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Determination of ephedrine alkaloid stereoisomers in dietary supplements by capillary electrophoresis☆

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Abstract

Three complementary capillary electrophoresis (CE) methods were developed for the separation and quantification of ephedrine and pseudoephedrine stereoisomers. Either single or dual cyclodextrin-based chiral selector systems provided enantioselective separation of the compounds of interest. The three methods were applied to the analysis of a suite of five standard reference materials (SRMs) containing ephedra. Use of a high-sensitivity UV detection cell enhanced quantification of the analytes of interest over the wide range of concentrations encountered in the SRMs. Results for (−)-ephedrine ranged from 0.31 to 76.43 mg/g, and for (+)-pseudoephedrine ranged from 0.049 to 9.23 mg/g in the materials studied. Results from the three methods agreed well with each other and with the results from other methods of analysis. The addition of known amounts of specific enantiomers was used to confirm the enantiomeric identity of the analytes. The results obtained by the three CE methods were utilized for value assignment of the ephedrine alkaloid content of these five SRMs.
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Keywords: Ephedrine; Stereoisomers; Capillary electrophoresis

1. Introduction

Ma huang is a traditional Chinese medicine derived from Ephedrae herba that is used as a stimulant, diaphoretic, and anti-asthmatic [1]. The stimulant effects of ma huang, also known as ephedra, are linked to the presence of six alkaloids: (−)-ephedrine, (+)-pseudoephedrine, (−)-*N*-methylephedrine, (+)-*N*-methylpseudoephedrine, (−)-norephedrine, and (+)-norpseudoephedrine [2,3]. Structures of these compounds are shown in Fig. 1. Each of these six compounds also has an enantiomer that does not occur naturally in the plant. Ephedrine and pseudoephedrine are generally the most abundant alkaloids found in *Ephedra sinica*, and they typically constitute more than 80% of the alkaloid content of the dried plant material [4,5].

The medicinal properties of these alkaloids have been recognized for many years, and synthetic forms of several of these compounds have been incorporated into a variety of medications. Ephedrine is a bronchodilator used to treat the symptoms of colds and asthma [6]. Pseudoephedrine is a milder stimulant than ephedrine and is widely used as a nasal decongestant [7]. Phenylpropanolamine (also known as (±)-norephedrine) was previously found in over-the-counter decongestants and appetite suppressants, but it has been removed from these products in the United States because of its potential link to hemorrhagic strokes [8].

Dietary supplements containing ephedra, either alone or in combination with other ingredients, have been marketed

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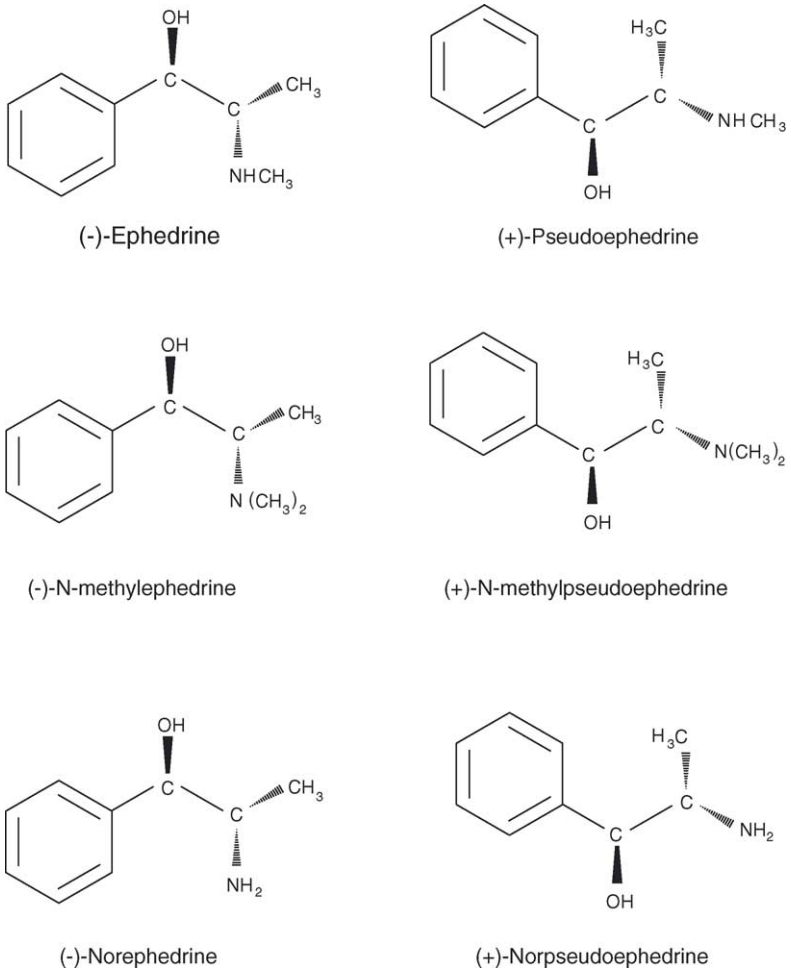

