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Simultaneous determination of *Ephedra sinica* and *Citrus aurantium* var. *amara* alkaloids by ion-pair chromatography

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Abstract

Ephedra sinica (Ma Huang) preparations have recently gained a lot of attention because of serious side effects associated with their prolonged consumption. *Citrus aurantium* var. *amara* is now used as an alternative, despite the fact that similar side effects are suspected. We have developed and validated the first analytical procedure for the simultaneous determination of all major alkaloids from both species. Using the ion-pairing reagent SDS, a C-18 stationary phase (3 μm material) and a pH-gradient for elution enabled the baseline separation of six alkaloids ((±)-octopamine, (±)-synephrine, tyramine, (−)-norephedrine, (+)-pseudoephedrine and (−)-ephedrine) within less than 30 min. The method is sensitive (LOD ≤ 4.6 ng and LOQ ≤ 16.2 ng on-column), selective (L-tyrosine and L-phenylalanine, two closely related amino acids did not interfere), accurate (recovery rates of spiked samples were between 97.5 and 102.0%), repeatable ($\sigma_{rel} \leq 4.6\%$) and precise (intra-day variation ≤7.7%, inter-day variation ≤7.0%). Without the need of a special sample treatment different matrices (plant material, commercial products) were successfully analyzed for their alkaloid content. Dominant alkaloids were (−)-ephedrine (0.9–1.6%) and/or (±)-synephrine (0.1–3.0%). Whether a product contained *Ephedra*-alkaloids or not could be determined in all investigated samples unambiguously.

Keywords: *Ephedra sinica*; Ma Huang; *Citrus aurantium*; Alkaloid; Ion-pair chromatography

1. Introduction

In April 2004 the Food and Drug Administration prohibited the sale of *Ephedra* containing dietary supplements in the US [1]. Using these products for longer periods of time (e.g. for weight loss) can cause serious adverse health outcomes (heart ailments, strokes); alkaloids are responsible for these side effects [2,3].

Major alkaloids in *E. sinica* Stapf (Ephedraceae; vernacular name: Ma Huang), a shrub-like plant distributed from Northern China to Inner Mongolia, whose dried, green haulms are medicinally used, are (−)-norephedrine (**4**), (+)-pseudoephedrine (**5**) and (−)-ephedrine (**6**; Fig. 1). The compounds are structurally closely related to catecholamines, thus they act as sympathomimetic stimulants; they are clinically used for the treatment of asthma, anaphylactic reactions or hypotension [4]. Fruits of *Citrus aurantium* L. var. *amara* (Rutaceae; vernacular name: bitter orange) also contain adrenergic amines ((±)-octopamine (**1**), (±)-synephrine (**2**), tyramine (**3**)); they too function as α- and β-agonists, but do not increase the diastolic blood pressure [5]. After the recent ban of Ma Huang in the US many products for weight loss and bodybuilding now contain *C. aurantium* extracts instead. Although no direct adverse events have been associated with their ingestion thus far, they also should be used with caution because of suspected cardiovascular side effects and possible interferences with CYP P450 enzymes [6].

Several analytical techniques utilizing i.e. HPLC [7–9], CE [10], CEC [11], GC [12] or ^{1}H-NMR [13] have been reported for the determination of *E. sinica* or *C. aurantium* alkaloids. For LC separations the use of ion-pairing reagents

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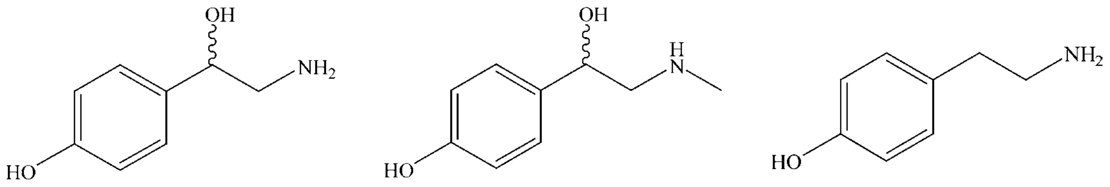
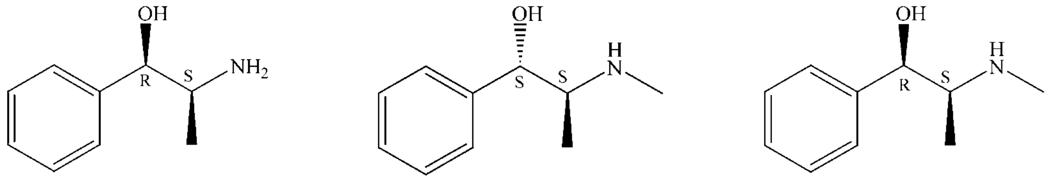


