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Journal of Pharmaceutical and Biomedical Analysis 48 (2008) 254–263
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
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# Determination of ephedrine alkaloids in *Ephedra* natural products using HPLC on a pentafluorophenylpropyl stationary phase

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Received 31 August 2007; received in revised form 15 October 2007; accepted 28 October 2007
Available online 6 November 2007

**Abstract**

In this study a pentafluorophenylpropyl (PFPP) stationary phase was applied to the fast and reliable qualitative and quantitative analysis of ephedrine alkaloids in *Ephedra* plant material and derivatives. A Discovery HS F5 column (150 mm × 4.6 mm i.d., 5 μm) was used, with an isocratic mobile phase composed of ammonium acetate (7 mM) in acetonitrile–water (90:10, v/v), at a flow rate of 1.0 ml/min. The column temperature was set at 45 °C. UV detection was set at 215 and 225 nm. The total analysis time was 16 min. The validation parameters, such as linearity, sensitivity, accuracy, precision and specificity, were found to be highly satisfactory. Sonication and microwave extractions were compared in order to optimize the yield of the target analytes. Under the optimized extraction conditions (based on two cycles of sonication with methanol at 40 °C for 15 min), different matrices containing *Ephedra* were successfully analyzed for their alkaloid content. The method was applied to the analysis of standard reference materials (SRMs) containing *Ephedra*. Furthermore, the developed technique allowed the simultaneous determination of ephedrine alkaloids and synephrine, the main phenethylamine alkaloid of *Citrus aurantium*, that has replaced *Ephedra* in dietary supplements used in the treatment of obesity. The results indicated that this procedure is suitable for the phytochemical analysis of *Ephedra* plant material and extracts, and can be applied to demonstrate the label claims for product content, including the absence of ephedrine alkaloids in *Ephedra*-free products.


*Keywords:* *Ephedra*; Ephedrine alkaloids; Synephrine; Pentafluorophenylpropyl stationary phase; Phytochemical analysis; HPLC

## 1. Introduction

*Ephedra sinica* Stapfs. (Ephedraceae family), also known as Ma Huang, is one of the oldest medicinal herbs in traditional Chinese medicine (TCM). Other species of this genus include *E. equisetina*, *E. intermedia* and *E. vulgaris* (syn. *E. distachya*) [1,2]. *E. sinica* preparations are obtained from the aerial parts of the plant and have been used for the treatment of asthma, bronchial spasms, as a stimulant and diaphoretic [3]. The ephedrine alkaloids (Fig. 1) are considered the active constituents of plants belonging to the genus *Ephedra*. (−)-Ephedrine is the major isomer; the minor alkaloids include (−)-norephedrine, (+)-norpseudoephedrine, (+)-pseudoephedrine and (−)-methylephedrine. The amount of ephedrine alkaloids varies from 0.02 to 3.40% in the aerial parts of the plant [2]. The pharmacological studies have indicated that ephedrine is a sympathomimetic agonist at both α- and β-adrenergic receptors, which determine an increase of cardiac rate and contractility, peripheral vasoconstriction, bronchodilatation and central nervous system (CNS) stimulation [2]. Ephedrine is not the only alkaloid used in commercial products, since decongestant preparations usually contain pseudoephedrine. In recent years, the number of dietary supplements containing *Ephedra*, either as powdered botanical or, more frequently, as a standardized extract, had increased dramatically. Most of these products have been sold for the treatment of obesity or for increasing performance in body building. Often these dietary supplements also contained caffeine, either synthetic or from botanical extracts, in addition to other ingredients [3]. Weight loss and enhanced performance in body building may be due to the CNS stimulation and thermogenic properties of ephedrine [2]. However, severe contraindications have been given for individuals with hypertension or other cardiovascular diseases, glaucoma, diabetes and hyperthyroidism. Products containing *E. sinica* (or another botanical source of ephedrine) were among the most popular dietary supplements on the market, until their sale was

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doi:10.1016/j.jpba.2007.10.034

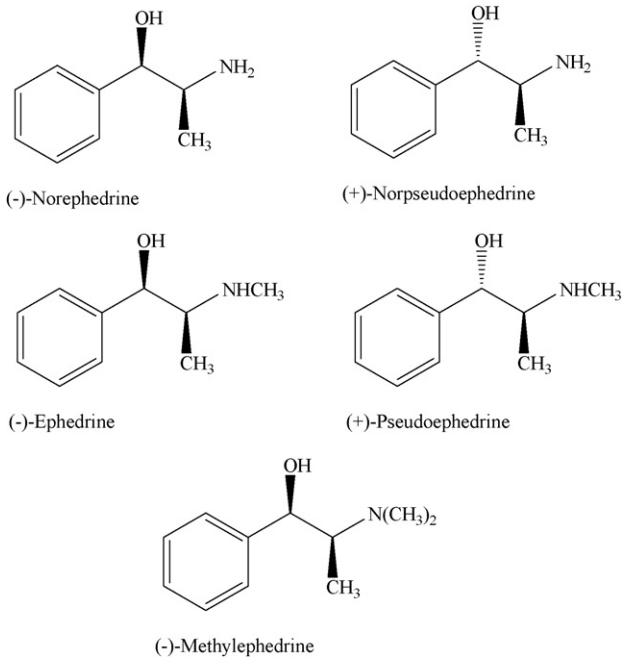


Fig. 1. Chemical structures of ephedrine alkaloids.

banned by the U.S. Food and Drug Administration (FDA) in April 2004. After the ban of *Ephedra* products, "*Ephedra*-free" dietary supplements for weight loss were introduced. However, *Ephedra*-free is not necessarily danger-free [4]. *Citrus aurantium* is an ingredient in many of these *Ephedra*-free dietary supplements. The main active constituent of *C. aurantium* fruit extracts is (−)-synephrine [4], a phenethylamine alkaloid similar in structure to ephedrine (Fig. 2). However, dietary supplements often contain *C. aurantium* in combination with concentrates of other herbs that are rich in caffeine and have the same potential to induce arrhythmia, hypertension, heart attacks and strokes as the combination of ephedrine and caffeine [4].

