and seeds) was based on the extraction of a weighed amount of ground sample (about 0.5 g) with 20 mL of water at room temperature using a magnetic stirrer (Velp Scientifica, Milan, Italy) for 15 min. After centrifugation at 15,000 rpm for 30 min, the extract was filtered under vacuum and then cleaned up by solid-phase extraction (SPE).

The samples from *C. aurantium* dry extracts were prepared by extraction of a weighed amount (30–50 mg) with 5 mL of water using a magnetic stirrer (15 min). The extract was then filtered under vacuum and cleaned up by SPE.

Regarding *C. aurantium* dietary supplements, a weighed amount (0.2 g) of sample (powdered tablets or the capsule contents) was extracted with 10 mL of water using a magnetic stirrer (15 min) and centrifuged at 5000 rpm for 15 min. The extract was then filtered under vacuum and cleaned up by SPE.

The SPE cartridge (500 mg LiChrolut SCX column from Merck, Darmstadt, Germany) was prepared by pre-washing with 2 mL methanol, followed by 2 mL water using a vac-

uum manifold (Adsorbex Sample Preparation Unit, Merck, Darmstadt, Germany). The *C. aurantium* sample solution was then added and the column was washed with 5 mL water, followed by 2 mL water–methanol (75:25, v/v), all eluates being discarded. (±)-Synephrine was then eluted with 6 mL methanol–0.5 M HCl (9:1, v/v) and the solution diluted to volume (25 mL) with the mobile phase. All the extracts were filtered through a 0.45 μm cellulose acetate filter into an LC vial and capped.

2.7. LC analysis of C. aurantium samples

In *C. aurantium* samples, peaks of synephrine enantiomers were identified on the basis of their retention time (t_R) values and UV spectra by comparison with those of the reference standard solution. Peak identity was also confirmed by spiking the extracts with pure synephrine enantiomers. Peak purity test was performed using a photodiode array detector coupled to the LC system and comparing the UV spectra of each peak with those of authentic reference samples.

Quantification was performed by integrating the areas of the peaks due to synephrine enantiomers. The peak areas were used to calculate the amount of synephrine enantiomers present in the samples by applying the linear equation obtained from the external standard calibration.

2.8. Statistical analysis

Means, standard deviation data and bivariate regression analyses were performed using Microcal Origin (Version 4.1).

3. Results and discussion

3.1. Identity of synephrine enantiomers

Racemic synephrine was resolved with (−)- and (+)-3-bromocamphor-8-sulfonic acid, followed by fractional crystallization of the diastereoisomeric salts and ion-exchange to afford the optically active hydrochloride salts. The optical rotation ($[\alpha]_D^{20}$) in water at a concentration of 10 mg/mL was found to be −45.8° (ca. 1.06, H_2O) for (−)-synephrine hydrochloride and +46.4° (ca. 1.00, H_2O) for (+)-synephrine hydrochloride.

The identity of synephrine enantiomers was confirmed on the basis of ^{1}H NMR and FT-IR. The following results for (−)-synephrine hydrochloride were obtained.

^{1}H NMR (200 MHz, $DMSO_{d6}$,) of (−)-synephrine hydrochloride: δ 2.55 (s, 3H), 2.95 (m, 2H), 4.77 (dd, 1H, J = 9.22; 4.10 Hz), 5.94 (d, 1H, J = 4.00 Hz), 6.76 (d, 2H, J = 8.60 Hz), 7.16 (d, 2H, J = 8.60 Hz), 8.81 (s, 2H), 9.46 (s, 1H).

FT-IR (KBr-disks, wave number (cm^{-1})) of (−)-synephrine hydrochloride: 3408–3333 ν(OH), 3087–2342 $\nu(^+NH_2$, CH), 1614 $\delta(^+NH_2)$, 1591–1459 ν(aromatic C=C), 1258–1036 ν(C–N, C–O), 828 δ_{oop}(=C–H).

The same results were obtained for (+)-synephrine hydrochloride.

3.2. Method development and optimization

To develop a suitable LC method for the separation of synephrine enantiomers, three chiral columns, namely, LiChroCART Chiradex, Sumichiral OA-6000 and Chiral-CBH, were used. Various experiments were conducted to select the best stationary and mobile phases that would give optimum resolution and selectivity for the two enantiomers.

The stationary phase of the LiChroCART Chiradex column is based on spherical particles of silica gel with β-cyclodextrins (β-CD) bonded covalently. β-CD are naturally occurring oligosaccharides with hydrophobic cavities, which enable them to form inclusion complexes with organic substances in aqueous solutions. As β-CD are built from chiral glucose units, it is possible to use them as the chiral selectors for the separation of racemic mixtures: enantiomers, for instance, can form diastereomeric inclusion complexes with β-CD.