(±) - octopamine (**1**) (±) - synephrine (**2**) tyramine (**3**)



(-) – norephedrine (**4**) (+) – pseudoephedrine (**5**) (-) – ephedrine (**6**)

L – tyrosine (A) L – phenylalanine (B)

Fig. 1. Structures of the main alkaloids in *C. aurantium* var. *amara* (**1**–**3**) and *E. sinica* (**4**–**6**), together with the amino acids tyrosine (A) and phenylalanine (B).

(such as SDS) or cation exchange columns is often described. Despite the number of available methods (especially for Ma Huang), none of them except one describe the simultaneous analysis of the alkaloids from both species. This recently published method enables the determination of the major *Ephedra* alkaloids and synephrine in commercial products, but it requires a complicated sample clean-up by HPLC column-switching, not readily available in many laboratories [14].

With reduction of weight being a profitable market segment (interesting for fraud), validated methods for a simple, fast and simultaneous determination of all relevant alkaloids in *C. aurantium* and *E. sinica* are essential to assure quality and safety of commercial products. Thus we developed an HPLC-method fulfilling these requirements.

2. Experimental

2.1. Materials

All standard compounds (purity ≥98%) used in this study represent the naturally occurring isomers or racemic mixtures, and were purchased from Sigma (**1**, **2**, **3**, **5**, **6**) or Aldrich (**4**; St. Louis, MO, USA). Solvents (acetonitrile, methanol, water) and reagents (concentrated phosphoric acid, concentrated hydrochloric acid, sodium dodecyl sulphate (SDS)) were of analytical grade and came from Merck (Vienna, Austria).

Plant material (*E. sinica* herb, *C. aurantium* var. *amara* peel) was purchased from Kögl Pharmaceuticals (Innsbruck, Austria), commercial products were bought in 2001 and 2003 from Nutrimart (Diamond Bar, CA, USA) via the internet. Voucher specimens of all samples are deposited at the Department of Pharmacognosy, University of Innsbruck.

2.2. Sample preparation

Samples (500 mg of a product or 1.00 g of the finely powdered plant material) were extracted three times with 3 ml 0.37% hydrochloric acid (concentrated hydrochloric acid/water = 1/99; v/v) by sonication at ambient temperature for 10 min. After centrifugation (10 min at 3000 rpm), the extracts were combined in a 10 ml volumetric flask and filled up to the final volume with extraction solvent. Prior to use, all samples were filtered through a 0.45 μm nylon membrane filter (SRP 15, Machery Nagel, Düren, Germany).

2.3. Calibration

An amount of 10 mg of each compound **1**–**6** was placed in a 10 ml volumetric flask and dissolved in methanol (stock

Table 1
Calibration data for compounds **1**–**6**, including regression equation, relative standard deviation of slope ($n = 3$), correlation coefficient (R^2) and limit of detection and quantitation (LOD and LOQ, respectively; values in nanograms on-column)

	Regression equation	σ_{rel} of slope	R^2	LOD	LOQ
1	$y = 1.49\mathrm{E}{-}5X + 0.472$	6.5	1.0000	4.6	16.2
2	$y = 1.38\mathrm{E}{-}5X - 0.010$	4.3	0.9999	2.3	8.0
3	$y = .40\mathrm{E}{-}5X - 0.250$	6.0	0.9999	2.3	8.0
4	$y = 1.25\mathrm{E}{-}5X - 2.782$	3.2	0.9998	2.3	8.0
5	$y = 1.35\mathrm{E}{-}5X - 0.165$	2.8	0.9999	2.3	8.0
6	$y = 1.29\mathrm{E}{-}5X - 0.709$	5.0	0.9999	2.3	8.0

solution). Beginning with this solution, five additional calibration levels were prepared by a 1:2 serial dilution with methanol. Standard solutions were stable for at least 30 days if stored at 4 °C (confirmed by re-assaying the solution). Within the range of concentrations injected (4.1–1000 μg ml^{-1}) the detector response was linear; see Table 1 for combined calibration data (limits of quantitation and detection were determined by serial dilution and calibration curves were generated by linear regression based on peak area). All data were recorded and processed by Chemstation software Rev. A09.01 (Agilent, Waldbronn, Germany).

