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Determination of Ephedrine Alkaloids in Botanicals and Dietary Supplements by HPLC-UV:

Collaborative Study

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Abstract

An international collaborative study was conducted of a high-performance liquid chromatography (HPLC)-UV method for the determination of the major (ephedrine [EP] and pseudoephedrine [PS]) and minor (norephedrine [NE], norpseudoephedrine [NP], methylephedrine [ME], and methylpseudoephedrine [MP]) alkaloids in selected dietary supplements representative of the commercially available products. Ten collaborating laboratories determined the ephedrine-type alkaloid content in 8 blind replicate samples. Five products contained ephedra ground herb or ephedra extract. These 5 products included ground botanical raw material of *Ephedra sinica*, a common powdered extract of *Ephedra sinica*, a finished product containing only *Ephedra sinica* ground botanical raw material, a complex multicomponent dietary supplement containing Ma Huang, and a high-protein chocolate flavored drink mix containing Ma Huang extract. In addition, collaborating laboratories received a negative control and negative control spiked with ephedrine alkaloids at high and low levels for recovery studies. Test extracts were treated to solid-phase extraction using a strong-cation exchange column to help remove interferences. The HPLC analyses were performed on a polar-embedded phenyl column using UV detection at 210 nm. Repeatability relative standard deviations (RSD_r) ranged from 0.64–3.0% for EP and 2.0–6.6% for PS, excluding the high protein drink mix. Reproducibility relative standard deviations (RSD_R) ranged from 2.1–6.6% for EP and 9.0–11.4% for PS, excluding the high protein drink mix. Recoveries ranged from 84.7–87.2% for EP and 84.6–98.2% for PS. The data developed for the minor alkaloids are more variable with generally unsatisfactory HORRATS(i.e., >2). However, since these alkaloids generally add little to the total alkaloid content of the products, the method gives satisfactory results in measuring total alkaloid content (RSD_r 0.85–3.13%; RSD_R 2.03–10.97%, HORRAT 0.69–3.23, exclusive of the results from the high protein drink). On the basis of these results, the method is recommended for Official First Action for determination of EP and PS in dietary supplements exclusive of the high protein drinks.

Ephedra is a shrub-like evergreen plant that is found in arid regions of Europe, central Asia, and other parts of the world. Major species of ephedra include *Ephedra sinica* Stapf., *E. equisetina* Bunge, *E. intermedia*, and *E. distachya*. The traditional Chinese medicine Ma Huang is derived from aerial parts of ephedra (1–3), and has been used for the treatment of asthma, bronchial spasms, and as a stimulant and diaphoretic (4). Ephedra is known to contain up to 6 bioactive alkaloids: (-)-ephedrine (EP), (+)-pseudoephedrine (PS), (-)-methylephedrine (ME), (+)-methylpseudoephedrine (MP), (-)-norephedrine (NE), and (+)-norpseudoephedrine (NP). These alkaloids constitute about 1 to 2.5 wt % of the plant on a dry weight basis, with (-)-

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ephedrine accounting for between 30 to 90% of this Total (5). Typically EP and PS combined account for >90% of the total alkaloids in ephedra. The species *E. nevadensis, E. antisyphilitica,* and *E. trifurca,* all found in North America, have thus far been found to be free of these alkaloids (6).

EP and PS are available as single-entity or combination prescription and over-the-counter drugs in the United States. NP is a Schedule IV controlled substance requiring a Drug Enforcement Agency (DEA) permit in the United States. In recent years, the number of dietary supplements containing ephedra, either as powdered botanical, or more commonly, as a standardized extract, has increased dramatically. Most of these products are sold as dietaids or energy boosters due to their "thermogenic" effect—the ability to raise the rate at which calories are burned. Often these dietary supplements will also contain caffeine, either synthetic or from botanical extracts, in addition to other ingredients.

There have been a number of severe adverse events associated with the use of ephedra products reported in recent years (7,8). Often these ad verse events can be attributed to over doses of ephedrine alkaloids, and recently there have been several well-publicized deaths of professional athletes in which ephedra products have been implicated. products containing ephedra are currently banned in the National Football League (NFL).

Because of the health and legal implications associated with the use of products containing ephedra, it is desirable to have a chromatographic method that can accurately and reproducibly quantify EP and PS in botanical products and dietary supplements. Ephedrine alkaloids present a number of challenges in their analysis. All 6 ephedrine alkaloids are structurally very similar. They are very hydrophilic amine compounds that have poor retention on traditional reversed-phase high-performance liquid chromatography (HPLC) systems. Their basic nature often leads to excessively broad peaks and peak tailing on chromatographic systems. Lastly, they have poor UV absorption above about 210 nm. Hurlbut and Carr developed an HPLC procedure utilizing solid-phase extraction (SPE) to quantify ephedrine alkaloids in dietary supplements (9). This method, however, utilizes a detection wavelength of 255 nm, necessitated by the presence of acetate in the mobile phase, and therefore the method is unsuitable for determining levels of individual ephedrine alkaloids below ~3000 ppm. Gurley et al. (10) presented anion-pairing HPLC method for the determination of ephedrine alkaloids in dietary supplements, but the method used D-amphetamine (a DEA Schedule II controlled substance) as an internal standard, and the closeness in retention times of the ephedrine alkaloids could potentially result in misidentification of peaks. Betz et al. (11) developed a chiral gas chromatography (GC) method, and Liu and Sheu (12) and Flurer etal.(13) developed capillary electrophoresis methods for the determination of ephedrine alkaloids. None of these methods, however, is easily transferable to most analytical laboratories. The U.S. Pharmacopeia (USP) and current AOAC INTERNATIONAL methods for determining (-) ephedrine are nonchromatographic methods and therefore are unsuitable for determining the levels of both EP and PS in the presence of other ephedrine alkaloids and other endogenous plant components.

Because of the need for an accurate and reproducible method for the determination of EP and PS in botanical and dietary supplement samples, an HPLC-UV method was developed utilizing strong-cation exchange (SCX) SPE as a cleanup step and short-wavelength UV detection. The method is able to separate all 6 ephedrine alkaloid diastereomers, and quantify EP and PS, but can not separate the individual enantiomers. This method was submitted for a collaborative study, and the results of this collaborative study are expected to support the validation of this method.

