DIETARY SUPPLEMENTS

# Determination of Ephedra Alkaloids by Liquid Chromatography/Tandem Mass Spectrometry

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**In conjunction with an AOAC Task Group on dietary supplements, a liquid chromatography/tandem mass spectrometry (LC–MS/MS) method was validated for measurement of 6 major alkaloids in raw ephedra sinica herb, ephedra extracts, ephedra tablets, complex dietary supplements containing ephedra, and a high-protein drink mix containing ephedra. The amount of ephedrine-type alkaloids present was determined by LC with mass selective detection. Six replicates of each matrix were analyzed on 3 separate occasions. The presence of 6 ephedrine-type alkaloids was detected at a level >0.5 μg/g based on a 0.5 g sample. The standard curve range for this assay is from 0.02 to 1.0 μg/mL. Appropriate dilutions covered a wide range of specific alkaloid concentrations. The calibration curves for all 6 analytes had correlation coefficients >0.995.**

An accurate method for quantification of ephedrine-type alkaloids in dietary products is essential for regulatory compliance and for use as a quality control tool. These compounds have been associated with a significant number of adverse health incidents; as a result, accurate and robust methods are needed to determine and measure their presence in a broad range of matrixes. In conjunction with an AOAC Task Group on dietary supplements, a liquid chromatography/tandem mass spectrometry (LC–MS/MS) method was validated for measurement of these compounds in raw ephedra sinica herb, ephedra extracts, ephedra tablets, complex dietary supplements containing ephedra, and a high-protein drink mix containing ephedra. The method may be used to measure amounts of ephedrine (EP), pseudoephedrine (PE), norephedrine (NE), norpseudoephedrine (NPE), methylephedrine (ME), and methylpseudoephedrine (MPE). The task group agreed that the U.S. Food and Drug Administration's (FDA) LC–MS/MS ephedra method should be validated for all dietary supplements and ingredients.

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The original method developed by the FDA used LC for separation and MS for detection [Gay, M.L., White, K.D., Obermeyer, W.R., Betz, J.M., & Musser, S.M. (2001) *J. AOAC Int.* **84**, 761–769]. The method has been modified to use a dual-stage MS (LC–MS/MS) system. This modification facilitates optimum chromatographic parameters and a mode of detection that improves precision and accuracy and allows measurement of both the precursor and product ions for positive analyte identification. The ephedrine-type alkaloids are extracted from dietary supplements with methanol and water (80 + 20) and prepared by using solid-phase extraction (SPE) cartridges.

## Experimental

### *Chemicals and Reagents*

(**a**) *Acetonitrile.*—LC grade (Fisher Scientific, Pittsburgh, PA).

(**b**) *Methanol.*—LC grade (Fisher Scientific).

(**c**) *Glacial acetic acid, 100.0%.*—Fisher Scientific.

(**d**) *Ammonium acetate.*—Reagent grade, 98% (Fisher Scientific, and Acros Organics, Fair Lawn, NJ).

(**e**) *Milli-Q water, purification system.*—ASTM Type deionized (DI) water containing <0.5 ppb total organic carbon (Millipore Corp., Bedford, MA).

### *Reference Standards*

(**a**) *1R, 2S-(-)-Norephedrine (NE), 99%.*—Acros Organics.

(**b**) *(+)-Norpseudoephedrine hydrochloride (NPE).*—[Cathine hydrochloride (DEA Schedule IV)], 98% (RBI, a subsidiary of Sigma-Aldrich Chemical Co., Milwaukee, WI).

(**c**) *1R, 2S-(-)-Ephedrine hydrochloride (EP), 99%.*—Aldrich Chemical Co.

(**d**) *1S, 2S-(+)-Pseudoephedrine (PE), 99%.*—Acros Organics.

(**e**) *1R, 2S-(-)-N-methylephedrine (ME), 99%.*—Acros Organics.

(**f**) *1S, 2S-(+)-N-methylpseudoephedrine (MPE), 99%.*—Acros Organics.

(**g**) *1R, 2S-(-)-Ephedrine-$d_5$ hydrochloride.*—Supplied by the FDA (Washington, DC).

(**h**) *"Red Rose" black tea and cocoa powder.*—Purchased from a local grocery store; used as negative controls.