Adverse events attributed to consumption of products containing ephedrine alkaloids led to the development of many analytical methods for their determination. Because of the health and legal implications associated with products containing *Ephedra*, it is desirable to have a reliable analytical method that can quantify ephedrine alkaloids in plant material and derivatives. A variety of chromatographic and electrophoretic separation techniques have been used for the determination of ephedrine alkaloids in *E. sinica* and related species. Several analytical methods involving GC [5,6] and HPLC with UV [2,3,7–13] or MS [12–15] detection have been reported. HPLC–MS/MS has also been recently applied to the analysis

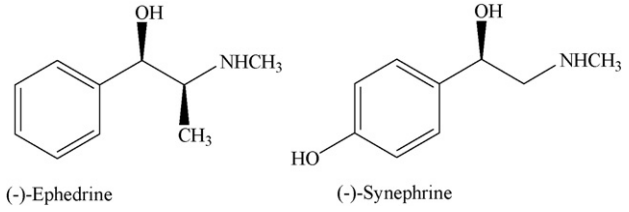


Fig. 2. Structures of ephedrine and synephrine.

of ephedrine alkaloids in biological fluids [16]. Furthermore, CE has been successfully employed for the analysis of these analytes [13,17–19]. HPLC is the preferred technique in phytochemical analysis, owing to its sensitivity, precision and specificity. HPLC with UV detection is an apparatus generally present in analytical laboratories and it is one of the analytical technique applied most frequently in the analysis of natural products; the same is not for HPLC coupled to MS or MS/MS. Furthermore, UV detection offers sufficient selectivity and sensitivity for determination of ephedrine alkaloids in plant extracts [2,3,7–13]. Since ephedrine alkaloids are hydrophilic amine compounds, they are characterized by poor retention on traditional reversed-phase columns. Furthermore, their basic nature often leads to excessively broad peaks and peak tailing on conventional chromatographic systems. In some methods, the mobile phase contains an ion-pair reagent to increase the peak symmetry and resolution of the target analytes [8,10,11]. The main limitation of ion-pair reagents is that their poor volatility and ion-suppressing effects make methods using them less amenable to MS analysis. Previous methods involving HPLC [2,3,7–15] require time-consuming sample preparation procedures, based on SPE or multiple extraction steps, or lengthy analysis time. In this study, an HPLC method with UV detection on a pentafluorophenylpropyl (PFPP) stationary phase coupled with a simplified sample preparation was optimized and validated for the rapid and reliable separation and quantitative determination of ephedrine alkaloids in *E. sinica* natural products and related species (*E. vulgaris*). For controlling the retention, several influencing factors, such as the mobile phase counter ion concentration and column temperature, were investigated and the optimum separation conditions were determined. To demonstrate the practicality of the technique, the validated method was applied to determine the levels of the active compounds in *Ephedra* plant material (*E. vulgaris* and *E. sinica*) and natural products. Furthermore, the developed method allowed the simultaneous determination of ephedrine alkaloids and synephrine. The NIST *Ephedra* standard reference materials (SRMs) were selected for evaluating the applicability of this study. The *Ephedra*-containing SRMs represent a variety of natural, extracted and processed sample matrices that are certified for the levels of ephedrine alkaloids [13,19,20]. Therefore, these products are particularly recommended in method validation and as control materials for analytical techniques used in the determination of ephedrine alkaloids. For the extraction procedure, sonication and microwave extractions were compared. The effects of several variables (solvent, temperature and time) on the extraction yield of the above-mentioned compounds were investigated.

## 2. Experimental

### 2.1. Chemicals and solvents

Norephedrine, norpseudoephedrine, methylephedrine, ephedrine and pseudoephedrine were purchased from Cerilliant (Round Rock, TX, USA). The compounds were received as a 1.0 mg/ml solution in methanol (as free base), except norpseu-

doephedrine that was available at 0.1 mg/ml concentration (as free base). Synephrine was purchased from Sigma (Milan, Italy).

Hydrochloric acid (37%), ammonium formate and ammonium acetate were from Fluka (Milan, Italy). HPLC grade methanol and acetonitrile were from Sigma (Milan, Italy). Water was purified using a Milli-Q Plus 185 system from Millipore (Milford, MA, USA).

The mobile phases used in this study were prepared by dissolving ammonium formate or acetate in a mixture of acetonitrile–water (90:10, v/v) to obtain the desired molar concentration of ammonium counter ion. All mobile phases were pre-mixed. The pH values of the mobile phases were unadjusted (pH 6.9, prior to the addition of organic modifier).

### 2.2. Plant material

*E. vulgaris* Rich. aerial parts were kindly donated by a local herb company. The dried sample was protected from light and humidity until required for chemical analysis. The plant material was ground to obtain a homogeneous powder immediately before extraction with an IKA grinder (Staufen, Germany). SRM 3245 *Ephedra* standard reference material was purchased from the National Institute of Standards and Technology (Gaithersburg, MD, USA) and contains: *E. sinica* Stapf. aerial parts (SRM 3240), *E. sinica* Stapfs. native extract (SRM 3241), *E. sinica* Stapfs. commercial extract (SRM 3242), *Ephedra*-containing solid oral dosage form (SRM 3243) and *Ephedra*-containing protein powder (SRM 3244). As indicated in the certificate of analysis provided by the manufacturer, the native extract (SRM 3241) and the commercial extract (SRM 3242) were obtained by extraction with hot water under pressure of the *E. sinica* powdered botanical raw material (SRM 3240). A portion of the water extract was filtered, concentrated and spray-dried to produce the native extract. A second portion of the water extract was filtered, concentrated and then fortified with ephedrine to yield nominally 8% total ephedrine alkaloids prior to spray drying to produce the commercial extract. SRM 3243 *Ephedra*-containing solid oral dosage form was prepared from several different commercially available products (both tablets and capsules) that were purchased in the marketplace, from multiple vendors to obtain material of different production lots. SRM 3244 *Ephedra*-containing protein powder was prepared from several brands of commercially available products that were purchased in the marketplace, from multiple vendors to obtain material of different production lots; these materials were primarily milk-based products, although some egg protein was present. Individual amino acids, flavorings, botanicals (including *E. sinica*), vitamins and elements were among the other ingredients in the products that were combined.

### 2.3. Chromatographic apparatus

Chromatography was performed using 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 using an Agilent ChemStation for LC and LC–MS systems (Rev. B.01.03) and a Pentium IV personal computer.

### 2.4. HPLC method

For HPLC analysis, a Discovery HS F5 column (150 mm × 4.6 mm i.d., 5 μm) (Supelco, Bellefonte, PA, USA) was used, coupled to a Discovery HS F5 guard column (20 mm × 4.0 mm i.d., 5 μm). The mobile phase was ammonium acetate (7 mM) in acetonitrile–water (90:10, v/v), under isocratic conditions. The flow rate was 1.0 ml/min. The column was thermostatted at 45 °C. The sample injection volume was 5 μl. The detector monitored the eluent at 215 nm (for ephedrine alkaloids) and 225 nm (for synephrine). The total analysis time was 16 min. Three injections were performed for each sample.