Fig. 1. Structures of ephedrine alkaloids.

extensively in USA in recent years for weight reduction, increasing energy, and enhancing athletic performance. A 6-month study of an herbal ephedra/caffeine combination for weight loss indicated that this combination could be beneficial for overweight individuals who are otherwise healthy [9]. However, the use of products containing ephedra has also been associated with adverse health effects in certain individuals. The adverse events range from mild hypertension and palpitations to stroke and even death [10,11]. The number and severity of adverse effects prompted the U.S. Food and Drug Administration (FDA) to warn consumers to stop using dietary supplements that contain ephedra, and a ban on sales of these supplements in the USA was recently implemented [12].

Concerns about the safety of ephedra-containing products have led to the development of a variety of analytical methods to assess the alkaloid content. Methods to quantify these alkaloids are important for verifying label claims for product content, including claims for “ephedra-free” products, and for quality control purposes. Significant variations in the alkaloid content of ephedra-containing products have been previously reported, in part because of differences in product formulation [13,14]. Techniques that have been utilized to measure ephedra alkaloid content in dried plant, plant extracts, and dietary supplements include gas chromatography (GC) [15,16], liquid chromatography (LC) [17,18], and capillary electrophoresis (CE) [19,20]. Some of these methods have specifically focused on the identification of the ephedrine stereoisomers [2,21]. In this work, we developed three complementary CE methods for the determination of ephedrine and pseudoephedrine enantiomers in dietary supplements and related materials. We believe this is the first work to utilize CE in conjunction with a high-sensitivity UV absorbance detection cell to quantify these alkaloids in a wide range of ephedra-containing materials. Results were found to be comparable to data from other measurement techniques, and only minimal sample preparation was required for CE analysis. The CE methods were applied successfully to the value assignment of the ephedrine alkaloid content of five standard reference materials (SRMs) containing ephedra.

2. Experimental[1]

2.1. Reagents

Sodium phosphate monobasic, β-phenylethylamine hydrochloride (internal standard), and (±)-ephedrine hydrochloride were obtained from Sigma (St. Louis, MO, USA). Heptakis(2,6-di-*O*-methyl)-β-cyclodextrin (DM-β-CD), hydroxypropyl-β-cyclodextrin (HP-β-CD, molar substitution = 0.8), sulfated β-cyclodextrin (S-β-CD), and (1*R*,2*R*)-(−)-pseudoephedrine were obtained from Aldrich (Milwaukee, WI, USA). According to the vendor information, the sulfated cyclodextrin has a typical substitution of 7–11 mol/mol β-CD. (1*R*,2*S*)-(−)-Ephedrine hydrochloride (EP) and (1*S*,2*S*)-(+)-pseudoephedrine hydrochloride (PE) were obtained from ChromaDex (Santa Ana, CA, USA). (+)-Ephedrine hydrochloride was from Fluka (Buchs, Switzerland).

2.2. Preparation of run buffers

Run buffers were prepared by dissolving 75 mg NaH_2PO_4 and 1000 mg of the appropriate chiral selector in 19 mL water and adjusting the pH to 2.5 with 0.1 mol/L HCl. The mixture was transferred to a 25 mL volumetric flask and diluted to volume with water. Run buffers were filtered through 0.2 μm nylon filters prior to use.