2.4. Analytical method

Analytical experiments were performed on a HP 1100 HPLC system, equipped with photodiode array detector, autosampler and column heater (Agilent, Waldbronn, Germany). A HyperClone C18 BDS 1 column (100 mm × 4.6 mm, 3 μm particle size) from Phenomenex (Torrance, CA, USA) was used for all separations. The mobile phase comprised of three solvents, 3 mM aqueous SDS solution with pH 4.0 (A), 0.1% aqueous phosphoric acid (v/v) containing 3 mM SDS (B) and an acetonitrile–methanol mixture in the ratio of 2/1 (C; v/v); the pH-value of solvent A was adjusted with 5% phosphoric acid (v/v), solvent B showed a pH of 2.0. Elution started with 75A/0B/25C, changed linearly over 10 min to 0A/65B/35C, to reach 0A/60B/40C after five more minutes. In ten additional minutes it changed to 0A/55B/45C (linear gradient), to stay at that composition for 5 min (total runtime 30 min). After each analysis the column was washed for 5 min with 100C, followed by an equilibration period of 10 min. The flow rate was adjusted to 1.0 ml min^{-1}, the detection wavelength set to 210 nm and 10 μl of sample were injected. All separations were performed at ambient temperature (25 °C). Peaks were assigned by spiking the samples with standard compounds, and comparison of their UV-spectra and retention times.

2.5. Method validation

The HPLC-method was validated for linearity (see Section 2.3), limit of quantitation and detection, peak purity, accuracy, precision and repeatability.

Table 2
Intra- and inter-day precision of developed assay, if sample I is extracted and assayed five-fold per day on three consecutive days (values in μg ml^{-1}, relative standard deviation in parentheses)

Compound	Intra-day ($n = 5$)			Inter-day ($n = 3$)
	Day 1	Day 2	Day 3	
1	26.7 (7.7)	27.0 (6.0)	27.0 (4.1)	26.9 (0.6)
2	85.1 (3.9)	84.7 (4.5)	95.6 (4.2)	88.5 (7.0)
5	98.0 (1.6)	96.8 (1.1)	92.9 (1.0)	95.9 (2.8)
6	456.0 (0.5)	451.8 (1.4)	466.9 (1.0)	458.2 (1.7)

Limit of quantitation (S/N ratio of 10) and limit of detection (S/N ratio of 3) were determined by serial dilution of standard solutions containing **1**–**6** and determined to be 16.2 and 4.6 ng on-column (**1**) and below (Table 1), peak purity was confirmed by using the “peak purity evaluation” option included in the Chemstation software package. Based on the spectral information at hand (DAD-data), all relevant peaks in the sample chromatograms were found to be free of impurities (threshold value set to 975). Accuracy was determined by spiking sample II (500 mg each) with three concentrations of the standard compounds; the spiked samples were assayed under optimized conditions and recovery rates (data not shown in detail) were between 97.5 and 102.0%.

Precision (intra- and inter-day) of the assay was verified by analyzing sample I five-fold on three consecutive days; for detailed results see Table 2. Repeatability was confirmed by evaluating consistency of retention times and standard deviations. Retention times shifted only marginally and a maximum relative standard deviation of 4.6% was observed for triplicate injections (Table 3).

3. Results and discussion

The addition of an ion-pairing reagent to the mobile phase is required if biogenic amines are to be analyzed on C-18 material, otherwise the compounds are poorly retained on the stationary phase and cannot be resolved [15]. When determining these compounds in dietary supplements for weight loss, the complex nature of the matrix (usually mixtures of up to seven plants or extracts, amino acids and vitamins) further hampers the analysis. Thus, time-consuming or sophisticated sample preparation procedures are usually required in order to obtain satisfactory results.