### 2.5. Identification of constituents and peak purity

Peaks were identified on the basis of their retention time ($t_R$) values and UV spectra by comparison with those of the single compound in the standard solution. Peak identity was also confirmed by spiking the extracts with pure standards (standard addition method). Peak purity tests were performed using a photodiode array detector coupled to the HPLC system, comparing the UV spectra of each peak with those of authentic reference samples.

### 2.6. Extraction methods

#### 2.6.1. Sonication extraction

The sample preparation from *Ephedra* plant material (*E. vulgaris* and *E. sinica* aerial parts) involved a sonication extraction in an ultrasonic bath (Sonorex RK-100 H, Bandelin, Berlin, Germany) of a weighed amount of ground sample (0.5 g) with 10 ml of solvent (methanol or a mixture of hydrochloric acid (37%)–methanol (0.8:99.2, v/v)) at different temperatures (room temperature, 40 or 50 °C) for 15 min. The extract solution was filtered in a vacuum into a 25-ml volumetric flask. The residue was re-extracted in the same way. The filtrates of the two extractions were combined and the solvent was then added to the final volume.

Regarding *Ephedra* natural products, a weighed amount of sample (0.25 g of *E. sinica* native extract; 0.1 g of *E. sinica* commercial extract; 0.5 g of *Ephedra*-containing solid oral dosage form; 2.5 g of *Ephedra*-containing protein powder) was extracted twice by sonication with 10 ml of solvent as described above, and the final volume of the extract was 25 ml. In the case of *Ephedra*-containing protein powder, a centrifugation step (at 4000 rpm for 3 min) was applied before the filtration of the extract solution.

All the extracts were filtered through a 0.45-μm PTFE filter into a HPLC vial and capped. The extraction procedure was repeated twice for each sample.

#### 2.6.2. Microwave extraction

Regarding microwave extraction, a weighed amount of ground sample (0.25 g of *E. vulgaris* aerial parts) was extracted with 5 ml of solvent (methanol or a mixture of hydrochloric acid (37%)–methanol (0.8:99.2, v/v)) by using a monomode microwave apparatus with a closed vessel system (Discover instrument, CEM, Metthews, NC, USA) and subjected to different temperatures for different times of irradiation (40 °C for 15 min or 60 °C for 4 min or 80 °C for 1 min). Continuous microwave treatment was used (50 W). During the extraction, magnetic stirring was applied. After the extraction time had elapsed, the vessels were allowed to cool at room temperature before opening. The extract solution was filtered in a vacuum into a 25-ml volumetric flask. The residue was re-extracted in the same way. The filtrates of the two extractions were combined and the solvent was then added to the final volume. All the extracts were filtered through a 0.45-μm PTFE filter into a HPLC vial and capped. The extraction procedure was repeated twice for each sample.

### 2.7. Method validation

The method validation was carried out to show compliance with international requirements for analytical methods for the quality control of pharmaceuticals. For validation of the analytical method, the ICH guidelines were followed [21].

Concerning linearity, each compound was purchased in a methanol stock standard solution at 1.0 mg/ml concentration (as free base), with the exception of norpseudoephedrine (0.1 mg/ml, as free base). Further calibration levels were prepared by diluting each stock solution with methanol. For each compound, the external standard calibration curve was generated using six data points, covering the concentration ranges reported in Table 1. Five-μl aliquots of each standard solution were used for HPLC analysis. Injections were performed in triplicate for each standard solution. The calibration curve was obtained by plotting the peak area of the compound at each level versus the concentration of the sample. To evaluate if the normal distribution is a good model for these compounds, the normal probability plot of the residuals was calculated for each calibration and the residuals were graphically examined.

The LOD of the method was evaluated considering the analyte concentration that would yield a signal-to-noise (S/N) value of 3; the LOQ represents the analyte concentration that would yield a S/N value of 10. The LOD and LOQ values were experimentally verified by injections of standard solutions of the compounds at the LOD and LOQ concentrations.

The accuracy of the analytical procedure was evaluated using the recovery test. This involved the addition of known quantities of reference standard compounds to half the sample weight of *E. vulgaris* plant material. In the case of norephedrine, an amount of standard compound corresponding to the LOQ value was spiked. The fortified samples were then extracted and analyzed by the proposed HPLC method. Regarding *E. sinica* samples, the method accuracy was evaluated by comparing the results of the proposed method with the certified values provided by the manufacturer for *E. sinica* aerial parts. Value assignment of alkaloid content was certified through the application of multiple analytical methods, which included measurements by NIST and collaborating laboratories.

The precision of the chromatographic system was tested by performing intra- and inter-day multiple injections of a standard solution containing ephedrine alkaloids and synephrine, and then checking the %R.S.D. of retention times and peak areas. Ten injections were performed each day for three consecutive days.

The precision of the extraction procedure was validated by repeating the extraction procedure on the same sample of *E. vulgaris*. An aliquot of each extract was then injected and quantified. This parameter was evaluated by repeating the extraction in duplicate on three different days with newly prepared mobile phase and samples.

Specificity was tested by using the HPLC method to analyze the *Ephedra*-containing solid oral dosage form to demonstrate the capacity of the technique to discriminate the target analytes from the other constituents of the formulation.

Stability was evaluated with *E. vulgaris* and *E. sinica* plant extracts that were stored in amber glass flasks at 4 °C and at room temperature (about 25 °C) and analyzed every 12 h.

## 3. Results and discussion

### 3.1. Method development and optimization

Ephedrine alkaloids, a group of natural compounds widely distributed in *Ephedra* species, are a class of small and polar molecules that are particularly difficult to analyze by RP-HPLC. Several chromatographic methods published in the literature

Table 1
Statistical analysis for the calibration curves of ephedrine alkaloids and synephrine[a]

| Compound | Linearity range (μg/ml) | Slope (*a*) | Intercept (*b*) | $r^2$ |
|---|---|---|---|---|
| Norephedrine | 1.00–100.00 | 10.069 (±0.011) | −0.299 (±0.514) | 1.0000 |
| Norpseudoephedrine | 1.00–100.00 | 9.104 (±0.004) | 0.781 (±0.200) | 1.0000 |
| Synephrine | 1.00–100.00 | 13.788 (±0.019) | −0.373 (±0.914) | 1.0000 |
| Methylephedrine | 1.00–100.00 | 8.856 (±0.008) | −0.967 (±0.386) | 1.0000 |
| Ephedrine | 1.00–1000.00 | 8.609 (±0.003) | 4.680 (±1.170) | 1.0000 |
| Pseudoephedrine | 1.00–1000.00 | 8.664 (±0.003) | 0.097 (±1.152) | 1.0000 |

Experimental conditions as in Section 2.4.