For the separation of synephrine enantiomers with the LiChroCART Chiradex column, as reported in a previous study [3], mixtures of methanol and water or buffer (methanol–water or buffer from 5:95 (v/v) to 60:40 (v/v)) were tried, as suggested by the manufacturer, but no separation of synephrine enantiomers was achieved. With regard to the organic solvent, it is known that it plays an important role in enantioselectivity: the higher its polarity (methanol > ethanol > acetonitrile), the better the separation of enantiomers. Therefore, methanol was used as the organic solvent in this study.

Enantioseparation was then improved by adding tetrabutylammonium hydrogen sulfate, as the ion pair reagent, to the mobile phase [18]. The effects of methanol concentration, buffer concentration, ion pair reagent concentration, pH and flow rate on retention times (t_R), selectivity (α) and resolution (R_s) were examined to determine the optimal mobile phase composition. In particular, an increase in the concentration of methanol in the mobile phase reduced retention times, with the loss of resolution of the analytes of interest.

Furthermore, it is known from binding studies that an increase in temperature decreases the extent of complexation between the guest molecule and β-CD [19]. Therefore, it is expected that separations on chemically bonded β-CD at different temperatures should give different results. In fact, the enantioseparation of (±)-synephrine on such a stationary phase was improved by decreasing the column temperature.

The optimal mobile phase conditions were found to be methanol–NaH_2PO_4 (pH 3.5; 25 mM) (2:98, v/v) and tetrabutylammonium hydrogen sulfate (10 mM), at a flow rate of 0.4 mL/min. The column temperature was set at 2 °C and detection at 225 nm.

However, under these chromatographic conditions, a partial enantioseparation was obtained [3] (Fig. 3a): the retention

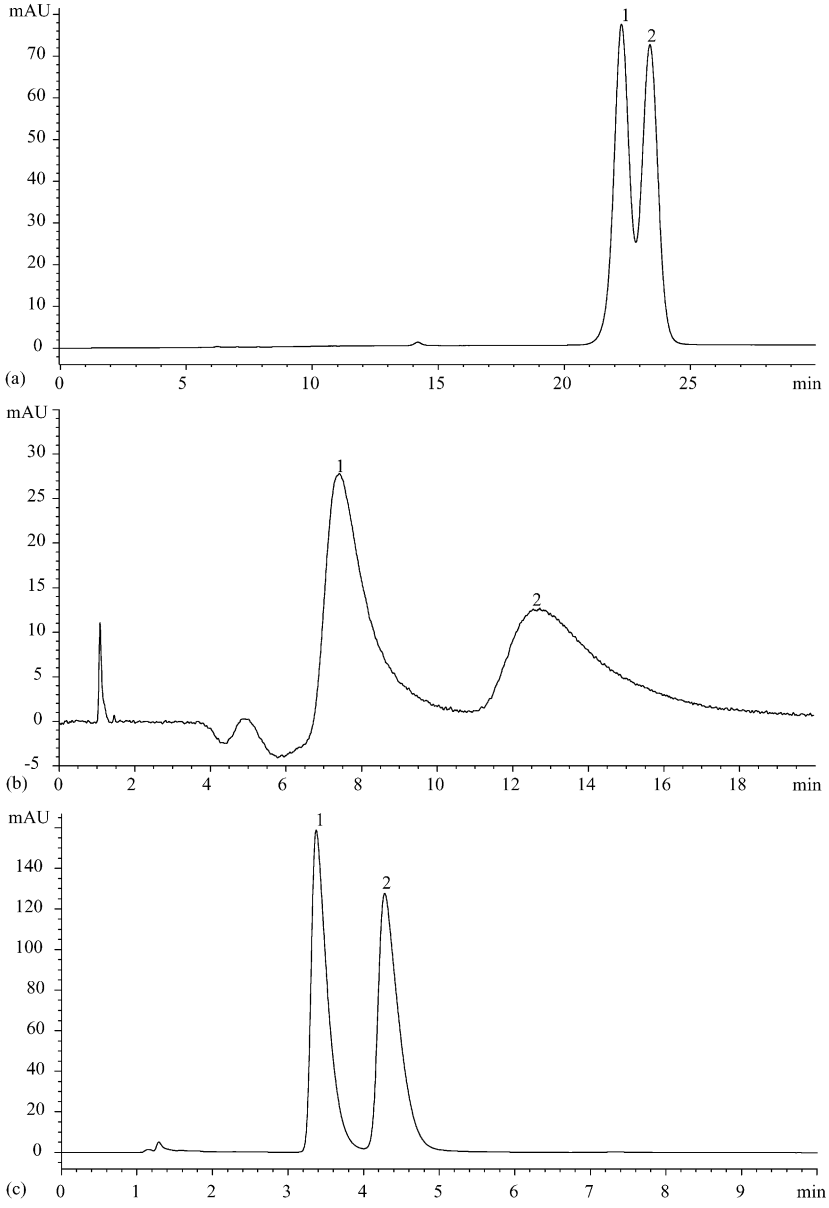

Fig. 3. Enantioselective separation of (±)-synephrine on: (a) LiChroCART Chiradex column, (b) Sumichiral OA-6000 column, (c) Chiral-CBH column. Peak identification: (1) (−)-synephrine; (2) (+)-synephrine.