Initial screening experiments showed that a separation of compounds **1**–**6** is only feasible with particular stationary phases (Discovery Bio Widebore C 18 from Supelco or HyperClone C-18 BDS-1 from Phenomenex). The latter, being of 3 μm particle size, was chosen because of a slightly better resolution of **4**–**6**. Other materials tested (Hypersil C18, Hypersil C18 (ODS), Luna C-8(2), Luna Phenyl-Hexyl, Lichrosphere C18) gave unsatisfactory results. Additionally, the solvent system has to be acidic (to convert the analytes in the cationic form) and the less polar part of the mobile phase has to comprise acetonitrile and methanol. Best results were

Table 3
Analysis of alkaloids in different commercial products (samples I–VII) and in *E. sinica* and *C. aurantium* var. *amara* plant material; values in percent, relative standard deviation in parentheses ($n = 3$)

Sample	**1**	**2**	**3**	**4**	**5**	**6**
I[a]	–	0.16 (0.7)	–	–	0.22 (0.3)	0.94 (0.1)
II[a]	–	0.08 (0.4)	–	0.01 (2.7)	0.22 (0.1)	1.50 (0.3)
III[b]	0.02 (2.8)	1.03 (0.4)	0.06 (2.7)	–	–	–
IV[b]	–	1.11 (0.5)	–	–	–	–
V[b]	–	1.27 (0.2)	–	–	–	–
VI[b]	0.02 (1.3)	1.28 (0.1)	0.01 (3.8)	–	–	–
VII[b]	0.09 (1.0)	3.05 (1.9)	–	–	–	–
E. sinica	–	–	–	–	0.70 (0.3)	1.63 (0.3)
C. aurantium	0.02 (4.6)	0.21 (0.9)	–	–	–	–

[a] Commercial product claiming to contain *E. sinica* and *C. aurantium* var. *amara*.

[b] Commercial product claiming to contain *C. aurantium* var. *amara*.

obtained when using a 2:1 mixture of these solvents. Of great importance revealed to be the SDS concentration as well. Increasing the ion-pairing reagent concentration from 1 to 3 mM steadily improved the result but also prolonged the required separation times (higher concentrations of surfactant result in higher capacity factors of organic analytes due to more effective ion pair formation) [16]. With the addition on 5 mM SDS the *Ephedra* alkaloid signals became unsymmetrical and started to overlapped in part, therefore 3 mM of the surfactant was considered to be the optimum concentration. As temperature was of minor influence on the obtained results, analyses were performed at 25 °C.

The method described above worked very well for separating standard mixtures of the six reference compounds. When analyzing some of the real-life samples (commercial products) a strange phenomenon occurred, as the signal for “octopamine” increased with multiple extractions of the sample. This is due to the presence of the barely soluble amino acid L-tyrosine (A), overlapping with the corresponding octopamine peak. Attempts to quantitatively remove this amino acid by anionic exchange SPE cartridges failed, but by introducing a third mobile phase (3 mM SDS solution with pH 4.0) this problem of co-elution could be resolved. Starting with the less acidic aqueous phase and applying a pH-gradient for elution, facilitated the separation of the amino acids L-tyrosine and L-phenylalanine from compounds **1**–**6** within less than 30 min (Fig. 2).

When extracting *Ephedra*-type alkaloids from plant material the use of different solvents (water, acetone, and methanol) and techniques (e.g. SPE, supercritical fluid extraction) is described in literature [17–20]. As acidic solvents were preferably used, we tested 0.37% hydrochloric acid in