2.3. Calibration solutions

Four calibration solutions were independently prepared by weighing approximately 8 mg of (−)-EP and 2 mg of (+)-PE into a vial and adding 2 mL of internal standard solution (2 mg/mL). An aliquot (100 μL) of this solution was transferred to a 10 mL volumetric flask. Methanol (900 μL) was added to the flask, and the solution was diluted to volume with water. A single internal standard solution was used for the preparation of all calibration solutions and samples. The calibrant solutions were prepared at concentrations that approximated the levels of the analytes in the samples as closely as possible. By preparing calibration solutions that closely bracket the analyte concentrations, the effect of possible nonlinear detector response can be minimized.

2.4. Instrumentation

Electrophoretic experiments were performed on an HP^{3D} capillary electrophoresis system (Hewlett Packard, Wilmington, DE, USA) with a photodiode array detector and a high-sensitivity UV detection cell (Agilent Technologies, Wilmington, DE, USA). Separations were performed in unmodified fused silica capillaries (80.5 cm × 75 μm internal diameter, effective length 72.0 cm) from Polymicro Technologies (Phoenix, AZ, USA). The cartridge temperature was maintained at 25 °C, and injections were performed by pressure (2.5 kPa, 5 s). Applied voltages were in the range of 15–30 kV. Detection was performed at 210 nm. At the beginning of each day, the capillary was conditioned with 0.03 mol/L H_3PO_4 (10 min), water (2 min), and run buffer (15 min). Between injections, the capillary was purged with 0.03 mol/L H_3PO_4 (1 min), water (1 min), and run buffer (2 min). The applied pressure for rinsing the capillary was 90 kPa.

Table 1
Listing of ephedra-containing materials studied

Identifier	Description of material
SRM 3240	*Ephedra sinica* Stapf aerial parts
SRM 3241	*Ephedra sinica* Stapf native extract
SRM 3242	*Ephedra sinica* Stapf commercial extract
SRM 3243	Ephedra-containing solid oral dosage form
SRM 3244	Ephedra-containing protein powder

2.5. Samples

Samples used in this work were SRMs in development at NIST. The five materials are listed in Table 1. SRM 3240 consists of dried plant material. SRM 3241 and SRM 3242 are extracts from the same plant material as used for SRM 3240. SRM 3243 is an ephedra-containing oral dosage form. SRM 3244 is an ephedra-containing protein drink mix powder. These materials were all solids and had previously been homogenized. The five materials differed in the levels of the ephedrine alkaloids as well as in their overall chemical composition.

2.6. Sample preparation

Samples were extracted with methanol by sonication extraction. Sample sizes were 0.1 g for SRM 3242, 0.25 g for SRM 3241, 0.5 g for SRM 3240 and SRM 3243, and 2.5 g for SRM 3244. All samples except for the protein powder were extracted by weighing an appropriate amount of material into a vial, adding 2 mL of internal standard solution (2 mg/mL) and 18 mL methanol, and sonicating the mixture for 30 min. The extracts were filtered through 0.2 μm nylon filters, and 1 mL of the extract was transferred to a 10 mL volumetric flask. The solution was diluted to volume with water. A slightly different procedure was used for the protein powder samples because the level of ephedra alkaloids was significantly lower in these samples than in the other materials. Protein powder samples were weighed into vials, and 2 mL of internal standard solution (0.2 mg/mL) was added. Methanol (18 mL) was added to the vial, and the mixture was sonicated for 30 min. The extract was filtered, and 1 mL of

[1] Certain commercial equipment, instruments, or materials are identified in this article to specify adequately the experimental procedure. Such identification does not imply recommendation or endorsement by NIST, nor does it necessarily imply that the materials or equipment identified are necessarily the best available for the purpose.

the filtered extract was transferred to a 2 mL glass vial. The volume was reduced to 100 μL under a stream of nitrogen. A 1 mL portion of a 10% volume fraction of methanol in water was added to the extract. All extracts were filtered through 0.2 μm nylon filters prior to CE analysis.