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

use the ion-pairing technique to increase retention and peak symmetry [8,10,11]. However, ion-pairing is not ideal, because it reduces the robustness, reproducibility and the HPLC–MS compatibility of the method.

RP-HPLC phases that contain polar groups as part of their structure often succeed in retaining and resolving compounds that $C_{18}$ phases do not, because they can interact with analytes by mechanisms not available with the $C_{18}$ alkyl chains. An earlier study had demonstrated that the PFPP stationary phase of the Discovery HS F5 column can overcome the problems of basic compound separation, such as peak tailing, low retention and low resolution [22]. However, the authors evaluated the chromatographic performance of this stationary phase on a set of pure compounds for demonstration purposes only; no validation and application on complex matrices, such as natural products, were reported. In this study, the chromatographic performance of the Discovery HS F5 column was investigated for the separation and quantitative determination of ephedrine alkaloids in *Ephedra* natural products. Synephrine was also included in the target analytes, since it is the major phenethylamine alkaloid of *C. aurantium*, that is now used as an alternative of *Ephedra* in dietary supplements indicated for weight loss [4]. The chromatographic conditions were optimized with the aim of obtaining chromatograms with a good resolution of adjacent peaks within a short analysis time. It is known that retention on a PFPP stationary phase depends mainly on the mobile phase counter ion concentration and column temperature [22]. Therefore, mobile phase counter ion concentration and temperature are powerful tools for optimization of analyte retention and selectivity.

Regarding the mobile phase composition, two solvents were used – acetonitrile and water (90:10, v/v) – containing 2–6 mM ammonium formate or 4–8 mM ammonium acetate. A reduction in analyte retention with the increase in mobile phase counter ion concentration (ammonium in this case) was observed for both ammonium formate and acetate. With a mobile phase containing 6 mM ammonium formate or 8 mM ammonium acetate in 90% acetonitrile, the retention times of the compounds were shorter, resulting in a more rapid separation.

The effect of temperature on the analyte retention and selectivity was tested by setting the column temperature at 20, 30 and 45 °C. Greater retention with increasing temperature was observed. The effect of temperature was weak with ammonium formate and it was particularly strong by using ammonium acetate in the mobile phase. Furthermore, by using ammonium acetate in the mobile phase, temperature was shown to be an effective parameter for the optimization of selectivity and resolution of the analytes.

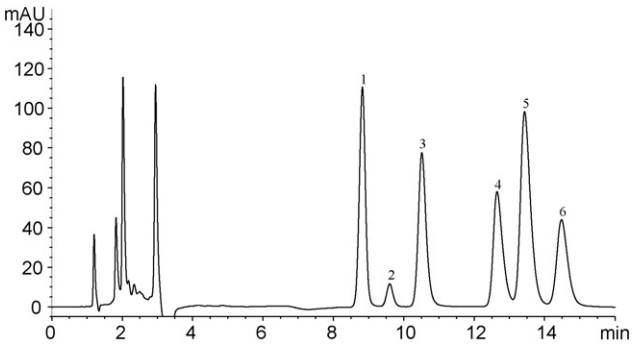


Fig. 3. Chromatogram of a standard mixture of ephedrine alkaloids and synephrine. Peak identification: (1) norephedrine; (2) norpseudoephedrine; (3) synephrine; (4) methylephedrine; (5) ephedrine; (6) pseudoephedrine. Experimental conditions as in Section 2.4.

By considering the simultaneous effects of mobile phase composition and column temperature, the optimum chromatographic conditions for the separation of ephedrine alkaloids and synephrine were determined to be 7 mM ammonium acetate in acetonitrile–water (90:10, v/v) at 45 °C. When these chromatographic conditions were used, ephedrine alkaloids and synephrine were well separated within 16 min using the Discovery HS F5 column (Fig. 3). The chromatogram in Fig. 3 also shows that the analytes, although polar and basic in nature, elute with excellent peak shape using the Discovery HS F5 column. The system-suitability report for the analyte separation is shown in Table 2.

### 3.2. Optimization of the extraction conditions

A variety of solvents and extraction methods have been used for the extraction of ephedrine alkaloids from *Ephedra* plant material [13]. In this study, the optimization procedure was performed on *E. vulgaris* aerial parts, because of the higher amount of sample available. Two extraction methods were evaluated and compared: sonication and microwave extractions. Both selected methods were performed under neutral and acidic conditions (by using methanol and a mixture of hydrochloric acid (37%)–methanol (0.8:99.2, v/v)) at different temperatures, in order to exhaustively extract the analytes of interest. Two sequential extraction cycles for each sample under each experimental condition were performed. Other techniques, such as refluxing with a Soxhlet apparatus, were not applied because

Table 2
System-suitability report for the separation of ephedrine alkaloids and synephrine

| Compound | $t_R$ (min) | Theoretical plate number ($n$) | Resolution ($R_s$) | Selectivity ($\alpha$) | Peak symmetry |
|---|---|---|---|---|---|
| Norephedrine | 8.82 | 12673 | – | – | 0.99 |
| Norpseudoephedrine | 9.60 | 13044 | 2.38 | 1.09 | 0.91 |
| Synephrine | 10.51 | 11242 | 2.49 | 1.10 | 0.79 |
| Methylephedrine | 12.65 | 10952 | 4.85 | 1.20 | 0.64 |
| Ephedrine | 13.43 | 10625 | 1.56 | 1.06 | 0.67 |
| Pseudoephedrine | 14.48 | 10451 | 1.92 | 1.08 | 0.75 |

Experimental conditions as in Section 2.4.