times were 22.27 min for (−)-synephrine and 23.40 min for (+)-synephrine. Selectivity (α) was 1.05 and resolution (R_s) was 0.96.

Even though the synephrine enantiomers were partly separated, the degree of separation was not sufficient to quantify these analytes, particularly if one of them was present at a low concentration. It was concluded that the LiChroCART Chiradex column did not provide sufficiently good separation.

Regarding the Sumichiral OA-6000 column, the stationary phase consists of a coordination compound of copper(II) ion and the chiral ligand (*R*,*R*)-tartaric acid mono-(*R*)-1-(α-naphthyl)-ethylamide. The basic principle of enantioselective ligand exchange chromatography (LEC) [20] was demonstrated by chemically binding a chiral selector, a copper(II) complex of an appropriate ligand, to the matrix of a polymeric particulate sorbent and conducting chromatography of the racemates under conditions suitable for the formation of ternary complexes composed of the stationary chiral ligand, central copper(II) ion and the analyte. Enantioselectivity of formation of the ternary complexes, i.e. the difference in thermodynamic stabilities of two diastereomeric structures incorporating the enantiomers of the analyte, was found to be extremely high.

In this study, enantioseparation was based on the difference in the coordination formation ability of the solutes, such as synephrine enantiomers, and the chiral ligand with copper(II) ion.

The composition of the mobile phase was optimized in accordance with the guidelines suggested by the manufacturer. Initial investigations using this stationary phase focused on a mobile phase consisting of copper(II) sulfate (2 mM) in water–acetonitrile (95:5, v/v). Under these conditions, synephrine enantiomers were not separated.

The initial mobile phase was then replaced with copper(II) acetate (0.5 mM) and ammonium acetate (10 mM) in water, obtaining partial enantioseparation. Further improvements in mobile phase composition, including the concentration of copper(II) acetate, the concentration of ammonium acetate, pH (5.5–7.0) and flow rate, were then applied. The equilibrium of the formation of the copper(II) complexes was affected by pH, owing to the competition for the complexing anion by hydrogen. At pH 6.5 or higher, a precipitate formed in the mobile phase. Consequently, the pH of the mobile phase could not be higher than 6.4.

Concerning the influence of temperature, it was found that changes in column temperature did not affect the separation.

The optimum composition of the mobile phase was found to be copper(II) acetate (1 mM) and ammonium acetate (10 mM) in aqueous solution (pH* 6.4), at a flow rate of 1.7 mL/min. The column was thermostatted at 26 °C and detection was set at 225 nm. The separation of synephrine enantiomers on the Sumichiral OA-6000 column using the mobile phase described above is shown in Fig. 3b. Although the peaks were somewhat broad and tailed, a satisfactory enantioseparation was achieved within a reasonable analysis time: the retention times of (−)- and (+)-synephrine enantiomers were 7.41 and 12.72 min, respectively; selectivity (α) was 1.72 and resolution (R_s) was 1.74.

With regard to the Chiral-CBH column, the stationary phase contains a stable enzyme, cellobiohydrolase (CBH), immobilized onto 5 μm spherical silica particles. A number of proteins have been used as the chiral selectors in LC stationary phases. A protein consists of amino acids, which are chiral compounds. All proteins have the ability to discriminate a chiral molecule. However, only a limited number of proteins have been investigated as LC chiral selectors [21].

The stationary phase of the Chiral-CBH column is used in the reversed-phase mode and is effective for the enantiomer separation of basic drugs from many compound classes [22–27]. The mobile phases are usually buffer solutions with a relatively low content of uncharged organic modifier. The chiral recognition site of the enzyme cellobiohydrolase is a 40 Å long tunnel in the core of the protein [28]. This tunnel contains acidic amino acids such as aspartic acid and other amino acids such as tryptophan, tyrosine and serine. Ion-exchange, hydrogen bonding and hydrophobic interactions can be involved in the retention mechanisms [28].