3. Results and discussion

Ephedra sinica contains only (−)-ephedrine and (+)-pseudoephedrine, and these are the isomers that would be expected in dietary supplements that contain plant extracts. Detection of (+)-ephedrine, (±)-ephedrine, or (−)-pseudoephedrine in a product containing ephedra would suggest that the content had been altered through the addition of synthetic alkaloids. Hence, the enantiomeric composition of these compounds is important in verifying the natural (plant) origin of the alkaloids.

A limited number of studies have sought to separate and quantify the ephedrine and pseudoephedrine enantiomers in ephedra-containing materials. A GC method utilizing a cyclodextrin-based chiral stationary phase has been reported, but this approach required extensive sample preparation and the analysis time was nearly 60 min [2]. Flurer et al. described a CE method utilizing a derivatized cyclodextrin as the chiral additive that was used for the separation, identification, and quantification of the stereoisomers of ephedrine and related compounds in nutritional supplements containing ephedra [21]. However, they reported that the use of a single CE method was not sufficient for conclusive verification of the enantiomeric identify of the analytes. They noted that the combination of two methods, each using a different chiral selector, provided more conclusive evidence. Because each chiral additive or combination of additives provides different selectivity, the likelihood of undetected peak overlap is reduced and additional confidence in the enantiomeric identity of the analytes is gained. The ease of changing chiral additives in CE makes such an approach simple to perform. We sought to expand upon this previous work by examining additional chiral selector systems and applying the resulting methodology to rigorous determinations of the alkaloid content of a variety of ephedra-containing materials. These measurements were used as part of the value assignment process for the ephedrine alkaloids in the five ephedra-related SRMs.

3.1. Chiral selectors

Initial work focused on identifying electrophoretic parameters for the separation of the four stereoisomers of ephedrine and pseudoephedrine because these two compounds are the major alkaloids found in ephedra. The ephedrine alkaloids are basic compounds (pK_a of ephedrine is 9.6) in aqueous solution. Low pH conditions were selected for analysis of the ephedra-containing materials to ensure full protonation of the analytes and to minimize the contribution of the electroosmotic flow to the observed resolution [22]. A number of neutral and ionic derivatives of β-cyclodextrin were evaluated as potential chiral selectors. Selection of the cyclodextrins used in this work was based upon previous experience with various functionalized cyclodextrins [23] as well as literature reports describing the enantioselective separation of ephedrine and related compounds [21,24–26]. Separations were optimized by altering the chiral selector concentration, the concentration of the background electrolyte, and the applied voltage. The resulting three sets of optimum electrophoretic parameters are shown in Table 2. Separation of the four stereoisomers plus the internal standard, β-phenylethylamine, using each of the three additive systems is shown in Fig. 2. β-Phenylethylamine was selected as the internal standard because it is structurally similar to the analytes, has a comparable pK_a value, and did not interfere with any of the components found in the samples.

Each of the three methods offered slightly different enantioselectivity, as can be seen in Fig. 2. DM-β-CD yielded better enantioresolution of ephedrine than HP-β-CD, but (−)-PE was not resolved from (−)-EP. However, it is unlikely that (−)-PE would be found in samples because (−)-PE is not found in ephedra plants or in any common pharmaceuticals. Both DM-β-CD and HP-β-CD provided good separations of pseudoephedrine enantiomers.

The combination of the negatively charged S-β-CD and the neutral DM-β-CD offered the best separation of the four stereoisomers, and the analytes were well resolved from the internal standard. The reversed polarity mode, with detection at the anode, was necessary when this selector combination was used. The ratio of the two chiral selectors was optimized through a series of electrophoretic experiments varying the concentrations of the two selectors. Separation of the four stereoisomers could also be achieved with just the S-β-CD, but resolution between the internal standard and (−)-PE de-