**Table 1. Concentration of calibration standards**

| Standard level | Concentration NE, NPE, EP, PE, ME, MPE, μg/mL | Concentration Ephedrine-$d_5$, μg/mL |
|---|---|---|
| 1 | 0.0200 | 0.100 |
| 2 | 0.0500 | 0.100 |
| 3 | 0.100 | 0.100 |
| 4 | 0.400 | 0.100 |
| 5 | 0.700 | 0.100 |
| 6 | 1.00 | 0.100 |

### *Apparatus*

(**a**) *SPE columns.*—Isolute, propylsulfonic acid (PRS), 6 mL, 500 mg, P/N 540-0050-C.

(**b**) *YMC phenyl column.*—3 μm, 250 × 2 mm (Waters Corp., Milford, MA).

(**c**) *LC injection vials.*—Target I-D, C4001-2W (National Scientific Co., Duluth, GA).

(**d**) *Pump.*—Agilent (Palo Alto, CA) 1100 Series.

(**e**) *Column heater.*—Agilent 1100 Series.

(**f**) *Autosampler.*—Agilent 1100 Series.

(**g**) *Mass selective (MS) detector.*—Micromass Quattro LC (Waters Corp.).

(**h**) *Data system.*—MassLynx Version 3.4 (Waters Corp.).

### *Preparation of Reagents*

The mobile phase was prepared by adding 3.80 g ammonium acetate, 30 mL acetonitrile, and 20 mL glacial acetic acid to 1000 mL volumetric flask containing ca 500 mL water. The solution was mixed and diluted to volume with water. The resulting solution was 2% HOAc, 3% ACN, and 50mM $NH_4OAc$, pH 3.77. The dilute mobile phase was prepared by diluting 50 mL mobile phase to 1 L with water.

The extraction solvent, MeOH–DI (80 + 20), was prepared by placing 200 mL water in a 1000 mL graduated cylinder and diluting to volume with methanol. The elution buffer was prepared by adding 1.20 g ammonium acetate, 0.5 mL glacial acetic acid, 30 mL acetonitrile, and 50 mL water to a 100 mL volumetric flask. The solution was mixed and diluted to volume with water. The resulting solution was 0.5% HOAc, 30% ACN, and 150mM $NH_4OAc$.