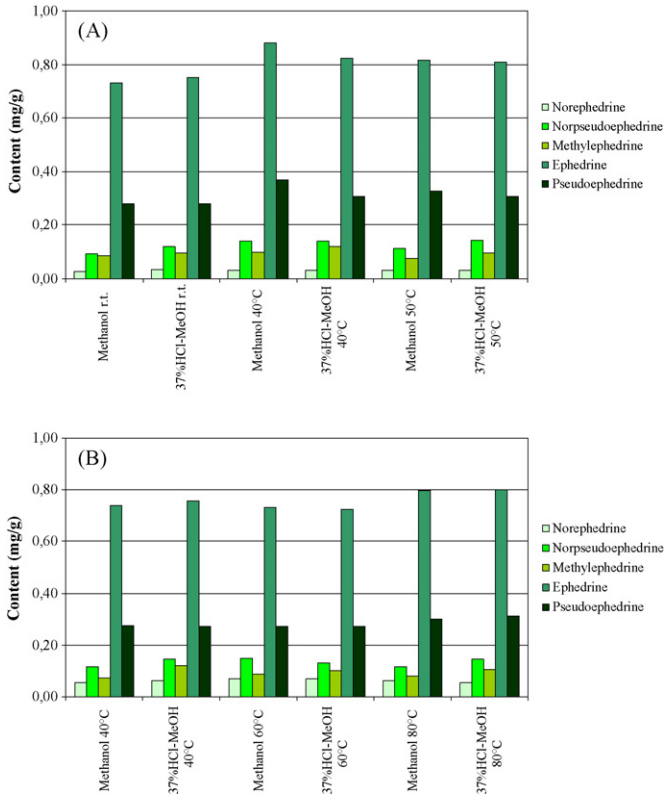


Fig. 4. Comparison between (A) sonication and (B) microwave techniques for the extraction of ephedrine alkaloids from *E. vulgaris*.

they require a long period of time (at least 18 h), as reported by Sander et al. [13]. Furthermore, Soxhlet extraction often causes a thermal degradation of the analytes.

Extraction by sonication is a powerful technique in phytochemical research: the destruction of the cell walls during this procedure can explain the frequently observed increase in the extraction yield. In this work, experiments were carried out at room temperature, 40 and 50 °C for 15 min. As shown in Fig. 4, the optimal extraction conditions for the target analytes were obtained by two successive sonication extractions with methanol at 40 °C for 15 min. Results obtained with methanol or acidic methanol solution at room temperature or at 50 °C were less satisfactory. The effect of time was evaluated by performing one experiment based on two cycles of sonication with methanol at 40 °C for 30 min, but the extraction yield became fairly constant (data not shown); so 15 min was chosen as the optimal extraction time.

Regarding microwave extraction, the main advantages of microwave extraction in comparison with other techniques are both the considerable reduction in time and the smaller solvent consumption, if compared to conventional extractions [23]. In recent years, many reports have been published on the application of microwave to the extraction of secondary metabolites from plants [23]. Nevertheless, to the best of our knowledge, no reports on microwave extraction of ephedrine alkaloids from plant material have been published. In order to investigate the effects of microwave on the compound yields, different temperatures and irradiation times were compared (40 °C for 15 min or 60 °C for 4 min or 80 °C for 1 min). The extractions were carried out in a closed vessel system, under controlled temperature and pressure, to minimize the loss of components due to volatilization. For this purpose, the sample was allowed to cool before the vessels were opened. In this study, the highest yields of ephedrine alkaloids from *E. vulgaris* were obtained with two sequential extractions at 80 °C for 1 min, with both methanol and acidic methanol as the extraction solvents. Degradation products were not observed in the HPLC chromatograms. However, under these conditions, the amount of the major alkaloids (ephedrine and pseudoephedrine) extracted from the plant material was lower compared with the results obtained by sonication with methanol at 40 °C.

In agreement with the above observations, in this study the extraction procedure was performed using sonication with methanol at 40 °C for 15 min. It was found that two successive extraction cycles for each sample were sufficient to obtain a complete extraction of the secondary metabolites from *E. vulgaris*; the high recovery obtained with two sequential cycles did not justify the carrying out of further extraction steps.

A clean-up of the extracts by strong-cation exchange or mixed-mode SPE [12,13] was not performed, since it has been reported that the capacity of the SPE cartridge is easily exceeded by the amount of the analytes present in *Ephedra* extracts [12]; furthermore, unwanted species from the sample matrix are also retained by the SPE cartridge, further reducing its capacity and limiting its usefulness [12].

### 3.3. Evaluation of validation data

For the target compounds, linear regression analyses were performed by the external standard method. The validating parameters of each calibration curve (slope ($a$), intercept ($b$), standard error of slope, standard error of intercept and correlation coefficient ($r^2$)) are shown in Table 1. Excellent linearity was observed for the analytes between peak areas and concentrations over the range tested. Graphical examination of the residuals, performed by evaluation of the normal probability plots of the residuals, demonstrated linearity for all of the compounds in the range tested.

The LOD and LOQ values of the compounds of interest were found to be 0.30 and 1.00 μg/ml (i.e. 0.02 and 0.05 mg/g in the sample), respectively. These results indicate that the proposed HPLC method is sufficiently sensitive for the determination of ephedrine alkaloids and synephrine in botanicals and commercial products.

Regarding accuracy, the percentage recovery values that were obtained by comparing the results from samples and fortified samples of *E. vulgaris* aerial parts are reported in Table 3. The recovery rates obtained were close to 100% in almost all cases. Considering the results of the recovery test, this method is considered accurate. In the case of *E. sinica* aerial parts, the amounts of ephedrine alkaloids determined by the proposed method are in good agreement with the certified values [13]. Table 4 shows the percentage recovery obtained by comparison between the results of the present method and the certified values for ephedrine alkaloids in *E. sinica* aerial parts.

Table 3
Recovery data of alkaloids from *E. vulgaris* aerial parts

| Compound | Spiked amount (mg/g) | Determined amount (mg/g) | Mean recovery (%) ($n = 3$) | R.S.D. (%) |
|---|---|---|---|---|
| Norephedrine | 0.05 | 0.10 | 98.7 | 2.5 |
| Norpseudoephedrine | 0.14 | 0.28 | 99.4 | 1.2 |
| Methylephedrine | 0.10 | 0.20 | 99.0 | 1.5 |
| Ephedrine | 0.87 | 1.75 | 99.2 | 0.3 |
| Pseudoephedrine | 0.37 | 0.73 | 99.1 | 0.5 |

Experimental conditions as in Section 2.4.