Collaborative Study

Study Design

An HPLC-UV method, validated in-house by ChromaDex Research & Development Laboratory, was submitted to 10 laboratories participating in the collaborative study. Each Laboratory was sent 8 materials as blind duplicates, for a Total of 16 samples. One blind duplicate was a negative control containing no ephedrine alkaloids. One blind duplicate was negative control spiked with ephedrine alkaloids at a low level. One blind duplicate was negative control spiked with ephedrine alkaloids at a high level. The remaining test samples consisted of blind duplicates of a botanical raw material (powdered herb), a common extract, a finished product containing only ephedra, a complex mixture of multiple dietary supplements containing ephedra, and a high protein chocolate flavored drink mix containing ephedra. Random identification numbers were assigned to each sample. Each test sample was blinded in terms of composition and Concentration of ephedrine alkaloids; however, the test samples were identified as botanical, extract, finished product, or high protein drink mix, as the nature of the product is important to the analyte isolation procedure.

Collaborators

Eleven laboratories originally agreed to participate in the collaborative study and received materials to conduct the study. Ten Laboratories completed the study in the allotted time. One Laboratory that was participating in the study was not able to complete the work due to other obligations. Of the 10 Laboratories that completed the study, 5 were from the United States, 2 were from Canada, 2 were from Asia, and 1 was from Europe.

Test Sample Preparation

a. *Botanical raw material*.—Authenticated *Ephedra sinica* was provided by Botanical Liaisons (Boulder, CO; www.botanicallaisons.com). This material was provided as a dry powder.

b. *Common extract*.—Ephedra powdered extract was supplied by Triarco (Wayne, NJ; www.triarco.com). The extract was prepared from *Ephedra sinica* stems, and the supplier test description indicated 8% total ephedrine alkaloids.

c. *Finished product containing only ephedra*.—Hard-shell gelatin capsules containing 375 mg powdered above-ground parts of *E. sinica* were supplied by American Herbal Products Association (AHPA; Silver Spring, MD; www.ahpa.org).

d. *Complex mixture of multiple dietary supplements including ephedra*.—Multicomponent capsules containing 334 mg Ma Huang (shrub stems, standardized to 20 mg ephedrine alkaloids/capsule), 100 mg guarana seed (standardized to 22 mg caffeine/capsule), 100 mg caffeine, 75 mg bitter orange extract (fruit, standardized to 4.5 mg synephrine/capsule), 50 mg kola nut extract (standardized to 10 mg caffeine/capsule), 25 mg white willow bark extract (standardized to 3.75 mg salicin/capsule), 5 mg ginger powder, and 5 mg passion flower extract were manufactured by Labrada Nutrition (Houston, TX; www.labrada.com) and supplied by The Vitamin Shoppe (North Bergen, NJ).

e. *High protein chocolate flavored drink*.—A “thermogenic” high protein powdered drink mix (Ripped Fuel) was supplied by Twin Laboratories Inc. (Hauppauge, NY). Each serving contained 334 mg Ma Huang extract (standardized for 20 mg ephedrine alkaloids), and 909 mg guarana extract (standardized for 22% caffeine). The bulk of the material was a low fat source of high quality milk and egg proteins. Each serving contained 40 g pure protein, vitamins, minerals, and other ingredients.

f. *Negative control*.—Authenticated *E. nevadensis*, which is generally agreed to contain no ephedrine alkaloids, was supplied by Botanical Liaisons. The material was milled to a powder by Covance Laboratories (Madison, WI; www.covance.com).*Low spike*.—A portion of the powdered negative control was spiked by Covance Laboratories with each of the 6 ephedrine alkaloids at a target Concentration of approximately 10mg/g total alkaloids (w/w).The concentration levels of each of the individual alkaloids were varied to approximate what might be encountered in a typical product. The spiked material was then mixed to obtain a uniform mixture. *High spike*.—A portion of the powdered negative control was spiked by Covance Laboratories with each of the 6 ephedrine alkaloids at a target concentration of approximately 100 mg/g Total alkaloids (w/w). The Concentration levels of each of the individual alkaloids were varied to approximate what might be encountered in a typical product.

Standards

Reference standards for (-)-ephedrine HCl, (+)-pseudoephedrine HCl, (-)-norephedrine, (-)-methylephedrine, and (+)-N-methylpseudoephedrine were obtained from commercial sources as indicated in the *Method* section. (+)-Norpseudoephedrine, a DEA Schedule IV restricted compound, was supplied to the U.S. collaborating Laboratories by Covance Laboratories. Because of DEA regulations, powdered NP could not be shipped out side of the United States to the international collaborating laboratories. These laboratories were supplied with 2 ampoules of 1 mL each containing 1.00 mg/mL NP in methanol. These ampoules were prepared and the NP content verified (1.00 ± 0.01 mg/mL) by Cerriliant (Austin, TX).

Preparation and Shipment

The individually prepared test samples, standards, chromatographic column, and SPE cartridges were provided to each collaborative laboratory. The standards and samples were shipped at ambient temperature to each Laboratory with a Return Receipt document. Collaborators were instructed to store the standards and samples at room temperature until use. Each collaborative laboratory prepared their own calibration and sample solutions according to the study protocol provided.

Single-Laboratory Validation Data(14)

a. *Concentration range*.—The calibration curves had a range of about 20 to 200 μg/mL for EP, 2.4 to 24 μg/mL for PS, and 0.5 to 50 μg/mL for the other ephedrine alkaloids. These values correspond to analyte Concentration ranges presented in Table 1.

b. *Validation data*.—The linearity of the proposed method was demonstrated from about 3 to 200 μg/mL for each ephedrine alkaloid. Recoveries at each linearity point were between 98.0 to 101.3% for all ephedrine alkaloids. Determination coefficients were equal to or greater than 0.9979 for all components (Table 2).

Red Rose black tea was spiked with the ephedrine alkaloids at 3 different concentration levels intriplicate. Average recoveries ranged from 78.4% for ME to 87.0% for NE (Table 3). Five different matrixes were tested for ephedrine alkaloids: botanical raw material, extract, capsules containing only botanical raw material, a granulated multicomponent ingredient containing ephedra, 2 multicomponent capsule finished products, and a high protein drink mix containing ephedra. Excluding the high protein drink mix, individual RSD_r ranged from 10.2% for ME in a multicomponent finished product (at 400 μg/g) to 0.30% for EP in ephedra extract (66 200 μg/g) as presented in Table 4. RSD_R for Total ephedrine alkaloids ranged from 0.35 to 5.4% including the protein drink mix.