Table 2
Summary of electrophoretic parameters used

	Method A	Method B	Method C
Chiral selector	2.8% Sulfated β-cyclodextrin + 1.2% heptakis(2,6-di-*O*-methyl)-β-cyclodextrin	4% Heptakis(2,6-di-*O*-methyl)-β-cyclodextrin	4% Hydroxypropyl-β-cyclodextrin
Background electrolyte	25 mmol/L NaH_2PO_4	25 mmol/L NaH_2PO_4	25 mmol/L NaH_2PO_4
pH	2.5	2.5	2.5
Voltage (kV)	−15	+30	+25
Temperature (°C)	25	25	25

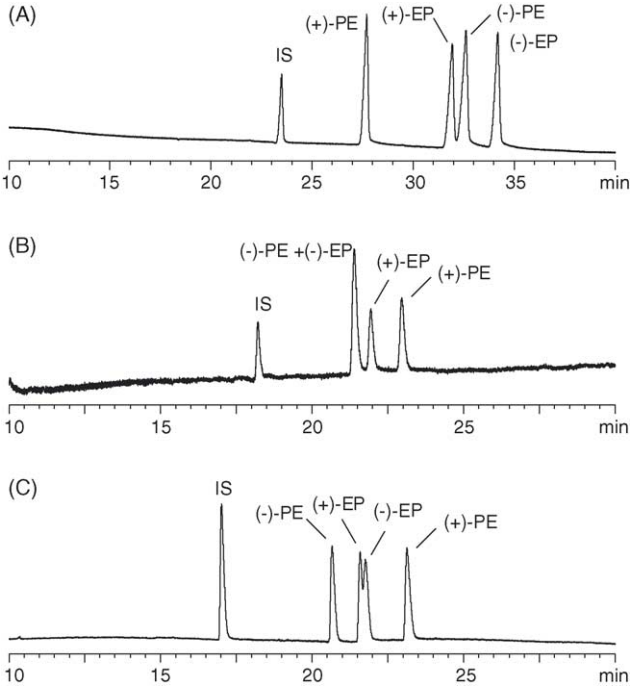

Fig. 2. Separation of racemic mixtures of ephedrine and pseudoephedrine with each of the three chiral selector systems. Details of methods (A), (B), and (C) are provided in Table 1 and in Section 2.

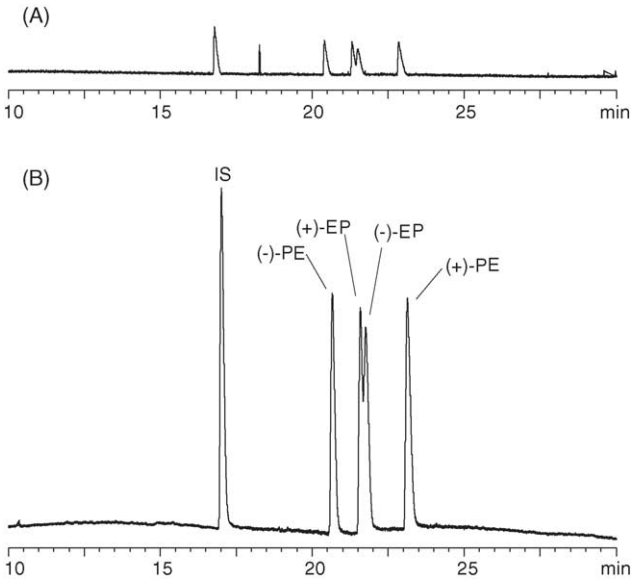

Fig. 3. Separation of ephedrine and pseudoephedrine enantiomers with a conventional UV detection cell (A) and with the high-sensitivity cell (B). The concentration of each enantiomer was 12.5 μg/mL. The run buffer was 4% hydroxypropyl-β-cyclodextrin in 25 mmol/L NaH_2PO_4, adjusted to pH 2.5.

teriorated to unacceptable levels. The use of 4% S-β-CD cyclodextrin did prove valuable for confirming the enantiomeric identity of the analytes, and this aspect will be described in detail later.

Replicate injections of calibration solutions were performed to gauge the variability in migration times for the three methods. No noticeable advantage in performance was observed among the three methods, and migration time precision was typically in the range of 1–3% (RSD) for all three methods. Higher current (~85 μA) was observed for the method utilizing the mixture of S-β-CD and DM-β-CD when compared to the neutral derivatives alone (~45–55 μA). This was not surprising given the charged nature of the S-β-CD chiral selector.