The optimization of the LC conditions for the enantioseparation of (±)-synephrine was carried out in accordance with the instructions provided by the manufacturer. The mobile phase parameters that may affect the enantioseparation are the concentration and the pH of the buffer solution and the concentration of the organic modifier. In this study, the concentration of the phosphate buffer (10–50 mM), the mobile phase pH (3–7) and the content of 2-propanol (5–15%, w/w) were the variables chosen for the optimization. Disodium EDTA (50 μM) was added to the mobile phase in order to complex the metal ions, which can deteriorate the column properties.

With a mobile phase consisting of 2-propanol (5%, w/w) in sodium phosphate buffer (pH 6.0; 10 mM) and disodium EDTA (50 μM), at a flow rate of 0.8 mL/min, a successful resolution of synephrine enantiomers was achieved using the Chiral-CBH column. The column temperature was set at 20 °C and detection at 225 nm. A typical LC chromatogram of the enantioselective separation of racemic synephrine is shown in Fig. 3c. The Chiral-CBH column provided very good separation of synephrine enantiomers with good peak shapes and short analysis time. The retention time was 3.37 min for (−)-synephrine and 4.28 min for (+)-synephrine. Selectivity (α) was 1.27 and resolution (R_s) was 2.11.

The enantiomeric elution order was determined by chromatographing the individual enantiomers of synephrine separately under the same chromatographic conditions. For all the three columns, the peak that eluted first was identified as (−)-synephrine and the second peak as (+)-synephrine. The enantiomeric purity determined on the Chiral-CBH column was found to be 98.6% for (−)-synephrine hydrochloride and 98.9% for (+)-synephrine hydrochloride.

The system-suitability results of the three LC methods are given in Table 1.

Of the three columns tested, the Chiral-CBH column provided excellent separation of synephrine enantiomers, and was therefore chosen for use in this study.

3.3. Method validation

The validation procedure was carried out on the Chiral-CBH column. In particular, the method was tested for linearity, limits of detection and quantification, precision, accuracy and specificity in accordance with the International Conference on the Harmonization of Technical Requirements for the Registration of Pharmaceuticals for Human Use (1996) and US Pharmacopeia 24 (2000) [29,30].

3.3.1. Linearity

Linearity test solutions were prepared from the stock solution of (±)-synephrine in water. External standard calibrations were obtained by three replicates of 8-point concentration levels under the optimal conditions described above. The calibration curves showed that there was a linear dependence of the peak area on the concentration of both enantiomers over the range of 0.40–40.14 μg/mL in the mobile phase. The equation used to describe the linear relationship between the peak area and the corresponding concentration was $y = ax + b$, where a and b are the coefficients of the regression equation (Table 2). The correlation coefficient (r^2) was 1.0000 ($P < 0.0001$) for both synephrine enantiomers.

Table 1
System-suitability report for synephrine enantiomer separation

Column	Compound	t_R (min)	Theoretical plates (N)	Resolution (R_s)	Selectivity (α)	Tailing factor (T)
LichroCART Chiradex	(−)-Synephrine	22.27	6002	–	–	0.86
	(+)-Synephrine	23.40	6074	0.96	1.05	1.18
Sumichiral OA-6000	(−)-Synephrine	7.41	267	–	–	2.61
	(+)-Synephrine	12.72	141	1.74	1.72	2.00
Chiral-CBH	(−)-Synephrine	3.37	1303	–	–	2.02
	(+)-Synephrine	4.28	1254	2.11	1.27	1.83

Table 2
Statistical analysis for the calibration curves of synephrine enantiomers[a]

Compound	Wavelength (nm)	Linearity range (μg/mL)	Slope (a)	Intercept (b)	r^2
(−)-Synephrine	225	0.40–40.14	60.068 (±0.075)	−4.820 (±1.416)	1.0000
(+)-Synephrine	225	0.40–40.14	60.838 (±0.071)	−5.645 (±1.351)	1.0000

[a] For each curve the equation is $y = ax + b$, where y is the peak area, x is the concentration of the analyte (μg/mL), a is the slope, b is the intercept and r^2 is the correlation coefficient. The P value was <0.0001 for all calibration curves. S.D. values are given in parenthesis.

3.3.2. Limits of detection and quantification

The limit of detection (LOD) represents the analyte concentration that would yield a signal-to-noise ratio of 3. The LOD was found to be 0.04 μg/mL for both synephrine enantiomers. The limit of quantification (LOQ) represents the analyte concentration that would yield a signal-to-noise ratio of 10. The LOQ was found to be 0.13 μg/mL for both synephrine enantiomers. These results suggest that the proposed LC method is sufficiently sensitive for the determination of synephrine enantiomers in *C. aurantium* samples.