AOAC Official Method 2003.13 Ephedrine Alkaloids in Botanicals and Dietary Supplements

High-Performance Liquid Chromatography-UV First Action 2003

This method is applicable for the determination of ephedrine (EP) and pseudoephedrine (PS) in *Ephedra sinica* raw herb and extracts, and finished products containing ephedra raw herb or extracts, in the presence of norephedrine (NE), norpseudoephedrine (NP), methylephedrine (ME), and methylpseudoephedrine (MP). *See* Table **2003.13A** for the results of the interlaboratory study supporting acceptance of the method.

A. Principle—The ephedrine alkaloids are extracted from the matrix with methanol—50mM potassium phosphate monobasic in water (3 + 97). The extract is treated by solid-phase extraction (SPE) on a strong-cation exchange (SCX) column, and the ephedrine alkaloids are eluted from the SPE column with methanol—ammonium hydroxide (95 + 5). After diluting the eluate with phosphoric acid solution to neutralize the ammonium hydroxide, the solutions are subjected to isocratic high-performance liquid chromatography (HPLC) on an ether-linked phenyl column with UV detection at 210 nm.

B. Apparatus—

- **a.** *HPLC system*.—Equipped with UV-Vis de tec tor.
- **b.** *Column*.—Phenomenex Synergi Polar RP HPLC column, 4.6 × 150 mm, 4 μm particle size (Phenomenex, Torrance, CA; www.phenomenex.com).
- **c.** *Analytical balance*.—Readability 0.01mg.
- **d.** *Filtration apparatus*.—0.45 μm nylon filter.
- **e.** *Ultrasonic bath*.
- **f.** *Syringe filter*.—0.45 μm PTFE.
- **g.** *SCX SPE cartridge*.—containing 500 mg resin; Phenomenex Strata SCX.
- **h.** *Pipettor*.—Dispensing 200–1000 μL.
- **i.** *Benchtop centrifuge*.

C. Reagents—

- **a.** *Deionized water*.
- **b.** *Methanol*.—HPLC grade.
- **c.** *Potassium phosphate, monobasic*.—KH_2PO_4; ACS reagent grade.
- **d.** *Ammonium hydroxide*.—ACS reagent grade.
- **e.** *Phosphoric acid*.—H_3PO_4; ACS reagent grade 85%.

D. Reference Standards—*Caution:* The alkaloid standards and test samples may be harmful by inhalation or skin adsorption, irritating to mucous membranes and upper respiratory tract. The target organs for acute toxicological effects are the central nervous system and the heart. For handling, avoid contact with eyes, skin, and clothing. For storage, keep tightly closed; keep away from heat and open flame. Store in a cool dry place. Refer to MSDS sheets for specific alkaloid standard.

- **a.** *Ephedrine·HCl*.—99%, ChromaDex (Santa Ana, CA; www.chromadex.com).

b. *Pseudoephedrine·HCl*.—99% (ChromaDex).

c. *Norephedrine*.—99% (ChromaDex).

d. *Methylephedrine*.—98% (ChromaDex).

e. *Methylpseudoephedrine*.—99% (ChromaDex).

f. *Norpseudoephedrine*.—Cathine·HCl, 98% (C222; RBI, a subsidiary of Sigma-Aldrich Chemical Co., Milwaukee, WI; www.sigmaldrich.com). *Note*: NP is a DEA Schedule IV Restricted Compound.

g. *Norpseudoephedrine*.—1.00 mg/mL (ChromaDex).

E. Preparation of Reagents—

a. *Mobile phase buffer solution*.—50mM. Dissolve 13.6 g KH_2PO_4 in 1000 mL water. Prepare fresh weekly.

b. *Mobile phase*.—Mix 30 mL methanol with 970 mL mobile phase buffer solution. Filter through a 0.45 μm nylon filter and degas under vacuum. Prepare fresh weekly.

c. *Diluent*.—Mix 30 mL methanol with 970 mL water. Add 1.3 g KH_2PO_4 and stir until all salt is dissolved; then degas. Prepare fresh weekly.

d. *Phosphoric acid*.—50mM. Add about 345 μL 85% H_3PO_4 to 100 mL water and mix well. Prepare fresh every 2 weeks.

e. *Phosphoric acid*.—500mM. Add about 3.5 mL 85% H_3PO_4 to 100 mL water and mix well. Prepare fresh every 2 weeks.

f. *SPE elution solvent*.—Mix 5 mL NH_4OH with 95 mL methanol. Prepare fresh every 2 weeks.

F. Preparation of Standard: U.S. Laboratories—

a. *Stock related alkaloid solution*.—Accurately weigh 12.5 ± 0.2 mg each NE, ME, and MP into separate 50 mL volumetric flasks. Record exact weights. Add 20 mL methanol and 20 mL diluent, and sonicate until all standards are dissolved (about 5–10 min). Dilute to volume with diluent and mix well. Store solutions in a refrigerator at 2°–8°C and protect from light. Prepare fresh at least monthly.

b. *Stock standard solution*.—Accurately weigh 62.5 ± 2 mg EP·HCl reference standard (corresponding to about 51 mg EP free base; *see J. Calculations* section); 7.5 ± 0.2 mg PS·HCl reference standard (corresponding to about 6.1 mg PS free base; *see J. Calculations* section); and 2.5 ± (0.1 mg) NP·HCl reference standard (corresponding to about 2.0 mg NP free base) and transfer all into a 25 mL volumetric flask. Record exact weights. Pipet 5 mL each of the stock related alkaloid solutions, **F**(**a**), into the same flask, add about 5 mL diluent, and sonicate until all reference standards are dissolved (about 5 min). Dilute to volume (25 mL) with diluent and mix well. This is the stock standard solution, containing about 2.1 mg/mL EP free base; 0.25 mg/mL PS free base; 0.08 mg/mL NP free base; and 0.05 mg/mL each NE, ME, and MP.

c. *Linearity standard solutions*.—Pipet the indicated volume of stock standard solution in Table **2003.13B** into separate 50 mL volumetric flasks and dilute to volume with diluent. The linearity standards are stable for at least 2 weeks when stored at ambient laboratory conditions.