3.2. Application to ephedra-containing dietary supplements

CE is an attractive approach for the analysis of dietary supplements containing ephedra because simple sample preparation methods can be used and its high efficiency makes it suitable for complex samples. Methanol sonication had previously been evaluated in our laboratory for the extraction of ephedrine alkaloids from dietary supplements [27], and this approach was selected for preparation of samples for CE analysis. Initial attempts to quantify the alkaloid stereoisomers revealed a lack of sensitivity that was particularly problematic for materials such as the protein powder that had lower levels of the alkaloids. A high-sensitivity detection cell dramatically improved our ability to quantify components of the supplements. The path length of the high-sensitivity cell is 1.2 mm, and the internal volume is 12 nL. This cell fits into the standard capillary cartridge for the CE instrument. A comparison of standard solutions with a normal detection cell and with the high-sensitivity cell is shown in Fig. 3. Use of the cell improved signal-to-noise ratios by a factor of five, and peak areas increased more than tenfold. Therefore this cell was incorporated into measurements of the supplements.

Six samples of each of the five ephedra-containing materials in Table 1 were analyzed with each of the three methods, and the results are shown in Table 3. Duplicate injections of each calibrant and single injections of each sample extract were performed. Figs. 4 and 5 illustrate the results obtained for the analysis of SRM 3243 and SRM 3244, respectively, with each of the three methods. As shown in the figures, the analytes of interest were well resolved from other sample constituents. The broad range of ephedrine and pseudoephedrine concentrations that could be determined with these methods is particularly noteworthy. Ephedrine concentrations ranged from a high of 76.43 mg/g (based on an average of the three methods) for SRM 3242, to a low of 0.31 mg/g in SRM 3244. Pseudoephedrine concentrations ranged from 9.23 mg/g in SRM 3242 to 0.049 mg/g in SRM 3244. Method precision was also quite good, even at lower levels of the alkaloids. Table 3 also illustrates the level of agreement observed among the three methods. Hence, although a single CE method could be used to quantify the analytes, additional confidence in the results can be gained through the combination of methods used in this work. The process

Table 3
Summary of results (mg/g) for the analysis of ephedra-containing SRMs by each of the three CE methods[a]

Sample	Method A	Method B	Method C
SRM 3240			
(−)-EP	11.63 (7.7)	12.36 (7.2)	11.76 (7.2)
(+)-PE	3.71 (10.8)	3.65 (9.1)	3.69 (8.8)
SRM 3241			
(−)-EP	28.32 (5.2)	28.15 (7.2)	28.65 (3.9)
(+)-PE	11.21 (2.4)	10.80 (6.7)	11.28 (2.9)
SRM 3242			
(−)-EP	78.31 (1.8)	75.84 (0.8)	75.13 (1.0)
(+)-PE	9.63 (5.0)	9.04 (1.0)	9.03 (1.0)
SRM 3243			
(−)-EP	10.68 (2.3)	10.91 (1.4)	10.90 (0.8)
(+)-PE	2.73 (4.8)	2.67 (3.8)	2.72 (3.4)
SRM 3244			
(−)-EP	0.30 (5.1)	0.31 (3.2)	0.31 (4.3)
(+)-PE	0.048 (3.5)	0.049 (1.7)[b]	0.048 (5.0)

[a] Results shown represent an average of six measurements unless otherwise indicated. Coefficients of variation (CV) are indicated in parentheses after each value. The values shown are not reported on a dry-mass basis. The percentage moisture was 4.52% for SRM 3240, 4.3% for SRM 3241, 4.255% for SRM 3242, 4.63% for SRM 3243, and 3.57% for SRM 3244.
[b] Value is an average of four determinations.

of changing run buffers is readily automated with current CE instrumentation.