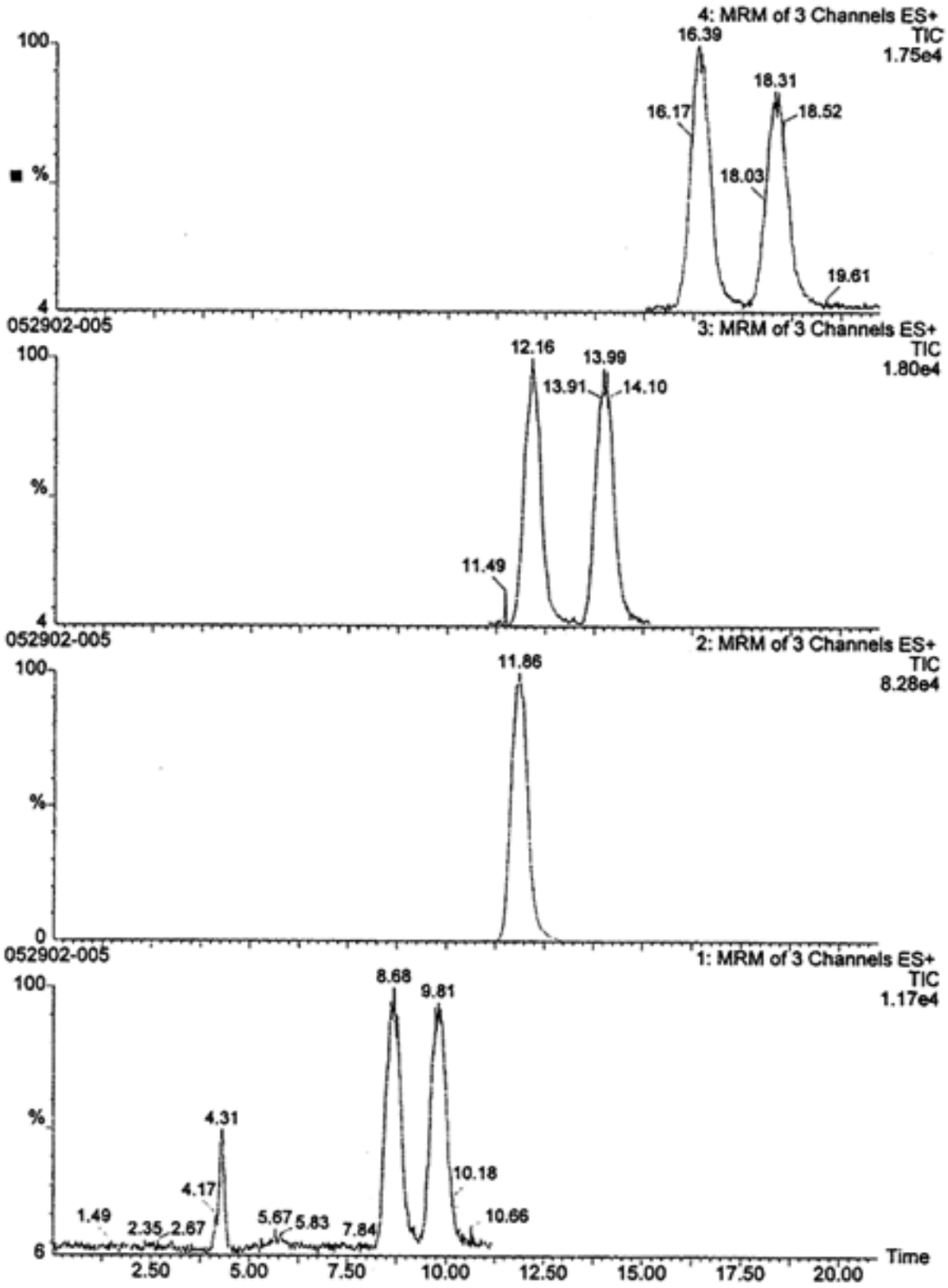


**Figure 1. Chromatogram of 0.02 μg/mL mixed standard containing the 6 alkaloids of interest as well as the ephedrine-$d_5$ internal standard. The peaks displayed are, in order, NE, NPE, EP-$d_5$, EP, PE, ME, and MPE.**