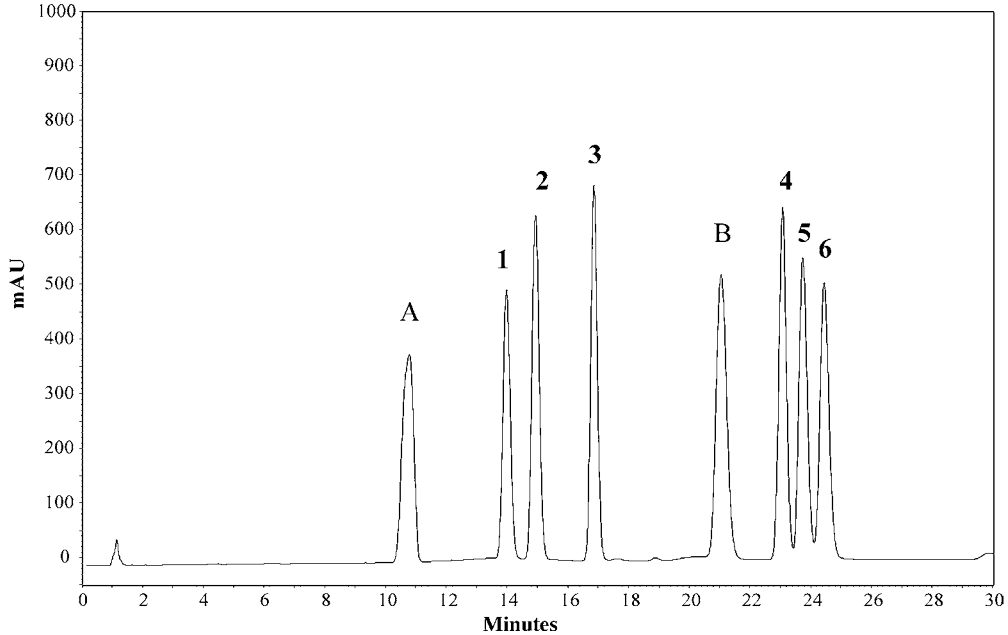


Fig. 2. Separation of standard compounds **1**–**6** and two amino acids (A, B) under optimized conditions (column: HyperClone C18 BDS1, 100 mm × 4.6 mm, 3 μm; mobile phase: 3 mM aqueous SDS solution, pH 4.0 (A), 0.1% phosphoric acid (v/v) with 3 mM SDS (B), acetonitrile/methanol = 2/1 (C); gradient: 75A/0B/25C in 10 min to 0A/65B/35C, in 5 min 0A/60B/40B, in 10 min to 0A/55B/45C, and left at that composition for 5 min; flow rate: 1.0 ml min^{-1}; sample volume: 10 μl; temperature: ambient; detection: 210 nm); peak assignment is according to Fig. 1.

water and a three-fold extraction by sonication at room temperature for its applicability. One sample (500 mg of product II) was extracted four times with 3 ml of 0.37% hydrochloric acid, and the extract obtained after every sonication step was analyzed. After the third repetition a minimum of 98.6% (compound **6**) of each standard compound was already in solution. The chosen approach can therefore be considered to be efficient and exhaustive.

The developed method was validated in order to show its compliance with international requirements for analytical methods for the quality control of pharmaceuticals. From the calibration data obtained (Table 1), linearity of the detector response for **1**–**6** was confirmed between 4.1 and 1000 μg ml^{-1}, and a detection limit of at least 4.6 ng on-column (compound **1**) was determined. Peak purity was investigated by using the respective option in the operating software; no indications for impurities or signs for co-elution could be found in the sample chromatograms (Fig. 3). All standards and samples were injected in triplicate. A maximum relative standard deviation of 4.6% (compound **1**, in *C. aurantium* plant material) and very stable retention times over the whole study period (300 injections) confirmed the precision of the method (Table 3); intra- and inter-day variation (five individual specimens of sample I were assayed on each of three consecutive days) showed to be lower than 7.7% (**1**, intra-day on day 1) for all compounds (Table 2).

Accuracy of the assay was determined by spiking sample II with three concentrations of **1**–**6**, representing 50, 100 and 150% of the expected value. Samples were extracted and assayed under optimized conditions, and the recovery rates obtained were close to 100% in most cases. Maximum deviations were observed for compounds **5** (97.5% recovery at 50%