Table 4 shows a comparison between the CE results obtained in this work and the certified values for ephedrine and pseudoephedrine in the five SRMs. The certified values were based upon a combination of different analytical

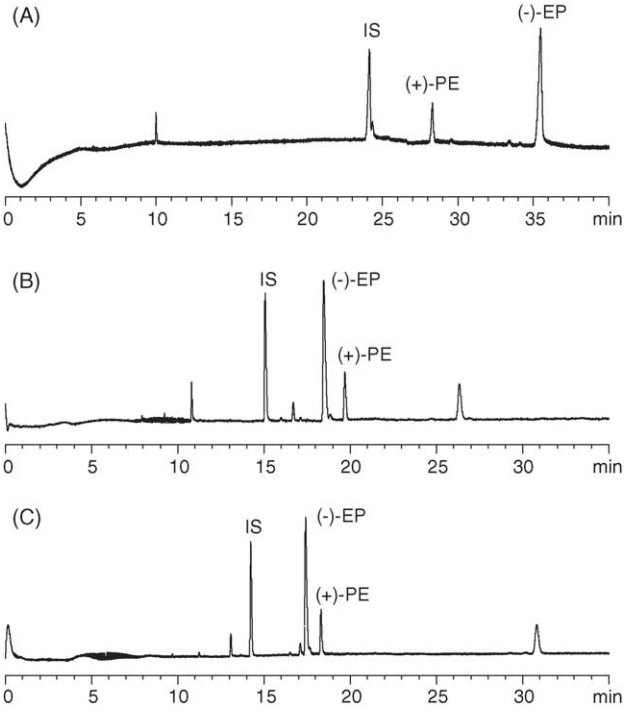

Fig. 4. Analysis of SRM 3243 by each of the three CE methods.

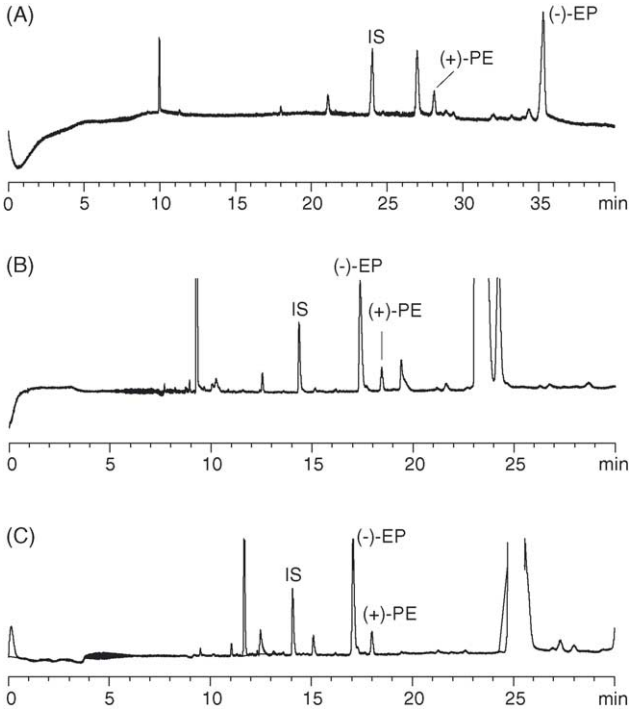

Fig. 5. Analysis of SRM 3244 by each of the three CE methods.

Table 4
Comparison of values obtained by CE to certified values for ephedra-containing materials (mg/g)

Sample	CE[a]	Certified values[b]
SRM 3240		
EP	12.39 (0.93)	11.31 ± 0.76
PE	3.83 (0.35)	3.53 ± 0.26
SRM 3241		
EP	29.43 (1.56)	28.86 ± 1.17
PE	11.51 (0.52)	10.74 ± 1.11
SRM 3242		
EP	79.24 (1.74)	78.1 ± 2.3
PE	9.57 (0.41)	9.27 ± 0.94
SRM 3243		
EP	11.27 (0.21)	11.21 ± 0.42
PE	2.82 (0.11)	2.81 ± 0.11
SRM 3244		
EP	0.32 (0.01)	0.243 ± 0.038
PE	0.0499 (0.0019)[c]	0.036 ± 0.009

[a] Results shown represent an average of the data from all three CE methods. Concentrations are expressed on a dry-mass basis and have been corrected for the purity of the calibrants. Standard deviations ($N = 18$) are shown in parentheses.
[b] Each certified concentration value, expressed as a mass fraction on a dry-mass basis, is an equally weighted mean of the results from six to nine analytical methods carried out at NIST and at collaborating laboratories. The uncertainty in the certified value is expressed as an expanded uncertainty (U) about the mean ($\bar{x}$) following the ISO Guide to the Expression of Uncertainty in Measurement [28].
[c] $N = 16$.