Table 4
Comparison of values obtained by the proposed HPLC method to certified values for *E. sinica* aerial parts (SRM 3240)

| Method | Content dry weight (mg/g) | | | | | |
|---|---|---|---|---|---|---|
| | Norephedrine | Norpseudoephedrine | Methylephedrine | Ephedrine | Pseudoephedrine | Total alkaloids |
| HPLC[a] | 0.36[b] | 0.65[b] | 1.28 ± 0.04 | 10.81 ± 0.05 | 3.42 ± 0.08 | 16.51 ± 0.05 |
| Certified values[c] | 0.44 ± 0.09 | 0.65 ± 0.14 | 1.18 ± 0.14 | 11.31 ± 0.76 | 3.53 ± 0.26 | 17.00 ± 1.20 |
| Recovery (%) | 81.8 | 100.0 | 108.5 | 95.6 | 96.9 | 97.1 |

[a] Data are expressed as mean ± S.D. For each sample $n = 6$. Experimental conditions as in Section 2.4.
[b] S.D. < 0.01.
[c] Data are expressed as mean ± S.D. Each certified value is an equally weighed mean of the results from multiple analytical methods carried out at NIST and collaborating laboratories.

Table 5
Intra- and inter-day precision data for retention time ($t_R$) and peak area of ephedrine alkaloids and synephrine

| Compound | Intra-day precision ($n = 10$, mean) | | | | | | Inter-day precision ($n = 30$, mean) | |
|---|---|---|---|---|---|---|---|---|
| | Day 1 | | Day 2 | | Day 3 | | $t_R$ (min) | R.S.D. (%) |
| | $t_R$ (min) | R.S.D. (%) | $t_R$ (min) | R.S.D. (%) | $t_R$ (min) | R.S.D. (%) | | |
| Norephedrine | 8.71 | 0.14 | 8.73 | 0.05 | 8.75 | 0.15 | 8.73 | 0.25 |
| Norpseudoephedrine | 9.50 | 0.14 | 9.51 | 0.06 | 9.54 | 0.15 | 9.52 | 0.23 |
| Synephrine | 10.45 | 0.17 | 10.44 | 0.19 | 10.50 | 0.22 | 10.47 | 0.31 |
| Methylephedrine | 12.60 | 0.14 | 12.62 | 0.05 | 12.66 | 0.15 | 12.63 | 0.23 |
| Ephedrine | 13.42 | 0.15 | 13.42 | 0.14 | 13.47 | 0.18 | 13.44 | 0.25 |
| Pseudoephedrine | 14.44 | 0.15 | 14.43 | 0.17 | 14.49 | 0.18 | 14.46 | 0.25 |

| Compound | Area (mAU s) | R.S.D. (%) | Area (mAU s) | R.S.D. (%) | Area (mAU s) | R.S.D. (%) | Area (mAU s) | R.S.D. (%) |
|---|---|---|---|---|---|---|---|---|
| Norephedrine | 352.64 | 0.50 | 354.90 | 0.67 | 362.31 | 0.54 | 356.87 | 1.32 |
| Norpseudoephedrine | 35.95 | 1.87 | 36.07 | 1.91 | 37.01 | 0.94 | 36.38 | 2.04 |
| Synephrine | 463.35 | 0.55 | 472.06 | 0.66 | 474.24 | 0.42 | 470.08 | 1.14 |
| Methylephedrine | 288.14 | 0.74 | 288.13 | 0.73 | 293.74 | 0.71 | 290.17 | 1.17 |
| Ephedrine | 409.93 | 0.87 | 413.13 | 0.49 | 418.27 | 0.76 | 413.98 | 1.10 |
| Pseudoephedrine | 295.00 | 0.59 | 295.01 | 0.89 | 297.35 | 0.71 | 295.86 | 0.80 |

Experimental conditions as in Section 2.4.

Table 6
Intra- and inter-day precision data for the extraction of alkaloids from *E. vulgaris* aerial parts

| Compound | Intra-day precision ($n = 6$, mean) | | | | | | Inter-day precision ($n = 18$, mean) | |
|---|---|---|---|---|---|---|---|---|
| | Day 1 | | Day 2 | | Day 3 | | Content (mg/g) | R.S.D. (%) |
| | Content (mg/g) | R.S.D. (%) | Content (mg/g) | R.S.D. (%) | Content (mg/g) | R.S.D. (%) | | |
| Norephedrine | <LOQ | – | <LOQ | – | <LOQ | – | <LOQ | – |
| Norpseudoephedrine | 0.15 | 1.02 | 0.14 | 1.48 | 0.14 | 1.00 | 0.14 | 1.56 |
| Methylephedrine | 0.10 | 1.12 | 0.10 | 1.16 | 0.10 | 1.31 | 0.10 | 1.21 |
| Ephedrine | 0.88 | 0.35 | 0.88 | 0.37 | 0.88 | 0.28 | 0.88 | 0.32 |
| Pseudoephedrine | 0.37 | 1.11 | 0.37 | 0.97 | 0.37 | 1.01 | 0.37 | 1.05 |

Experimental conditions as in Section 2.4.

The precision of the chromatographic system is described in Table 5 that shows the intra- and inter-day %R.S.D. values of retention times and peak areas. The low values of intra- and inter-day %R.S.D. values for both retention times and peak areas indicate the high precision of the chromatographic system.

Concerning the precision of the extraction procedure, the intra- and inter-day %R.S.D. data of the repeated analysis are shown in Table 6. The low values of %R.S.D. indicate a high level of precision of the method for the predominant analytes, ephedrine and pseudoephedrine, and also for the minor components, such as norpseudoephedrine and methylephedrine. Norephedrine amount was below the LOQ value in *E. vulgaris* extracts and, therefore, it was not quantified.

Compared with the assay results of *E. sinica* aerial parts, native and commercial extracts, the chromatogram obtained from the oral dosage form indicated that the HPLC method is specific for the analysis of ephedrine alkaloids (Fig. 5): the other compounds present in the formulation were not retained on this stationary phase and were eluted close to the solvent front without interfering with the separation of the analytes of interest. Furthermore, peak purity tests were performed using the photodiode array detector to demonstrate that the analyte chromatographic peak is pure and not attributable to more than one component. There were no indications of impurities or signs of co-elution in the sample chromatograms.

The analytes in solution did not show any appreciable change in chromatographic profile over 72 h. No degradation products were detected.

The validation data highlighted the suitability of the proposed HPLC method for the analysis of ephedrine alkaloids.