G. Preparation of Standards: International Laboratories—

a. *Stock related alkaloid solutions*.—Accurately weigh 12.5 ± 0.2 mg each NE, ME, and MP into separate 50 mL volumetric flasks. Record exact weights. Add 20 mL methanol and 20 mL diluent, **E**(**c**), and sonicate until all standards are dissolved (about 5–10 min). Dilute to volume with diluent and mix well. Label and date each stock (0.25 mg/mL target alkaloid). Store solutions in a refrigerator set to maintain at 2°–8°C and protect from light. Prepare fresh at least monthly.

b. *Working stock solution*.—Accurately weigh 62.5 ± 2 mg EP·HCl reference standard (corresponding to about 51 mg EP free base) and 7.5 ± 0.2 mg PS·HCl reference standard (corresponding to about 6.1 mg PS free base) and transfer all into a 25 mL volumetric flask. Record exact weights. Quantitatively transfer the contents of 1 ampoule NP reference standard solution (1.00 mg/mL) into the same 25 mL volumetric flask. Rinse the empty ampoule twice with diluent, and transfer the rinsings to the 25 mL volumetric flask. Pipet 5 mL each of the stock related alkaloid solutions, **F**(**a**), into the same flask, add about 5 mL diluent, and sonicate until all reference standards are dissolved (about 5 min). Dilute to volume (25 mL) with diluent and mix well. This is the stock standard solution, containing about 2.1 mg/mL EP free base; 0.25 mg/mL PS free base; 0.08 mg/mL NP free base; and 0.05 mg/mL each NE, ME, and MP.

c. *Preparation of standard curve*.—Pipet the indicated volume of stock standard solution in Table **2003.13C** into separate 50 mL volumetric flasks and dilute to volume with diluent.

Note: The linearity standards are stable for at least 2 weeks when stored at ambient laboratory conditions.

H. Preparation of Test Samples—

a. *Raw herb*.—Accurately weigh 2.0 ± 0.2 g ground ephedra herb and transfer into a 100 mL volumetric flask. Add about 50 mL diluent and shake on a mechanical shaker for about 15 min. Sonicate flasks for an additional 45 min (no temperature control is used). Allow solution to equilibrate to ambient temperature, dilute to volume with diluent, and mix well.

b. *Standardized (common) extract*.—Accurately weigh 280 mg (±30 mg) ephedra-standardized extract and transfer into a 100 mL volumetric flask. Add about 50 mL diluent and shake on a mechanical shaker for about 15 min. Sonicate flasks for an additional 10 min at ambient temperature. Dilute to volume with diluent and mix well.

c. *Capsules/tablets containing raw herb*.—Weigh 20 capsules or tablets. For hardshell capsules, empty contents of all 20 capsules into a container and reweigh empty capsule shells. Grind tablets to a fine powder in a coffee grinder or other mill.

Weigh powdered capsule or tablet material equivalent to 2.0 g (± 0.2 g) raw ephedra herb, and transfer into a 100 mL volumetric flask. Add about 50 mL diluent and shake on a mechanical shaker for about 15 min. Sonicate flasks for an additional 45 min (no temperature control is used). Allow the solution to equilibrate to ambient temperature, dilute to volume with diluent, and mix well.

d. *Capsules/tablets containing standardized (common) extract*.—Weigh 20 whole capsules or tablets. For hardshell capsules, empty contents of all 20 capsules into a container and reweigh empty capsule shells. Grind tablets to a fine powder in a coffee grinder or other mill.

Weigh powdered capsule or tablet material equivalent to 285 mg (±30 mg) ephedra-standardized extract, and transfer into a 100 mL volumetric flask. Add about 50 mL

diluent, and shake on a mechanical shaker for about 15 min. Sonicate flasks for an additional 15 min at ambient temperature. Dilute to volume with diluent and mix well.

e. *protein drink mixes containing ephedra*.—Accurately weigh 10 g (± 0.5 g) powdered protein drink mix, and transfer into a 100 mL volumetric flask. Add about 40 mL methanol and 1 mL 500mM H_3PO_4 and shake on a mechanical shaker for 15 min. Sonicate for an additional 45 min (no temperature control is used). Allow solution to equilibrate to ambient temperature. Dilute to volume with methanol and mix well. Allow solution to settle for about 1 h.

f. *SPE*.—Subject linearity standard solutions, test solutions, and blanks (s) to the following SPE procedure utilizing an SPE column containing 500 mg SCX resin. Use a vacuum manifold designed for SPE columns. The typical vacuum required to pull the solutions at a reasonable flow rate through the columns is approximately 5 in. Hg, although this value will depend on the particular columns used, and the amount of particulates in the solution.

g. *SPE conditioning*.—Condition an SPE cartridge by first passing 2 mL methanol through cartridge, followed by 1 mL 50mM H_3PO_4. Do not allow to dry.

h. *Linearity standard solutions*.—Pipet 10 mL each linearity standard solution and blank onto separate conditioned SPE columns. Pull solutions through columns at a rate not exceeding 2 mL per min. Allow columns to be pulled to dryness.

i. *Test solutions*.—Centrifuge approximately 10mL test solution at approximately 2000 rpm for 10 min. Alternately, allow solutions to settle for at least 1 h. Use 100% MeOH as the blank.

Pipet 5 mL test solution onto SPE column. Pull solution through column at a rate not exceeding 2 mL per min. Let column be pulled to dryness. Discard eluate. Run a 5.0 mL blank with each set of assays.

j. *Column wash*.—(*1*) Wash SPE column by first passing 1 mL 50mM H_3PO_4 solution through column. Let column be pulled to dryness. Discard eluate. (*2*) Next, pass 2 mL methanol through column. Let the column be pulled to dryness. Discard eluate.

k. *Ephedrine alkaloid elution*.—Place a 10 mL volumetric flask below the SPE cartridge. Pipet 1 mL SPE elution solvent onto cartridge and pull solvent into flask. Add another 1 mL SPE elution solvent onto cartridge and pull solvent into the flask. Add a third 1 mL aliquot SPE elution solvent onto cartridge and pull solvent into flask. Add about 5 mL 500mM H_3PO_4 to volumetric flask and allow solution to equilibrate to ambient temperature. Fill flask to volume with 500mM H_3PO_4 and mix well. transfer a portion to an HPLC autosampler vial. This is the working test solution.

Note: The standard and test solutions that have been treated by the SPE procedure are stable for at least 1 month when stored at ambient room temperature.