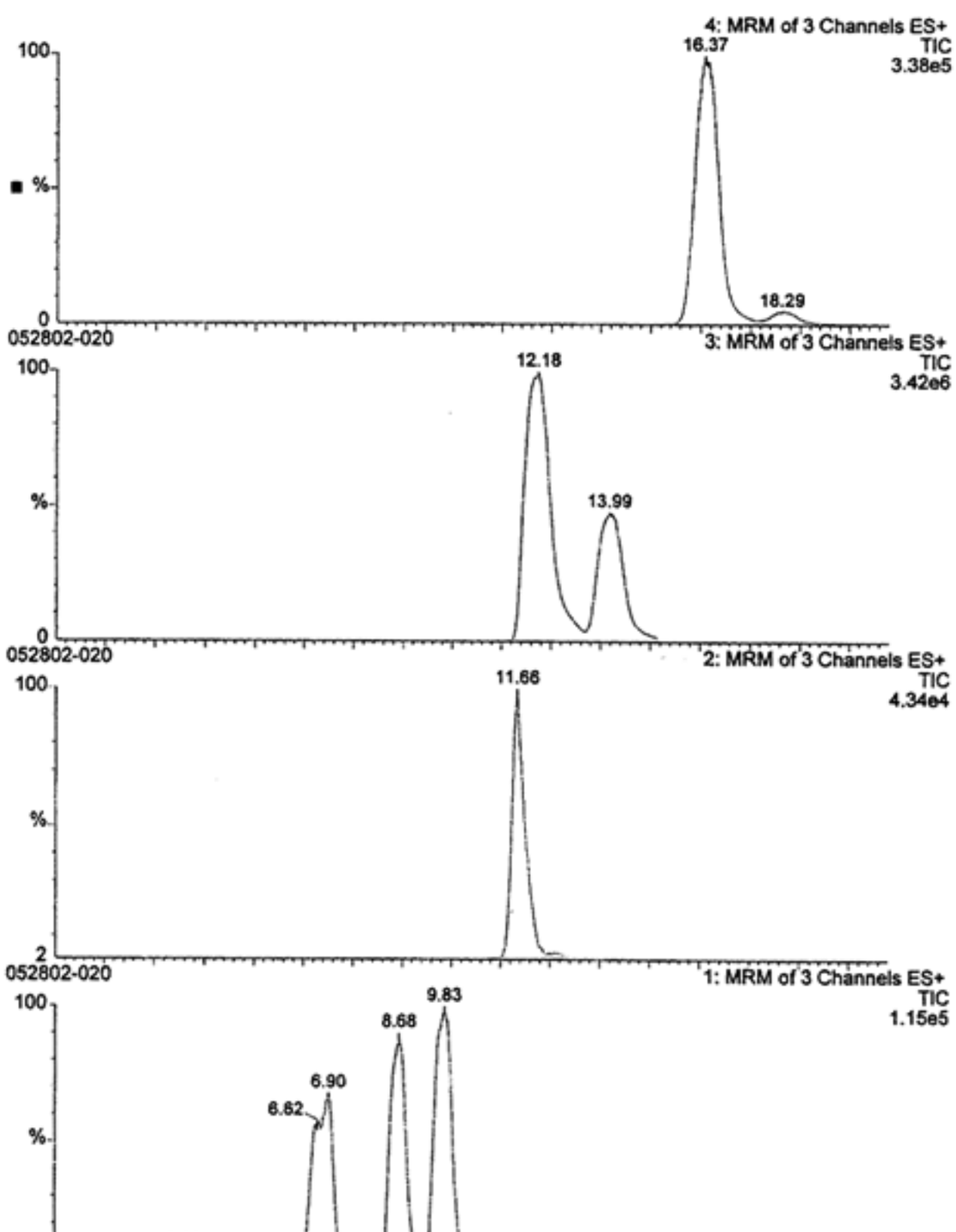


**Figure 2. Chromatogram showing the separation of the alkaloids contained within the Thermadrene capsule extract. The peaks displayed are, in order, NE, NPE, EP-$d_5$, EP, PE, ME, and MPE.**