### 3.4. Analysis of Ephedra plant material and natural products

The optimized method was applied to the phytochemical analysis of plant material from *Ephedra* species and *Ephedra*-containing natural products. Data are reported in Table 7 and expressed as mg/g of dry weight. As shown in Table 7 and in Fig. 5, the total amount of ephedrine alkaloids in *E. vulgaris* aerial parts was 10-fold lower in comparison with *E. sinica* plant material (1.49 mg/g vs. 16.51 mg/g). The content of the major alkaloids, ephedrine and pseudoephedrine, was 0.88 and 0.37 mg/g, respectively. The amount of the minor alkaloids was 0.14 mg/g for norpseudoephedrine and 0.10 mg/g for methylephedrine. In *E. sinica* aerial parts, the amount of the major alkaloids was 10.81 mg/g for ephedrine and 3.42 mg/g for pseudoephedrine, which is in good agreement with the literatures [13,19,20]. The other alkaloids were present only in small concentrations. In *Ephedra* natural products, ephedrine amount ranged from 0.23 mg/g in the *Ephedra*-containing protein powder to 78.47 mg/g in the commercial extract. The greater content of pseudoephedrine was determined in the native extract (9.69 mg/g) and the lower in the *Ephedra*-containing protein powder (0.03 mg/g). Synephrine was detected only in the *Ephedra*-containing solid oral dosage form (0.57 mg/g).

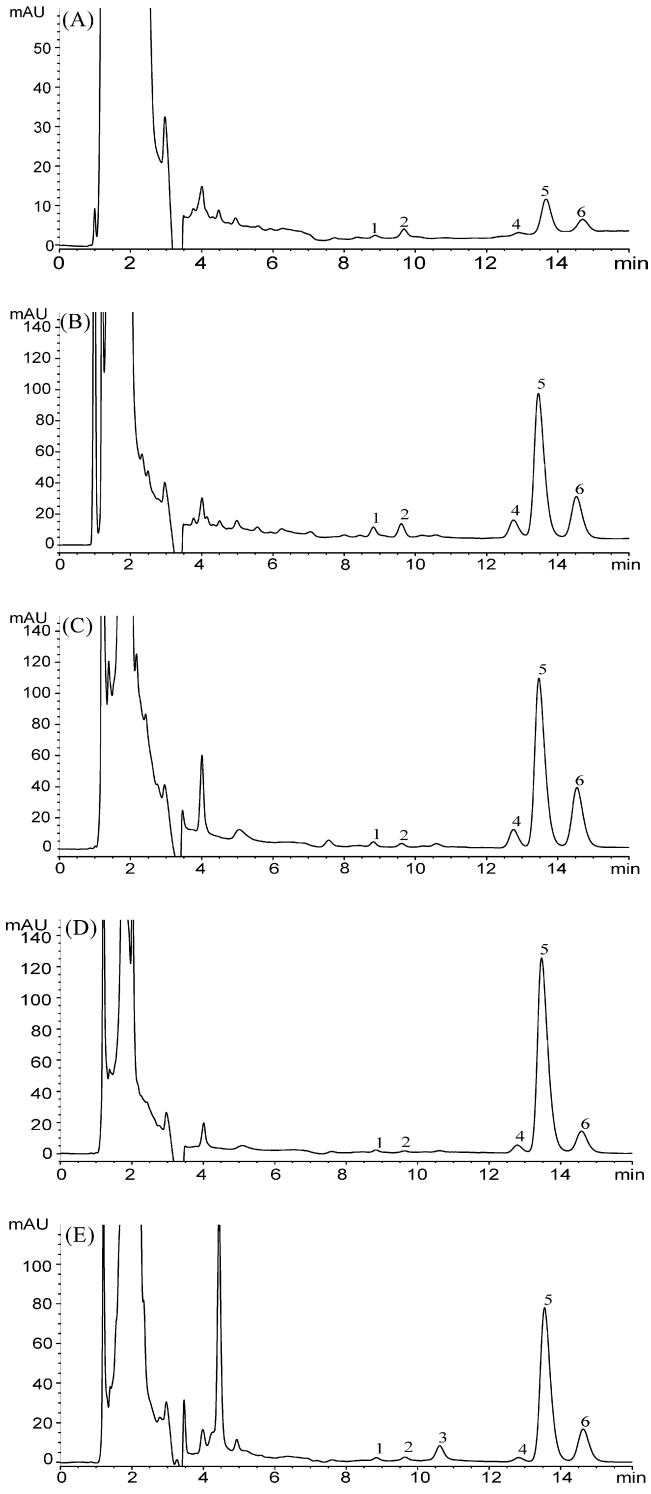


Fig. 5. Chromatograms of methanol extracts of: (A) *E. vulgaris* aerial parts; (B) *E. sinica* aerial parts (SRM 3240); (C) *E. sinica* native extract (SRM 3241); (D) *E. sinica* commercial extract (SRM 3242); (E) *Ephedra*-containing solid oral dosage form (SRM 3243). Peak identification: (1) norephedrine; (2) norpseudoephedrine; (3) synephrine; (4) methylephedrine; (5) ephedrine; (6) pseudoephedrine. Experimental conditions as in Section 2.4.

Table 7
Results obtained by HPLC analysis of ephedrine alkaloids and synephrine in *Ephedra* plant material and derivatives

| Sample | Content dry weight (mg/g)[a] | | | | | | |
|---|---|---|---|---|---|---|---|
| | Norephedrine | Norpseudoephedrine | Synephrine | Methylephedrine | Ephedrine | Pseudoephedrine | Total alkaloids |
| *E. vulgaris* aerial parts | <LOQ | 0.14[b] | <LOD | 0.10[b] | 0.88[b] | 0.37[b] | 1.49 ± 0.01 |
| *E. sinica* aerial parts (SRM 3240) | 0.36[b] | 0.65[b] | <LOD | 1.28 ± 0.04 | 10.81 ± 0.05 | 3.42 ± 0.08 | 16.51 ± 0.05 |
| *E. sinica* native extract (SRM 3241) | 0.32 ± 0.01 | 0.28 ± 0.02 | <LOD | 2.40 ± 0.05 | 27.98 ± 0.05 | 9.69 ± 0.05 | 40.68 ± 0.13 |
| *E. sinica* commercial extract (SRM 3242) | 0.49 ± 0.01 | 0.30 ± 0.01 | <LOD | 2.45 ± 0.03 | 78.47 ± 0.46 | 9.41 ± 0.14 | 91.12 ± 0.35 |
| *Ephedra*-containing solid oral dosage form (SRM 3243) | 0.15[b] | 0.19[b] | 0.57 ± 0.03 | 0.34 ± 0.01 | 10.25 ± 0.10 | 2.68 ± 0.02 | 14.19 ± 0.14 |
| *Ephedra*-containing protein powder (SRM 3244) | <LOD | <LOD | <LOD | 0.01[b] | 0.23 ± 0.02 | 0.03[b] | 0.27 ± 0.02 |

Experimental conditions as in Section 2.4.
[a] Data are expressed as mean ± S.D. For each sample $n = 6$. In the case of *E. vulgaris*, $n = 18$.
[b] S.D. <0.01.