3.3.3. Precision and accuracy of the chromatographic system

The precision and accuracy of the method were determined by replicate analyses of solutions with eight concentrations of synephrine enantiomers within the range of 0.40–40.14 μg/mL. Three replicates of each concentration were analyzed on each of the three separate days. The accuracy of the method was evaluated by the recovery, defined as the ratio of the analyte amount found to what was actually present in the solution at eight concentration levels, ranging from 0.40 to 40.14 μg/mL. The intra- and inter-day precision (calculated as %R.S.D.) and accuracy (calculated as %recovery) data of this method are listed in Table 3.

The intra-day precision values ranged from 0.03 to 0.24% for (−)-synephrine and from 0.03 to 0.35% for (+)-synephrine. The inter-day precision was 0.07–1.45% for (−)-synephrine and 0.06–1.26% for (+)-synephrine. The intra-day accuracy values of this method were in the range 97.4–100.6% for (−)-synephrine and 97.0–101.5% for (+)-synephrine. The inter-day accuracy values were in the range 98.0–101.6% for (−)-synephrine and 98.1–102.8% for (+)-synephrine.

The analytical method for the determination of synephrine enantiomers can thus be considered as precise and accurate within the overall concentration range investigated.

3.3.4. Precision of the extraction procedure

The precision of the extraction procedure was evaluated using one *C. aurantium* sample (whole fruits). Six samples, weighing about 0.5 g, were extracted as previously described. An aliquot of each extract was then injected and quantified. The amount of (−)-synephrine in these samples was 0.99 mg/g with an S.D. of 0.05. The low value of the S.D. suggests the good precision of the method.

3.3.5. Accuracy of the extraction procedure

To check the %analyte recovery of the SPE procedure, the standard addition method was applied. This involved the addition of known quantities of (+)- and (−)-synephrine enantiomers (0.5–1 mg) to known amounts of *C. aurantium* samples. The fortified samples were then extracted and analyzed with the proposed LC method. The %recovery was determined by subtracting the values obtained for the control matrix preparation from those samples that were prepared with the added standards, divided by the amount added and then multiplied by 100. Mean recoveries for (−)-synephrine were in the range 75.2–76.7% from *C. aurantium* fruits, 72.5–73.2% from the dry extracts and 75.2–76.1% from the dietary supplements. Mean recoveries for (+)-synephrine were in the range 68.3–73.8% from *C. aurantium* fruits, 75.1–75.3% from the dry extracts and 74.0–74.8% from the dietary supplements. Although recoveries were less than ideal, they were sufficient to address the questions of interest.

3.3.6. Specificity

The ability to assess unequivocally the analyte in the presence of closely related components that may be expected to be present in *C. aurantium* fruits and derivatives was tested by spiking a solution containing 40 μg/mL of (±)-synephrine with appropriate levels (40 μg/mL, each) of (±)-octopamine and tyramine. Fig. 4 shows the LC analysis of

Table 3
Intra- and inter-day precision and accuracy of the assay method

Enantiomer	Nominal concentration (μg/mL)	Intra-day analysis ($n = 3$)			Inter-day analysis ($n = 9$)		
		Mean concentration found (μg/mL)	Mean recovery[a] (%)	R.S.D. (%)	Mean concentration found (μg/mL)	Mean recovery[a] (%)	R.S.D. (%)
(−)-Synephrine	0.401	0.402	100.3	0.24	0.410	101.6	1.45
	0.803	0.804	100.2	0.11	0.810	101.0	0.86
	2.007	2.002	99.7	0.05	1.997	99.5	0.30
	4.014	3.911	97.4	0.03	3.932	98.0	0.48
	8.028	7.971	99.3	0.04	7.974	99.3	0.11
	20.070	20.076	100.0	0.05	20.074	100.0	0.07
	28.098	28.256	100.6	0.04	28.229	100.5	0.10
	40.140	40.047	99.8	0.05	40.015	99.7	0.09
(+)-Synephrine	0.401	0.407	101.5	0.35	0.413	102.8	1.26
	0.803	0.807	100.5	0.22	0.814	101.4	0.98
	2.007	2.016	100.5	0.13	2.010	100.2	0.28
	4.014	3.894	97.0	0.11	3.938	98.1	0.86
	8.028	7.954	99.1	0.06	7.963	99.2	0.10
	20.070	20.063	100.0	0.03	20.076	100.0	0.06
	28.098	28.219	100.4	0.05	28.256	100.6	0.11
	40.140	40.083	99.9	0.04	40.108	99.9	0.07

[a] %Recovery is defined as the found concentration/nominal concentration, expressed as a percentage.

the resulting solution: (−)-octopamine eluted just after (+)-synephrine, with a t_R value of 4.51 min ($\alpha = 1.08$; $R_s = 0.80$); (+)-octopamine ($t_R = 9.24$ min; $\alpha = 2.05$; $R_s = 7.17$) and tyramine ($t_R = 12.70$ min; $\alpha = 1.37$; $R_s = 3.32$) are well separated from the other peaks of the chromatogram.