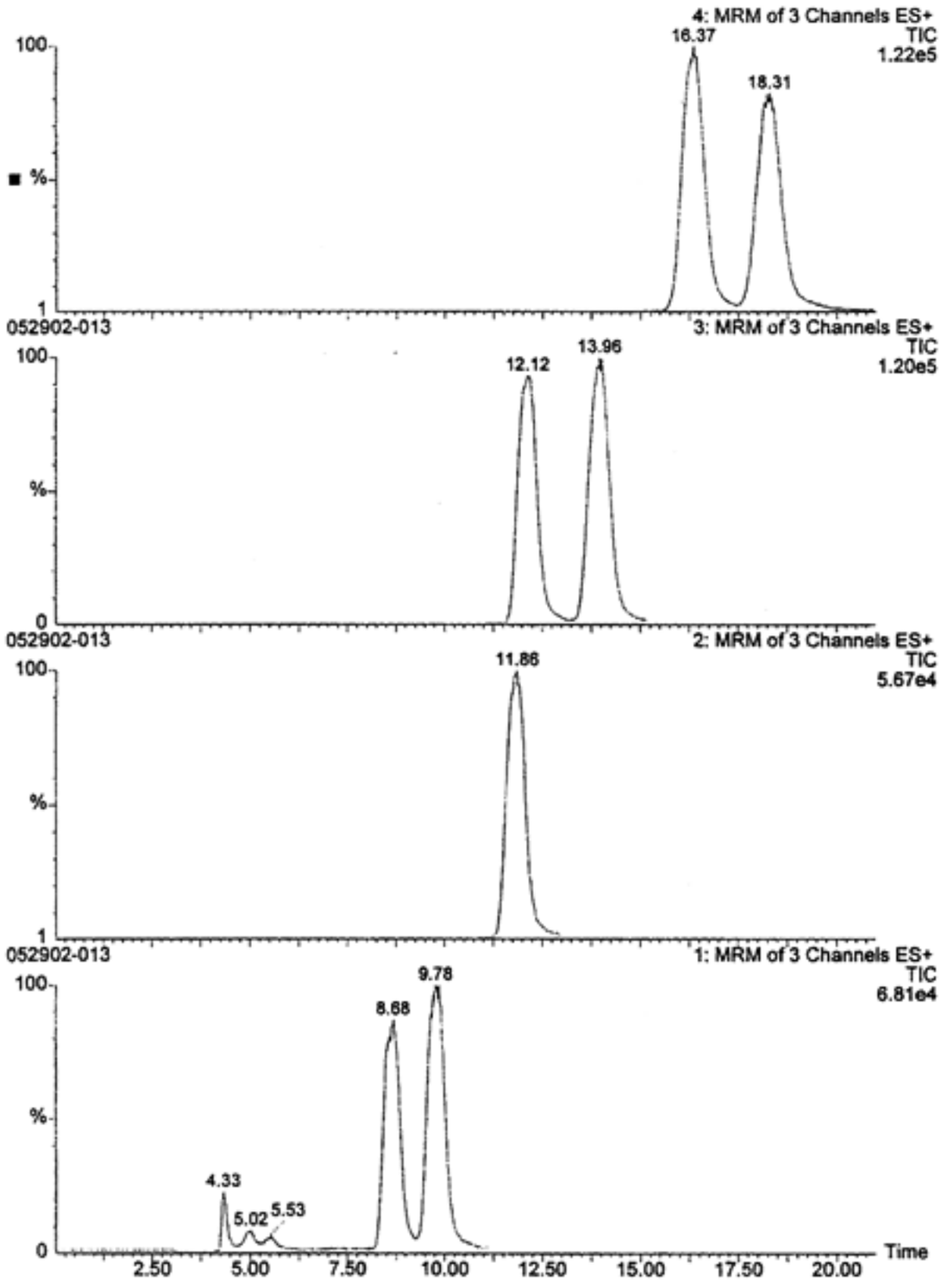


**Figure 3. Chromatogram of fortified "Red Rose" tea showing no significant interferences from the negative control (i.e., "Red Rose" tea). The peaks displayed are, in order, NE, NPE, EP-$d_5$, EP, PE, ME, and MPE.**

### *Preparation of Standard Solutions*

The internal standard was prepared by weighing ca 5 mg ephedrine-$d_5$ into a 10 mL volumetric flask and diluting to volume with extraction solvent. A portion of this solution was diluted with extraction solvent to a concentration of 10 μg/mL to make the working internal standard.

**Table 2. Ions to be monitored (ions shown in bold are used for quantification; others are used for confirmation)**

| Compound | Ions | Cone (V) | Collision (eV) |
|---|---|---|---|
| NE | 117, **134**, 152 | 10 | 15 |
| NPE | 117, **134**, 152 | 10 | 15 |
| EP | 133, **148**, 166 | 15 | 20 |
| PE | 133, **148**, 166 | 15 | 20 |
| ME | 147, 162, **180** | 22 | 20 |
| MPE | 147, **162**, 180 | 22 | 20 |
| EP-$d_5$ | **153** | 15 | 20 |

The stock standards were prepared by weighing ca 50 mg NE, NPE, EP, PE, ME, and MPE into separate 100 mL volumetric flasks. Each stock standard was diluted to volume with elution buffer. The calibration standards were prepared by mixing an appropriate aliquot of each stock standard with the internal standard and diluting with elution buffer to obtain the concentrations shown in Table 1. When not in use, all standards were stored in a refrigerator maintained at 2–8°C. A chromatogram showing the separation of the alkaloids at a concentration of 0.02 μg/mL is shown in Figure 1.

### *Sample Preparation*

The samples were prepared by weighing 0.1–1 g homogenous product, equivalent to 0.03–8 mg ephedrine-type alkaloid, into a 50 mL screw-capped polypropylene centrifuge tube. A 20 mL volume of extraction solvent was added. (*Note*: If preparing a spiked sample, adjust solvent accordingly.) The centrifuge tube was capped and sonicated at room temperature for at least 20 min. The sample was then mixed on a Vortex mixer for at least 1 min and centrifuged at 3000 rpm for at least 20 min.

A 50 μL volume of working internal standard was added to an accurately measured portion of supernatant and diluted with extraction solvent to 10 mL. The concentration of each alkaloid should range from 0.0100 to 0.500 μg/mL. This was accomplished with 2 dilutions: one for NE, NPE, ME, and MPE; another for EP and PE. Chromatograms showing separation of alkaloids in a sample extract and fortified control are shown in Figures 2 and 3.