I. Determination—

a. The standard and test solutions are analyzed using the chromatographic conditions shown in Table **2003.13D**.

b. *System suitability*.—*Repeatability*.—The relative standard deviation (RSD) of the EP peak area for at least 5 consecutive injections of the level 4 linearity standard solution must not be more than 2.0%. The RSD of each of the related compound peak areas for at least 5 consecutive injections of the level 4 linearity standard solution must not be more than 3.0%. *Resolution*.—The resolution (R) be tween PS and EP in the level 4 linearity standard solution chromatograms should not be less than 2.0.

The resolution between MP and ME in the level 4 linearity standard solution chromatograms should not be less than 1.3. There solution is calculated as follows:

$$R=2\times\frac{T_2-T_1}{W_1+W_2}$$

where T_2 = retention time of 2nd peak in the chromatogram; T_1 = retention time of 1st peak in the chromatogram; W1 = peak width 1st peak in the chromatogram; W2 = peak width of 2nd peak in the chromatogram.

Tailing.—The tailing factor (TF) must be no more than 1.5 for all the alkaloids in the linearity standard solution chromatograms. The tailing factor is calculated as:

$$TF=\frac{L+R}{2L}$$

where L = width from start of the peak to the peak apex at 5% of peak height; R = width from peak apex to peak end at 5% of peak height.

Linearity.—The R^2 for the regression line (peak area vs Concentration) for each ephedrine alkaloids must not be less than 0.995. The recovery at each linearity point for each ephedrine alkaloid must not be less than 95%. Adjust each standard concentration for its reported purity.

J. Calculations—

a. Concentration of EP and PS free bases in the standard preparation is calculated as follows:

$$C\times 0.8192$$

where C = concentration of EP·HCl or PS·HCl in standard preparation;0.8192 = molecular weight conversion between HCl salt and free base.

b. Amount of EP in the product, in micrograms/gram (μg/g), is calculated as follows:

$$\frac{A_E-b_E}{m_E}\times\frac{D}{W}\times 1000$$

where A_E = peak area of EP in the test chromatogram; m_E = slope of regression line for ephedrine; b_E = *y*-intercept of regression line for ephedrine; D = dilution factor, in mL = 180 for high protein drink mixes = 200 for all other products; W = weight of test portion, in mg; 1000 = conversion from mg to μg.

c. Amount of PS in the product, in micrograms/gram (μg/g), is calculated as follows:

$$\frac{A_P-b_P}{m_P}\times\frac{D}{W}\times 1000$$

where A_P = peak area of PS in the test chromatogram; m_P = slope of regression line for PS; b_P = *y*-intercept of regression line for PS; D = dilution factor = 180 for high protein drink mixes = 200 for all other products; W = weight of test portion, in mg; 1000 = conversion from mg to μg.

d. Total alkaloids in the product, in micrograms/gram (μg/g), is calculated as follows:

$$Total=EP+PS$$

where EP = EP calculated in the product; PS = PS calculated in the product.

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Results and Discussion

Eleven laboratories agreed to participate in the collaborative study. Ten Laboratories were able to submit data before the submission deadline. The remaining laboratory was not able to finish the study because of lack of time.

Collaborative Study Results

Results, in micrograms EP and PS per gram of product, for each of the 8 blind replicates are presented in Tables 5 and 6. Test samples were given random codes prior to shipment to the collaborators, and then decoded when the results were returned. Table **2003.13A** presents statistical summaries of these results. Statistical analysis to determine repeatability and reproducibility was performed using the AOAC Interlaboratory Statistical Program 2001 for Blind Replicates (15). Repeatability standard deviations (S_r), reproducibility standard deviations(S_R), repeatability relative standard deviations (RSD_r), reproducibility relative standard deviations (RSD_R), and number of statistical outliers are presented. HORRAT values are also presented in Table **2003.13A**, and are calculated as RSD_R (observed)/RSD_R (pre dicted), where the RSD_R (predicted) is calculated using the equation:

$$RSD_R = 2C^{-0.1505}$$

where C is the measured analyte Concentration in decimal mass units (16). Cochran's, Grubbs', and double Grubbs' tests were used to remove statistical outliers where appropriate. No more than 2 outlier results were removed from any data set in order to maintain statistical data from at least 8 collaborating Laboratories. Results for the low and high spike negative control samples as well as the high protein drink mix from collaborative Laboratory D were reported as a "greater than" concentration, as the results fell above the standard Concentration range; these results are not used in the statistical analysis. Collaborative Laboratory F found significant levels (>1700μg/g) of ephedrine alkaloids in the negative control blank, and did not detect any ephedrine alkaloids in the low spike negative control.

Total ephedrine alkaloid results (EP + PS) for each sample are also presented in Table **2003.13A**. Data from laboratories reporting values for individual alkaloids as a greater than or less than value are not included in Table **2003.13A**.

Recovery data for EP and PS in the low spike negative control and the high spike negative control are presented in Table 7.

Because 2 Laboratories (A and C) performed the work on multiple days, multiple calibration curves were generated. Determination coefficients (R_2) for all calibration curves are greater than 0.999.

Collaborators' Comments

Laboratory J noted that it was difficult to maintain a column temperature of 25°C with their column heater. In order to maintain constant temperature and ensure reproducible retention times, they increased the column temperature to 30°C. Resolution and selectivity of the method were not affected. The same Laboratory noted that the tailing factor they observed for EP was 1.54, and the system suitability requirement for tailing factor of EP was no more than 1.5. Laboratory I also noted that the tailing factor for EP was at the upper limit. The Study Director believes that the tailing factor of no more than 1.5 may be too stringent.

Laboratory C noted that the high buffer Concentration in the mobile phase (100mM) damaged their UV flow cell and pump pistons after 1 month of continuous use. No other laboratories noted any adverse effects from the high buffer concentration.

Laboratory H experienced a carry-over peak in the high protein chocolate drink mix samples and needed to extend the run time to eliminate the carry-over peak.

Performance Characteristics of the Study

Repeatability (RSD_r) and reproducibility (RSD_R) was generally good for EP and PS in all matrixes except the high protein drink mix and high spiked negative control samples. The results for the high protein drink mix will be discussed separately. The high spiked negative control had an RSD_R of >6% for EP and >10% for PS, which is not consistent with results for the other sample matrixes. HORRAT values for these samples were all under 2 except for PS (2.2) in the finished products and extract. For calculation of Total ephedrine alkaloids (EP + PS), RSD_R ranged from 0.85% for the common extract to 2.4% for the multicomponent capsules. RSD_R ranged from 2.0% for the common extract to 7.5% for the capsules containing only ephedra raw material, with HORRAT values for total ephedrine alkaloids ranging from 0.69 for the common extract to 1.8 for the capsules containing only ephedra (excluding the high spiked negative control and high protein drink mix). These results indicate very good reproducibility of the method for the analysis of botanical raw material, capsules containing only botanical raw material, common extract, and multicomponent capsules.