These results confirmed that the method is specific for the analysis of synephrine enantiomers.

3.3.7. Stability

The stability of synephrine enantiomers in aqueous standard solutions and in *C. aurantium* extracts was studied by keeping the samples in tightly capped volumetric flasks both at 4 °C and at room temperature. The samples were analyzed every 12 h and peak areas compared. Synephrine enantiomers were found to be stable in aqueous solution for at least 3 days. Degradation products were not observed in the chromatograms.

3.4. Application to C. aurantium fruits and derivatives

The chiral LC method was applied to evaluate the content of synephrine enantiomers in *C. aurantium* fruits and derivatives. The assay involved the use of SPE for sample clean-up prior to LC analysis. In the literature, there is little research on the clean-up of ephedrine-like alkaloids from natural products with SPE [31]. In this study, a simple, rapid and reliable SPE procedure was developed and applied: the samples were purified on strong cation-exchange columns before LC analysis. The amino group of the synephrine molecule ($pK_a = 9.3$), being easily protonated in aqueous solution, exists predominately in the cationic form, readily available for ion-exchange. This procedure enriched the sample of the analyte of interest and eliminated many of the interfering compounds.

By removing the SPE eluate by freeze-drying, followed by reconstitution of the residue in the mobile phase, racem-

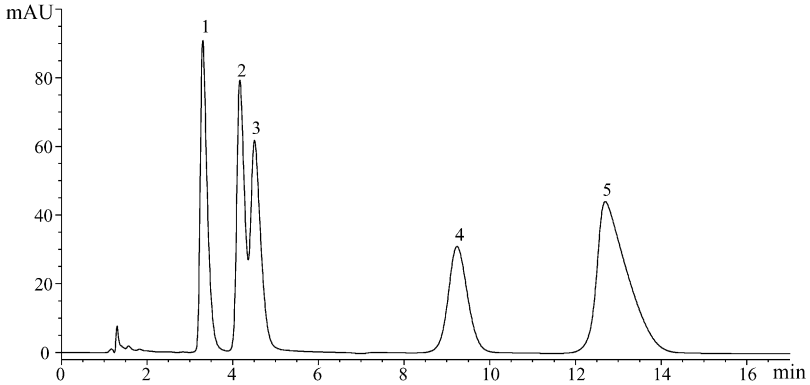

Fig. 4. Analysis of (±)-synephrine spiked with (±)-octopamine and tyramine. Peak identification: (1) (−)-synephrine; (2) (+)-synephrine; (3) (−)-octopamine; (4) (+)-octopamine; (5) tyramine.

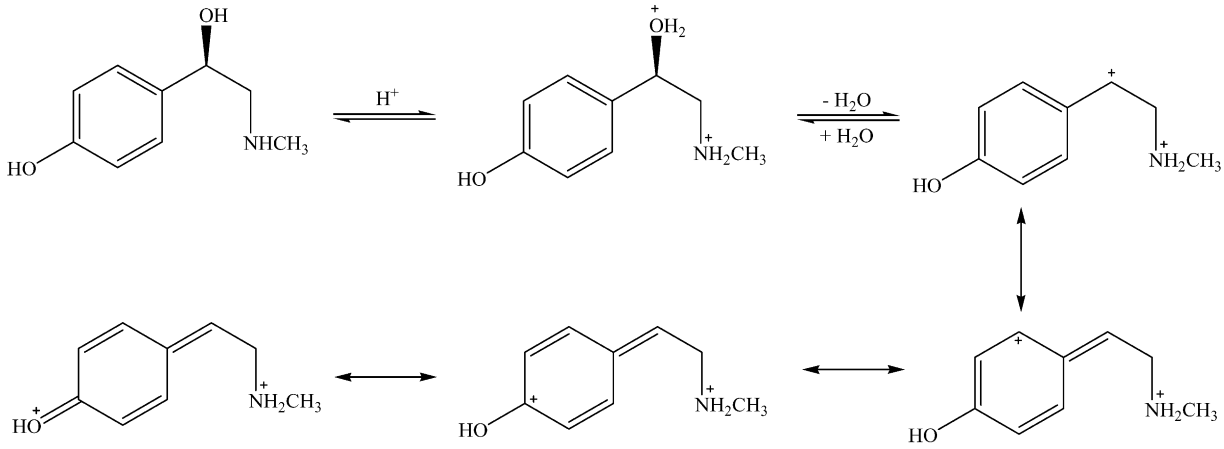

Fig. 5. Structure of synephrine dications in acidic media.

ization of (−)-synephrine was observed. The strong acidic media produced during the prolonged period of time needed to remove the solvent are believed to cause the racemization of the compound of interest, owing to the formation of the dicationic intermediates of synephrine [32] (Fig. 5).