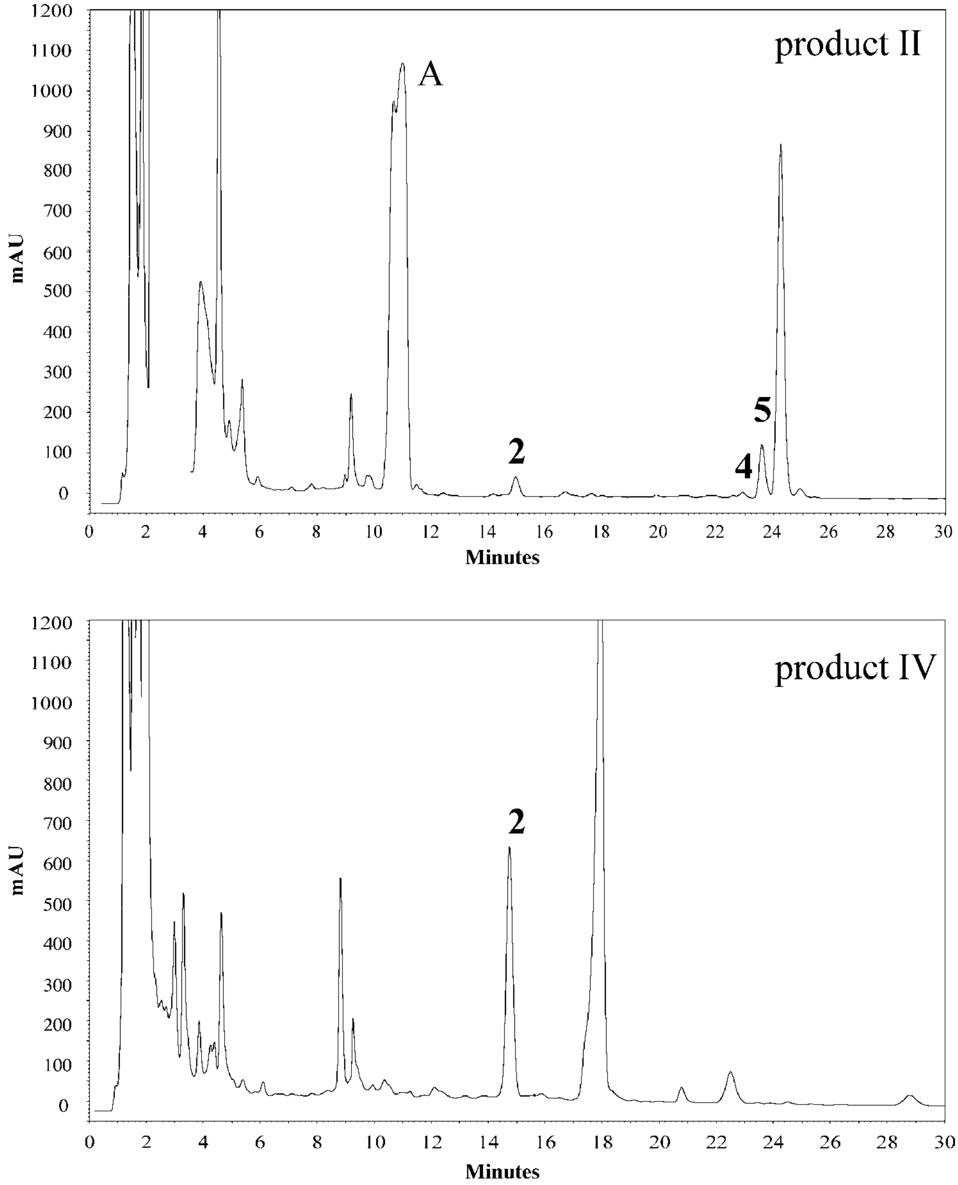


Fig. 3. Chromatogram of samples II and IV extracted and assayed under optimized conditions; peak assignment is according to Fig. 1.

spike level) and **6** (102.0% recovery at 100% spike level). These data not only confirm accuracy but also integrity of the extraction procedure, e.g. filtration of the sample through membrane filter prior to injection.

The HPLC method should have a wide range of possible applications, thus it was used for the analysis of plant material (*E. sinica* herb, *C. aurantium* var. *amara* peel) as well as a number of commercial products (Table 3). Some of the latter were bought in 2001 (samples I and II, they contained the extracts of both species), the rest was purchased in 2003 (and claimed to be *Ephedra* free). For all kinds of samples analyzed, the newly developed HPLC method easily allowed the determination of the contained species (*E. sinica* and/or *C. aurantium*) and the quantification of the appropriate major alkaloids (Fig. 3). Even in complex samples, consisting of multiple extracts and additives, no interfering peaks were observed. Compounds were assigned based on retention times and UV-spectra, a confirmation by LC-MS was not possible because of non-volatile constituents (phosphoric acid, SDS) in the mobile phase.

All samples analyzed followed the same pattern concerning their alkaloid content. If they contained Ma Huang, the most dominant alkaloid was (−)-ephedrine (0.94–1.63%), followed by (+)-pseudoephedrine (0.22–0.70%); (−)-norephedrine was detectable in one sample only. In samples containing bitter orange, (±)-synephrine was the major *Citrus* alkaloid (0.08–3.05%). Tyramin and (±)-octopamine were found, if present at all, in low concentrations (0.01–0.09%) only. The results were in good agreement to those reported in literature (e.g. 0.66–3.08% of **2** in commercial products [7]), and showed that all commercial products were labelled correctly.

The developed HPLC-method has significant advantages over existing methodologies. It allows the selective, quantitative analysis of all six major alkaloids in *E. sinica* and *C. aurantium* var. *amara* in one analytical run (the separation of other biologically not relevant enantiomers was not the aim of this study), with separation times comparable to previous methods using RP material. No time-consuming sample pretreatment is required, regardless of the analyzed specimen (plant material or commercial product). The method was validated in accordance to international standards, thus it should possess scientific as well as commercial applicability.

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