For the high protein chocolate flavored drink mix, high RSD_r and RSD_R were observed for both individual alkaloids. It is believed that these high RSDs are caused by a combination of low alkaloid concentration and matrix interferences. While this method appears capable of identifying these components in high protein drink mix, it does not appear reliable at providing reproducible results.

Because of poor recoveries of NE, NP, ME, and MP from the spiked negative controls, these components were not included in the total ephedrine alkaloid calculations. Repeatability and reproducibility results were obtained, however, and are presented in Table 8 for comparison.

Recoveries of EP were 84.7 and 87.2% for the low and high spike negative controls, respectively. Recoveries of PS were 84.6 and 98.2% for the low and high spike samples, respectively. Recoveries of the minor alkaloids are presented in Table 9. Causes of the poor recoveries of the minor alkaloids from the *E. nevadensis* negative control have not yet been determined. Previous single-laboratory validation work (14) showed recoveries >78% for all ephedrine alkaloids at all 3 spike levels when black tea was used as the matrix blank, and >85% for most of the ephedrine alkaloids at all 3 spike levels. The very high RSD_r and RSD_R for these samples also indicate an effect that is not observed in the actual botanical raw material, capsules containing only ephedra, common extract, or multicomponent capsules containing ephedra.

Eighty-one of the 83 R^2 values reported for the calibration curves exceeded 0.997. The two R^2 values that did not exceed this value were 0.99611 for NP from Laboratory A, and 0.99582 for ME from Laboratory C. Both these Laboratories generated 3 calibration curves resulting from analyses performed on 3 separate days. Both Laboratories reported R^2 values >0.997 for remaining 2 calibration curves.

Recommendations

On the basis of the results of this study, it is recommended that this method be adopted for Official First Action for the determination of EP and PS in botanical raw materials, extracts, finished products containing only ephedra, and multicomponent finished products (tablets/capsules) containing ephedra. It is not recommended for testing high protein drink mixes containing ephedra.

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Table 1

Concentration ranges for ephedrine alkaloids in calibration range[a]

	µg/g			
	Botanical raw material	**Extract**	**Finished dosage form**	**High protein drink mix**
NE	50–500	350–3500	67–670	10–100
NP	80–800	570–5700	100–1000	16–160
EP	2000–20000	15000–150000	2700–27000	400–4000
PS	250–2500	1800–18000	330–3300	50–500
ME	50–500	350–3500	67–670	10–100
MP	50–500	350–3500	67–670	10–100

[a]NE = Norephedrine; NP = norpseudoephedrine; EP = ephedrine; PS = pseudoephedrine; ME = methylephedrine;MP = methylpseudoephedrine.

J AOAC Int. Author manuscript; available in PMC 2008 November 18.

Table 2

Single-laboratory validation data: Linearity[a]

Alkaloid	Slope	Intercept	R^2	Avg. recovery, %
NE	0.4758	0.0620	0.9979	98.1
EP	0.5308	0.1410	0.9989	99.7
PS	0.5508	−0.0463	0.9995	100.0
ME	0.4690	−0.0074	0.9999	99.6
MP	0.4904	−0.0270	0.9998	100.2

[a] NE = Norephedrine; EP = ephedrine; PS = pseudoephedrine;ME = methylephedrine; MP = methylpseudoephedrine.

Table 3

Single-laboratory validation data: Accuracy[a]

NE		EP		PS		ME		MP	
Spike level, µg/g	**Recovery, %**	**Spike level, µg/g**	**Recovery, %**	**Spike level, µg/g**	**Recovery, %**	**Spike level, µg/g**	**Recovery, %**	**Spike level, µg/g**	**Recovery, %**
97.5	88.7	4045	86.6	488	87.2	109	80.4	103	83.0
195	85.6	8100	84.3	975	84.6	218	77.2	206	81.0
592	86.8	12100	85.8	1460	85.7	327	77.6	309	83.4

[a] NE = Norephedrine; EP = ephedrine; PS = pseudoephedrine; ME = methylephedrine; MP = methylpseudoephedrine. (*Note*: Accuracy of NP was not determined as laboratory did not have required DEA license.)

Table 4

Single-laboratory validation data: Repeatability, % RSD of replicate sample preparations[a]

Sample[b]	NE	NP	EP	PS	ME	MP	Total[c]
1	4.6	41	0.30	1.4	7.6	6.7	0.4
2	1.7	3.6	1.1	1.4	1.4	5.8	1.2
3	3.7	ND[d]	1.4	1.1	10.2	ND	1.4
4	ND	ND	0.30	0.68	12.0	ND	0.35
5	1.5	1.8	0.92	1.2	1.0	ND	0.96
6	8.2	47.1	1.0	4.5	1.8	ND	1.0
7	ND	ND	5.0	5.7	17.6	ND	5.4

[a] NE = Norephedrine; NP = norpseudoephedrine; EP = ephedrine; PS = pseudoephedrine; ME = methylephedrine;MP = methylpseudoephedrine.

[b] 1 = Multicomponent granulated material containing ephedra extract; 2 = *E. sinica* botanical raw material; 3 = multicomponent finished product 1; 4 = ephedra powdered extract; 5 = *E. sinica* capsules; 6 = multicomponent finished product 2; 7 = high protein powdered drink mix.

[c] Total = % RSD of total ephedrine alkaloid content.

[d] ND = None detected.

J AOAC Int. Author manuscript; available in PMC 2008 November 18.