Heating the SPE eluate would have expedited solvent removal, but was not carried out in view of the expected corresponding increase in analyte racemization.

In order to determine the level of synephrine enantiomers in *C. aurantium* samples without the risk of racemization, the acidic SPE eluate was diluted with the mobile phase and injected into the LC system. Under this extraction condition, racemization reaction did not occur. This was confirmed using (+)- and (−)-synephrine solutions separately.

Fig. 6a shows the typical chromatogram obtained from the analysis of synephrine enantiomers in *C. aurantium* fruits. The results showed that synephrine could be easily isolated from *C. aurantium* with this technique. Interfering peaks from herbal matrix composition were not observed in the chromatogram after SPE clean-up.

Plant biochemical pathways produce a series of phytochemicals whose stereochemical configurations are determined by precise enzymatic transformations [33]: it is noteworthy that some phytochemicals exist in only one enantiomeric form, whereas with others the optical rotation of the metabolite can vary between species. In this study, in accordance with the literature [2,3], (−)-synephrine was the only enantiomer isolated from *Citrus* fruits. (+)-Synephrine was not observed in any of the chromatograms from *C. aurantium* fruits. *C. aurantium* peels, pulps and seeds had the same chromatographic profile.

As shown in Fig. 6b, (−)-synephrine was the main component of *C. aurantium* dry extracts; however, a small amount of (+)-synephrine was detected in these samples. (+)-Synephrine may possibly be formed during the industrial production of *C. aurantium* hydroalcoholic extracts from fruits, using a high temperature and long period of refluxing.

The LC method for the enantioseparation of (±)-synephrine was applied to *C. aurantium* dietary supplements because this adrenergic amine is rare in plants except in *Citrus* fruits. Therefore, it is unlikely to occur in any of the other herbs present in the formulations analyzed. Fig. 6c shows a representative chromatogram of the analysis of synephrine enantiomers in a *C. aurantium* dietary supplement.

Table 4 reports the amount of synephrine enantiomers in *C. aurantium* fruits and phytoproducts. Quantification data are reported as mg/g of dry weight.

These quantification data were then compared with those reported in the literature, which were obtained with achiral LC methods. To make the comparisons easier, values were expressed in the same units (mg/g). Kusu et al. [34] described a level of synephrine of 2.31 mg/g for unripe fruits, and a level of 1.63 for nearly ripe fruits. Hashimoto et al. [35] analyzed the content of synephrine in crude Chinese drugs from *C.*

Table 4
Content of synephrine enantiomers in *C. aurantium* fruits, extracts and dietary supplements

Sample	Content dry weight (mg/g)[a]		
	(−)-Synephrine	(+)-Synephrine	Total
Whole fruits	0.99 ± 0.05	<LOD	0.99 ± 0.05
Peels	1.14 ± 0.02	<LOD	1.14 ± 0.02
Pulps	0.33[b]	<LOD	0.33[b]
Seeds	0.06[b]	<LOD	0.06[b]
Dry extract no. 1	34.98 ± 2.39	6.14 ± 0.16	41.12 ± 2.52
Dry extract no. 2	25.55 ± 0.39	1.43 ± 0.08	26.98 ± 0.36
Dietary supplement no. 1	20.88 ± 0.02	1.52[b]	22.39 ± 0.02
Dietary supplement no. 2	20.18 ± 0.04	1.04[b]	21.23 ± 0.04
Dietary supplement no. 3	5.58 ± 0.30	0.32 ± 0.02	5.90 ± 0.32
Dietary supplement no. 4	0.39[b]	0.10 ± 0.01	0.49 ± 0.02
Dietary supplement no. 5	5.67 ± 0.19	0.53 ± 0.03	6.19 ± 0.22

[a] Data are expressed as mean ± S.D. (standard deviation). For each sample $n = 6$.
[b] S.D. < 0.01.