### *Cleanup Procedure*

An SPE column was prepared by passing successive 2 mL portions of methanol, water, and dilute mobile phase through the column. A 10 mL portion of diluted sample was then added to the SPE column followed by two 3 mL portions of di-

**Table 3. Example ion ratios found in standards used for confirmation**

| Compound | Ion ratio | Peak area, % |
|---|---|---|
| NE | 117:134 | 15 |
| NE | 162:134 | 49 |
| NPE | 117:134 | 14 |
| NPE | 162:134 | 35 |
| EP | 133:148 | 12 |
| EP | 166:148 | 55 |
| PE | 133:148 | 12 |
| PE | 166:148 | 37 |
| ME | 147:180 | 9 |
| ME | 162:180 | 49 |
| MPE | 147:162 | 18 |
| MPE | 180:162 | 62 |

**Table 4. Levels of alkaloids found**

| Sample identification | Level found, μg/g | NE | NPE | EP | PE | ME | MPE |
|---|---|---|---|---|---|---|---|
| Endurance granulation raw material | 32400 | 553 | 515 | 23700 | 5720 | 1830 | 100 |
| Ephedra sinica stapf (ephedraceae) | 21300 | 448 | 1630 | 9140 | 8010 | 1920 | 138 |
| Optidrene tablet | 20500 | 389 | 444 | 15200 | 3510 | 904 | 38.6 |
| Ephedra powdered extract | 98600 | 240 | 282 | 83900 | 12400 | 814 | 1000 |
| Ephedra sinica capsules | 16500 | 867 | 1550 | 9580 | 3190 | 1290 | 42.2 |
| Thermadrene capsules | 34700 | 416 | 276 | 27200 | 3490 | 3220 | 134 |
| High-protein drink mix | 295 | 1.17 | 3.16 | 226 | 60.9 | 1.87 | 1.85 |
| "Red Rose" black tea | ND[a] | <0.500 | <0.500 | <0.500 | <0.500 | <0.500 | <0.500 |
| Instant chocolate powder | ND | <0.500 | <0.500 | <0.500 | <0.500 | <0.500 | <0.500 |

[a] ND = Not detected.

lute mobile phase. The SPE column was dried for 5 min by evacuating the reservoir apparatus; it was then wetted with 2 mL methanol, and all effluent was discarded. The SPE column was eluted with 4 mL extraction buffer into a 15 mL tube and diluted to 5 mL with elution buffer.

### *Chromatographic Parameters*

The LC column temperature was maintained at 30°C with a flow rate of 0.230 mL/min. Single injections were introduced with injection volume established at 10 μL. A sufficient number of injections (3–5) of sample or standard should be used to equilibrate the LC–MS/MS system. Sample introduction mode was used with a setting of electrospray positive (ES+). Mass range for the daughter scan for specific ion used a dwell setting of 0.25/s. The source temperature was set at 150°C with a desolvation gas flow rate of 600 L/h. The desolvation temperature was maintained at 350°C with a cone gas flow rate of 40 L/h. The ions detected and cone and collision voltages are shown in Table 2. The ion ratios used for confirmation are shown in Table 3.

### *Quantification and Confirmation*

Calibration curves were produced on 3 separate occasions during the course of this validation. At least one standard was injected at the beginning and one at the end of the batch run. In addition, LC calibration standards were included and interspersed among the samples in each assay batch. Because no certificates of analysis or substantiated label claims were available for the matrixes being tested, an initial range-finding analysis was performed to establish potential alkaloid levels. Based on these data, a calibration range was set to bracket the expected concentration for each test material or group of test materials (Table 1). For the validation, a calibration standard curve (minimum of 6 concentration levels) was run at a mini-

**Table 5. Method precision**

| | RSD, % | | | | | |
|---|---|---|---|---|---|---|
| Sample identification | NE | NPE | EP | PE | ME | MPE |
| Endurance granulation raw material | 7.72 | 7.84 | 7.05 | 6.68 | 6.61 | 7.20 |
| Ephedra sinica stapf (ephedraceae) | 0.469 | 3.07 | 6.67 | 6.48 | 1.20 | 3.04 |
| Optidrene tablet | 2.67 | 2.03 | 6.25 | 6.27 | 1.14 | 1.81 |
| Ephedra powdered extract | 2.88 | 7.48 | 6.32 | 7.34 | 2.60 | 0.800 |
| Ephedra sinica capsules | 3.53 | 4.65 | 3.65 | 4.45 | 0.465 | 3.22 |
| Thermadrene capsules | 7.21 | 7.25 | 2.57 | 2.87 | 6.61 | 4.40 |
| High-protein drink mix | 1.28 | 4.78 | 4.91 | 4.93 | 1.07 | 1.62 |
| "Red Rose" black tea[a] | 1.29 | 1.76 | 1.50 | 1.29 | 0.767 | 1.18 |
| Instant chocolate powder[a] | 2.73 | 3.38 | 2.37 | 3.06 | 2.95 | 2.64 |