Table 5

Results for blind replicates

	Results, µg/mL							
	Botanical raw material		Finished product containing only ephedra		Common extract		Complex mixture of multiple dietary supplements containing ephedra	
Lab ID	A1	A2	B1	B2	C1	C2	D1	D2
				Ephedrine				
A	6290	6328	7400	7180	64300	65200	23200	21600
B	6120	6180	6820	6870	66000	62400	21400	22200
C	6600	5780	7310	7590	64600	63800	22400	22300
D	6130	6045	6900	6560	65600	65500	23100	23000
E	6510	6385	7080	7020	62400	63100	21800	21500
F	6000	6390	8020	40200	66000	66400	21500	21300
G	6120	6090	7050	6990	65000	65100	21600	22400
H	6170	6079	6950	6800	62400	62100	20300	21200
I	5800	5664	6130	6040	56900	57200	18200	18300
J	6540	6465	7140	7100	65400	64700	21500	22500
				Pseudoephedrine				
A	1370	1380	2340	2270	9360	9130	1880	1980
B	1140	1080	2000	2000	9260	8480	1760	1700
C	1500	1520	2700	2780	11300	11200	2390	2380
D	1200	1210	2210	2060	9670	9500	2120	2200
E	1300	1290	2250	2240	9140	9220	1980	1920
F	1130	1300	2720	5040	9740	10300	1940	1940
G	1240	1260	2240	2220	9469	9470	1920	1990
H	1120	1250	2150	2110	8690	8900	1840	1970
I	1210	1170	1940	1950	7969	8300	1590	1570
J	1340	1340	2240	2230	9655	9540	2030	2100

Table 6

Results for blind duplicates

	Results, µg/mL							
	High protein drink mix		Low spike negative control		High spike negative control		Negative control	
Lab ID	E1	E2	F1	F2	G1	G2	H1	H2
				Ephedrine				
A	146	152	5990	5770	62065	63900	ND[a]	ND
B	145	142	5740	5600	56600	58000	ND	ND
C	248	217	5680	5780	61700	60400	ND	ND
D	[b]	[b]	7460	5600	[c]	[c]	ND	ND
E	262	206	5700	5660	58800	57800	ND	ND
F	140	136	ND	5600	55600	57400	ND	1160
G	143	157	5890	5780	60000	57800	ND	ND
H	225	237	6420	5840	44800	42900	ND	ND
I	89	79	4950	4720	51300	49400	ND	ND
J	158	164	5790	5760	59000	60300	ND	ND
				Pseudoephedrine				
A	ND	117	850	798	7120	10300	ND	ND
B	424	417	617	773	8410	7890	ND	ND
C	92	75	852	822	10600	10400	ND	ND
D	47	ND	696	961	[c]	[c]	ND	ND
E	52	48	788	755	8260	8740	ND	ND
F	26.5	28	ND	856	7680	8480	ND	412
G	36	41	792	780	9240	10100	ND	ND
H	43	45.5	828	797	8450	7610	ND	ND
I	28.2	43.5	766	769	7830	7700	ND	ND
J	41.6	43.5	629	674	8720	8810	ND	ND

[a] ND = None detected.

[b] Value below LOQ.

[c] Value above calibration range; data not used in statistical analysis.

Table 7

Recovery data for spiked negative controls: Ephedrine and pseudoephedrine

		Average amounts, µg/g[a]		
Sample[b]		**Negative control**	**Low spike**	**High spike**
EP	Amount added	0	6830	66700
	Amount found	0	5790	58100
	Recovery, %	NA[c]	84.7	87.2
PS	Amount added	0	926	8840
	Amount found	0	783	8683
	Recovery, %	NA	84.6	98.2

[a] Recovery data is from 9 laboratories. Two laboratories were determined to be outliers and not included in recovery results.

[b] EP = Ephedrine; PS = pseudoephedrine.

[c] NA = Not applicable.

Table 8

Statistical analysis of blind replicates: Repeatability and reproducibility (NE, NP, ME, and MP)

Sample[a]	Average, µg/g	S_r	RSD_r, %	S_R	RSD_R, %	HORRAT	Outlier labs	No. of labs used
			Norephedrine					
A	800	9	1.12	31.6	3.95	0.68	2	8
B	364	10.6	2.92	26.8	7.37	1.12	0	10
C	ND[b]	—	—	—	—	—	0	10
D	334	10.7	3.19	105	31.5	4.72	0	10
E	ND	—	—	—	—	—	0	10
F	72.3	9.28	12.8	22	30.4	3.61	0	10
G	563	144	25.6	166	29.5	4.79	0	10
H	ND	—	—	—	—	—	—	10
			Norpseudoephedrine					
A	748	37.4	4.99	46.7	6.25	1.06	2	8
B	588	13.6	2.31	31.1	5.29	0.86	0	9
C	[c]	—	—	—	—	—	—	9
D	186	7.9	4.22	93	50	6.87	1	8
E	ND	—	—	—	—	—	0	9
F	366	17.5	4.77	465	127	19.3	0	9
G	964	318	33	388	40.2	7.08	0	9
H	[d]	—	—	—	—	—	—	9
			Methylephedrine					
A	227	16.6	7.33	24.7	10.9	1.54	1	9
B	401	17.8	4.44	22.9	5.73	0.88	2	8
C	ND	—	—	—	—	—	0	10
D	668	10.3	1.55	32.6	4.88	0.81	1	9
E	ND	—	—	—	—	—	0	10
F	72.3	9.28	12.8	22	30.4	3.61	0	10
G	563	144	25.6	166	29.5	4.79	0	10
H	ND	—	—	—	—	—	—	10
			Methylpseudoephedrine					
A	ND	—	—	—	—	—	0	10
B	ND	—	—	—	—	—	0	10
C	[c]	—	—	—	—	—	—	10
D	[d]	—	—	—	—	—	—	10
E	ND	—	—	—	—	—	0	10
F	67	3.7	5.52	6.6	9.9	1.16	2	8
G	727	14.8	2.04	65.5	9.01	1.52	2	8
H	ND	—	—	—	—	—	0	10
			Total ephedrine alkaloids					
A	9290	89.8	0.97	403	4.34	1.07	1	9
B	10600	189	1.79	499	4.73	1.19	1	9
C	74900	634	0.85	1520	2.03	0.69	2	8
D	25100	590	2.36	816	3.26	0.93	1	9
E	259	36.6	14.2	136	52.6	7.58	1	9
F	7140	224	3.13	784	10.97	2.61	1	9

Sample[a]	Average, µg/g	S_r	RSD_r, %	S_R	RSD_R, %	HORRAT	Outlier labs	No. of labs used
G	70300	1400	1.99	6770	9.64	3.23	1	9
H	—	—	—	—	—	—	—	10

[a] A = Botanical raw material; B = finished product containing only ephedra; C = common extract; D = complex mixture of multiple dietarysupplements containing ephedra; E = high protein drink mix; F = low spike negative control; G = high spike negative control; H = negativecontrol.

[b] ND = None detected.

[c] Labs A, B, and H reported results for NP in these samples.