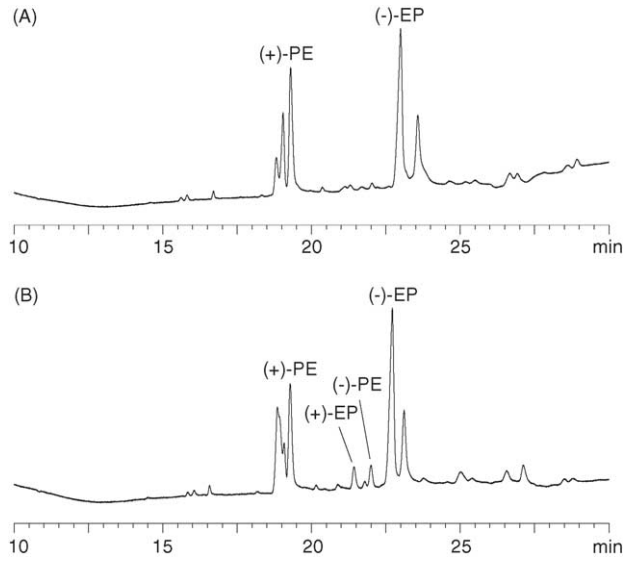

Fig. 6. Analysis of SRM 3244 before (A) and after (B) spiking with 20 μg each of (+)-EP and (−)-PE per gram of sample. The chiral selector was 4% S-β-CD in 25 mmol/L phosphate buffer, pH 2.5.

methods, including liquid chromatography with ultraviolet absorbance detection (LC/UV), liquid chromatography with mass spectrometric detection (LC/MS), liquid chromatography/tandem mass spectrometry (LC/MS/MS), high-field asymmetric waveform ion mobility spectrometry (FAIMS), and the CE results reported here, with measurements performed by NIST and by three collaborating laboratories [27]. It should be noted that the other methods were not enantioselective and therefore did not identify which enantiomers of the alkaloids were present.

3.3. Confirmation of enantiomer identity

As shown in Figs. 4 and 5, only the naturally occurring enantiomers of ephedrine ((−)-EP) and pseudoephedrine ((+)-PE) were detected in SRMs 3243 and 3244. Similar observations were made for the other three materials analyzed. Because slight shifts in migration time could result in potential misidentification of the enantiomeric identity of the analytes, we elected to confirm peak identities through the addition of specific enantiomers to the sample extracts. For this approach, a run buffer containing 4% S-β-CD in 25 mmol/L NaH_2PO_4 was prepared. Sample extracts were spiked with known amounts of (−)-PE and (+)-EP, the enantiomers that do not occur naturally in ephedra. Fig. 6 illustrates the analysis of SRM 3244 before and after the addition of the standards. As noted earlier, the use of this run buffer resulted in reduced resolution between the internal standard and (+)-PE. However, this did not prevent identification of the peaks of interest. The added components are readily distinguishable from the initial sample components in Fig. 6, and the enantiomeric identity of the alkaloid stereoisomers found in the samples was easily verified.

4. Conclusions

The detection of ephedrine alkaloids in dietary supplements is necessary to verify product label claims, including claims for “ephedra-free” products. Identification of the specific stereoisomers present in the product can also prove valuable for detecting product adulteration. We have demonstrated that CE is a viable approach to the determination of the predominant ephedrine alkaloid stereoisomers in a variety of ephedra-containing samples. A high-sensitivity UV detection cell dramatically improved sensitivity for these analytes. The use of complementary CE methods provides additional confidence in peak identity, and we anticipate that this approach can be applied to other analytical challenges.

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