The broad range of ephedrine and pseudoephedrine concentrations that could be determined with this method is particularly noteworthy. Method precision was also highly satisfactory, even at the lower levels of the target analytes, as indicated by the S.D. values. The comparison of the contents obtained in the present study with the certified values [13,20] indicated that the proposed method provides reliable results in the analysis of *Ephedra* alkaloids in complex matrices.

The developed HPLC technique for the analysis of *Ephedra* alkaloids has several advantages over existing methods [2,3,7–15], such as requiring no complex and time-consuming sample preparation regardless of the analyzed sample (plant material or derivatives). Furthermore, the optimized chromatographic conditions allow the simultaneous analysis of ephedrine alkaloids and synephrine in a very short analysis time, without the use of ion-pair reagents and gradient elution. The validation parameters, such as linearity, sensitivity, accuracy, precision and specificity, were found to be highly satisfactory. The applicability of the method was tested by the analysis of *Ephedra* plant material and derivatives. The results indicated that the proposed HPLC method can be successfully applied to monitor the quality of *Ephedra* plant material and extracts, and to determine the composition of commercial products and verify their label claims, including the absence of ephedrine alkaloids in *Ephedra*-free products.

## 4. Conclusion

The stationary phase of the Discovery HS F5 column allowed an excellent separation of ephedrine alkaloids and synephrine. Both mobile phase counter ion concentration and column temperature were optimized for the best analyte separation in a short time. The optimized method was fully validated in accordance with ICH guidelines. Regarding sample preparation, the comparison between sonication and microwave extractions indicated that sonication was the most efficient procedure, allowing the highest yield of all considered analytes in a short time. Under the optimized extraction and chromatographic conditions, a fast and reliable qualitative and quantitative analysis of *Ephedra* plant material and natural products was carried out. The results of the analysis of *Ephedra* SRMs indicated a good agreement with the certified contents. The proposed method can be considered suitable for the phytochemical analysis of a variety of *Ephedra*-containing samples, including plant material and derivatives. Furthermore, the developed technique can be employed to demonstrate the label claims for product content, including the absence of ephedrine alkaloids in *Ephedra*-free products.

## References

[1] Y.M. Liu, S.J. Sheu, S.H. Chiou, H.C. Chang, Y.P. Chen, Planta Med. 59 (1993) 376–378.
[2] B.T. Schaneberg, S. Crockett, E. Badir, I.A. Khan, Phytochemistry 62 (2003) 911–918.
[3] M.C. Roman, J. AOAC Int. 87 (2004) 1–14.
[4] F. Pellati, S. Benvenuti, J. Chromatogr. A 1161 (2007) 71–88.

[5] S.M. Wang, R.J. Lewis, D. Canfield, T.L. Li, C.Y. Chen, R.H. Liu, J. Chromatogr. B 825 (2005) 88–95.
[6] M. Wang, P.J. Marriott, W.H. Chan, A.W. Lee, C.W. Huie, J. Chromatogr. A 1112 (2006) 361–368.
[7] J.A. Hurlbut, J.R. Carr, E.R. Singleton, K.C. Faul, M.R. Madson, J.M. Storey, T.L. Thomas, J. AOAC Int. 81 (1998) 1121–1127.
[8] N. Okamura, H. Miki, T. Harada, S. Yamashita, Y. Masaoka, Y. Nakamoto, M. Tsuguma, H. Yoshitomi, A. Yagi, J. Pharm. Biomed. Anal. 20 (1999) 363–372.
[9] R.A. Niemann, M.L. Gay, J. Agric. Food Chem. 51 (2003) 5630–5638.
[10] B.T. Schaneberg, I.A. Khan, Pharmazie 59 (2004) 819–823.
[11] M. Ganzera, C. Lanser, H. Stuppner, Talanta 66 (2005) 889–894.
[12] J.W.H. Lam, G.J. Gardner, M. McCooeye, C.A. Fraser, R.E. Sturgeon, Anal. Bioanal. Chem. 383 (2005) 268–281.
[13] L.C. Sander, K.E. Sharpless, M.B. Satterfield, T. Ihara, K.W. Phinney, J.H. Yen, S.A. Wise, M.L. Gay, J.W. Lam, M. McCooeye, G. Gardner, C. Fraser, R. Sturgeon, M. Roman, Anal. Chem. 77 (2005) 3101–3112.
[14] M.L. Gay, K.D. White, W.R. Obermeyer, J.M. Betz, S.M. Musser, J. AOAC Int. 84 (2001) 761–769.
[15] M.L. Gay, R.A. Niemann, S.M. Musser, J. Agric. Food Chem. 54 (2006) 285–291.
[16] J. Beyer, F.T. Peters, T. Kraemer, H.H. Maurer, J. Mass Spectrom. 42 (2007) 150–160.
[17] J. Zhang, J. Xie, X. Chen, Z. Hua, Analyst 128 (2003) 369–372.
[18] H.Y. Chiang, S.J. Sheu, Electrophoresis 25 (2004) 670–676.
[19] K.W. Phinney, T. Ihara, L.C. Sander, J. Chromatogr. A 1077 (2005) 90–97.
[20] K.E. Sharpless, D.L. Anderson, J.M. Betz, T.A. Butler, S.G. Capar, J. Chen, C.A. Fraser, G. Gardner, M.L. Gay, D.W. Howell, T. Ihara, M.A. Khan, J.W. Lam, S.E. Long, M. McCooeye, E.A. Mackey, W.R. Mindak, S. Mitvalsky, K.E. Murphy, A. NguyenPho, K.W. Phinney, B.J. Porter, L.C. Sander, M.B. Satterfield, C. Scriver, R. Sturgeon, J. Brown Thomas, R.D. Vocke, S.A. Wise, L.J. Wood, L. Yang, J.H. Yen, G.C. Ziobro, J. AOAC Int. 89 (2006) 1483–1495.
[21] 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, pp. 1–13.
[22] D.S. Bell, H.M. Cramer, A.D. Jones, J. Chromatogr. A 1095 (2005) 113–118.
[23] E. Martino, I. Ramaiola, M. Urbano, F. Bracco, S. Collina, J. Chromatogr. A 1125 (2006) 147–151.