[d] Labs A and B reported results for NP in these samples.

Table 9

Recovery data for spiked negative controls: Minor ephedrine alkaloids

		Average amounts, µg/g		
Sample[a]		**Negative control**	**Low spike**	**High spike**
NE	Amount added	0	114	1450
	Amount found	0	72.3	563
	Recovery, %	NA[b]	63.4	38.8
NP	Amount added	0	103	614
	Amount found	[c]	366	964
	Recovery, %	NA	356	157
ME	Amount added	0	316	4990
	Amount found	0	200	2580
	Recovery, %	NA	63.3	51.7
MP	Amount added	0	95.4	1360
	Amount found	0	67.0	727
	Recovery, %	NA	70.2	53.5

[a] NE = Norephedrine; NP = norpseudoephedrine; ME = methylephedrine; MP = methylpseudoephedrine.

[b] NA = Not applicable.

[c] Laboratories A and B reported results for NP in this sample.

Table 2003.13A

Statistical analysis of blind replicates: Repeatability and reproducibility (ephedrine and pseudoephedrine)

Sample[a]	Average, µg/g	S_r	RSD_r, %	S_R	RSD_R, %	HORRAT	Outlier labs	No. of labs used
Ephedrine								
A	6180	60.8	0.98	246	3.98	0.92	2	8
B	7050	127	1.8	465	6.59	1.56	0	10
C	64400	410	0.64	1370	2.13	0.7	2	8
D	21900	560	2.56	761	3.47	0.98	1	9
E	169	16	9.47	53.3	31.5	4.26	0	10
F	5790	176	3.03	203	3.50	0.81	2	8
G	58100	1150	1.98	3840	6.6	2.15	2	8
H	[b]	—	—	—	—	—	—	10
Pseudoephedrine								
A	1270	51.6	4.08	122	9.64	1.77	0	10
B	2270	45.5	2.01	253	11.2	2.23	0	10
C	9410	242	2.58	848	9.0	2.23	0	10
D	1960	51	2.58	222	11.3	2.21	0	10
E	52.6	5.51	10.5	26.3	50	5.67	1	9
F	783	75.4	9.63	82.7	10.6	1.8	0	10
G	8683	408	4.7	987	11.4	2.78	2	8
H	[b]	—	—	—	—	—	—	10
Total ephedrine + pseudoephedrine								
A	7450	225	3.03	326	4.38	1.05	0	10
B	9320	171	1.83	695	7.46	1.84	0	10
C	74900	634	0.85	1520	2.03	0.69	2	8
D	23900	570	2.38	862	3.60	1.03	1	9
E	259	36.6	14.2	136	52.6	7.58	1	9
F	6570	182	2.76	230	3.50	0.82	1	9
G	65200	1680	2.57	6580	10.1	3.34	1	9
H	[b]	—	—	—	—	—	—	10

[a] A = Botanical raw material; B = finished product containing only ephedra; C = common extract; D = complex mixture of multiple dietary supplements containing ephedra; E = high protein drink mix; F = low spike negative control; G = high spike negative control; H = negative control.

[b] Lab F reported results for EP and PS in one of the blind replicates of the negative control.

Table 2003.13B

Preparation of standard solutions (U.S. laboratories)

Volume stock standard solution, mL	Final volume, mL	Alkaloid concentration, µg/mL					
		EP[a]	PS[a]	ME	MP	NE	NP[a]
0.50 [Level 1]	50	20.5	2.5	0.5	0.5	0.5	0.81
1.00 [Level 2]	50	41.0	4.9	1.0	1.0	1.0	1.6
1.50 [Level 3]	50	61.4	7.4	1.5	1.5	1.5	2.4
2.50 [Level 4]	50	102.4	12.3	2.5	2.5	2.5	4.0
3.50 [Level 5]	50	143.4	17.2	3.5	3.5	3.5	5.7
5.00 [Level 6]	50	204.8	24.6	5.0	5.0	5.0	8.1
Standard blank[b]	50	0.0	0.0	0.0	0.0	0.0	0.0

[a] Corrected for HCl form.

[b] Blank is 1 mL MeOH plus 99 mL diluent, E(c).

J AOAC Int. Author manuscript; available in PMC 2008 November 18.

Table 2003.13C

Preparation of standard solutions (international laboratories)

Volume stock standard solution, mL	Final volume, mL	Alkaloid concentration, µg/mL					
		EP[a]	PS[a]	ME	MP	NE	NP
0.50 [Level 1]	50	20.5	2.5	0.5	0.5	0.5	0.40
1.00 [Level 2]	50	41.0	4.9	1.0	1.0	1.0	0.80
1.50 [Level 3]	50	61.4	7.4	1.5	1.5	1.5	1.2
2.50 [Level 4]	50	102.4	12.3	2.5	2.5	2.5	2.0
3.50 [Level 5]	50	143.4	17.2	3.5	3.5	3.5	2.8
5.00 [Level 6]	50	204.8	24.6	5.0	5.0	5.0	4.0
Standard blank[b]	50	0.0	0.0	0.0	0.0	0.0	0.0

[a] Corrected for HCl form.

[b] Blank is 1 mL MeOH plus 99 mL diluent, E(c).

J AOAC Int. Author manuscript; available in PMC 2008 November 18.

Table 2003.13D

Chromatographic conditions

Column	Phenomenex Synergi PolarRP, 4.6 × 150 mm, 4 μm particle size
Mobile phase	Methanol—100mM KH_2PO_4 (3 + 97)
Flow rate	1.5 mL/min
Injection volume	20 μL
Detection	~0.10 AUFS at 210 nm
Injection needle wash solvent	Water
Column temperature	25°C
Run time	20 min[a]

Retention times

Marker compound	Retention time, min	Relative retention[b]
Norephedrine	4.9	0.61
Norpseudoephedrine	5.4	0.68
Ephedrine	8.0	1.0
Pseudoephedrine	9.4	1.2
Methylephedrine	12.8	1.6
Methylpseudoephedrine	13.2	1.7

[a] High protein drink mixes may require a run time of 30 min due to late eluting peaks. Products that contain caffeine require a 10 min column flush with acetonitrile-water-H_3PO_4 (50 + 50 + 0.1) after at least every 4th injection. Re-equilibrate the column with mobile phase for 10 min after each flush.

[b] Relative retention time is calculated as the retention time of the ephedrine alkaloid of interest divided by the retention time of ephedrine, and can be used to aid in peak identification.