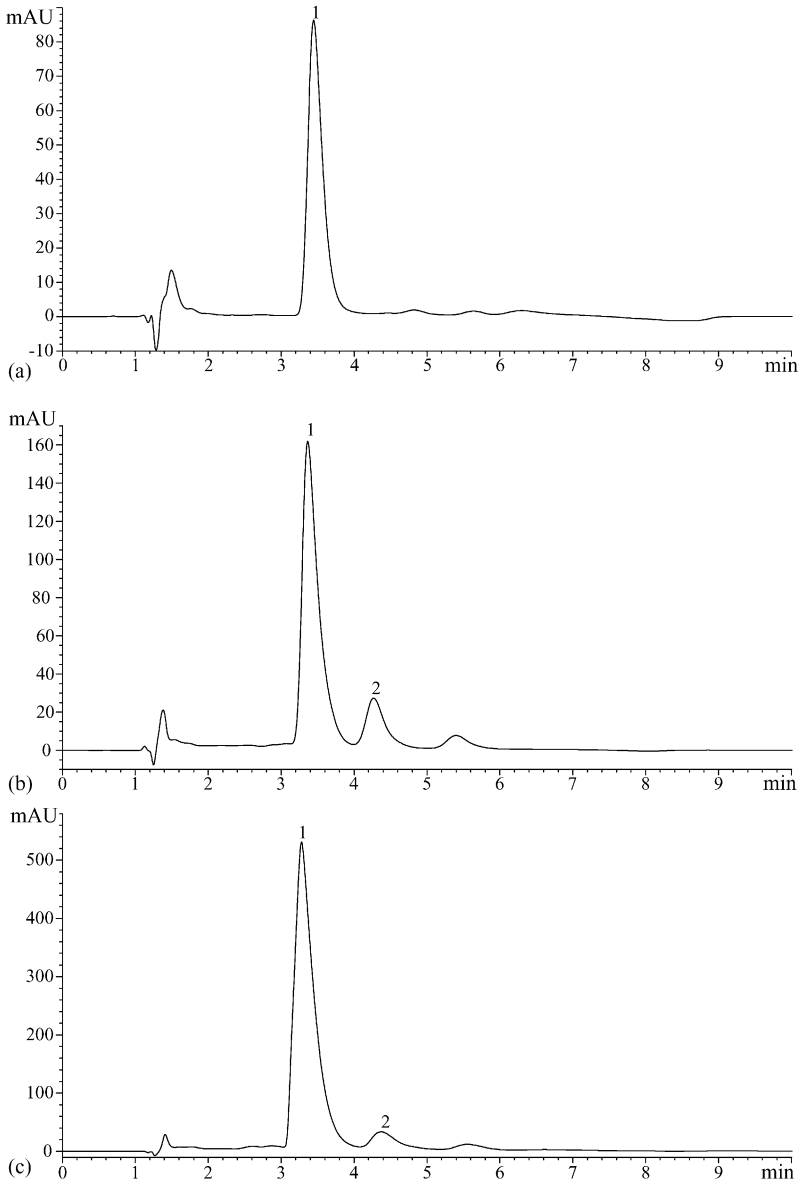

Fig. 6. Representative chromatograms of synephrine enantiomers in *C. aurantium*: (a) fruits, (b) dry extract (no. 1), (c) dietary supplement (no. 1). Peak identification: (1) (−)-synephrine; (2) (+)-synephrine.

aurantium fruits and they found a level of 1.12 mg/g. Takei et al. [36] applied an LC method to determine the content of synephrine in the peel of *Citrus* fruits (3.27 mg/g) and in unripe *Citrus* fruits (2.28 mg/g). Pellati et al. [3,4] determined 1.00 mg/g of synephrine in *C. aurantium* fruits.

Regarding the dietary supplements, in products in which *C. aurantium* extracts were combined with other drugs, no difficulties in the determination of synephrine enantiomers were encountered. This once more highlighted the fact that the method was specific for the determination of synephrine enantiomers in complex matrices. The products on sale on the Italian market showed a very different composition. This could be explained in the light of genetic variation and environmental factors, such as light, temperature, agronomic practices and so on, which may have contributed to the differences in the level of synephrine between the various samples. Furthermore, drying temperature, extraction methods, formulations and storage conditions may have occasioned this variability. Dietary supplements do not require pre-marketing approval and are therefore sold without undergoing extensive testing for safety and efficacy. The manufacturers must make sure that the ingredient list is accurate and that the ingredients are safe. The label must be truthful and not misleading. Since producers are not always aware of the quality of the raw material employed in the formulation of dietary supplements, the proposed LC method could be applied to monitor the quality of *C. aurantium* fruits, extracts and commercial products.

4. Conclusion

A chiral LC method was developed for the separation and quantitative determination of (−)- and (+)-synephrine in natural products. The validation procedure proved that the method has good linearity, accuracy, precision and sensitivity. The practical applicability of this procedure was tested by assaying synephrine enantiomers in *C. aurantium* fruits and phytoproducts. It was found that strong cation-exchange SPE is suitable for sample clean-up before LC analysis. The present method enabled us to measure synephrine enantiomers in *C. aurantium* samples and could be useful for further investigations on the possible racemization of (−)-synephrine during the extraction of *C. aurantium* fruits.

This assay provides a convenient method for the future investigation of the enantiomeric separation and detection of synephrine enantiomers in various vegetable matrices and biological fluids.

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