[a] Spike recovery data.

**Table 6. Method accuracy**

| | Recovery, % | | | | | |
|---|---|---|---|---|---|---|
| Sample identification | NE | NPE | EP | PE | ME | MPE |
| "Red Rose" black tea | 91.8 | 103 | 116 | 121 | 121 | 115 |
| Instant chocolate powder | 91.9 | 101 | 109 | 117 | 118 | 112 |

mum before and after each sample set. The relative response (peak area analyte/peak area internal standard) of analyte vs concentration was used to construct the calibration curve by using a least-squares linear regression method. The calibration curve had a target correlation coefficient (r) ≥0.995. The target relative standard deviation (RSD) for intra- and interassay replicates was ≤10%.

Samples of the negative controls (black tea, cocoa) were fortified in triplicate with the calibration standard mixture at 3 levels. Triplicate unfortified controls were analyzed concurrently. This was repeated on at least 2 additional occasions. The target average recovery was between 70 and 120%, and the target RSD between fortification level replicates was ≤10%.

A standard calibration curve was generated by using the ratio of the confirmation ion area vs the quantification ion area of the internal standard for each concentration level. A calibration curve was produced for each analyte. Weighting ($1/x$) was necessary to obtain acceptable percent deviation at lower standard concentrations.

$$y = mx + b$$

where $y$ is relative peak area (area of analyte/area internal standard), m is slope of the line generated by standard curve, $x$ is concentration of analyte found (μg/mL), and b is the $y$-intercept of line generated by standard curve.

The amount of analyte found in sample (μg/g) was calculated with the formula:

$$A = \frac{C \times V_F \times D}{W}$$

where A is μg/g ephedrine-type alkaloid found in sample, C is concentration (μg/mL) ephedrine-type alkaloid found in samples from standard curve, $V_F$ is final volume of extracts (5 mL), D is dilution (20 mL for extraction, include any other dilutions performed), and W is sample weight (g).

First, the peak area detected in the standards for each confirmation ion was divided by the peak area of the quantification ion. The mean of these ratios for all of the standards was then calculated. The sample ratio of confirmation to the quantification ion should be ±10% (arithmetic difference, not relative difference) of the mean standard ratio. For example, if a mean ratio for the standards is 50%, the range for the sample ratio would be 40–60% (not 45–55%). A summary of the validation data is shown in Table 4. The overall RSD results are shown in Table 5. Spike recovery results are shown in Table 6.

## Results and Discussion

The method detected the presence of 6 ephedrine-type alkaloids at a level >0.5 μg/g based on a 0.5 g sample. The actual sensitivity depends on sample size. The linearity range for this assay was from 0.02 to 1.0 μg/mL. Recoveries ranged from 80 to 125%. An additional concentration level at 0.005 μg/mL can be analyzed to provide lower limits of quantitation data.

As part of the validation, standard quality control practices were followed. These included external fortified recoveries of all alkaloids to monitor the extraction efficiency, the use of internal standards to monitor instrument performance, and analyte recovery through the SPE column. In addition, blanks were analyzed to monitor interferences, and repeated analyses were conducted on various matrixes to determine acceptable variation.

Although no interferences were observed, strict monitoring of the chromatographic parameters may be required for some matrixes. Analysis of the high-protein drink mix requires additional equilibration using a single injection of the actual sample matrix to improve precision of the analysis. In addition, an elution buffer blank must be injected after the high-protein samples. A decrease of retention times for all analytes occurred after multiple injections and extended use of the analytical system. For example, the retention time of the last analyte (MPE) decreased from 22.7 to 18.4 min over the course of 4–5 months of continued system/column use. No adverse separation or specificity problems were observed as a result of retention time changes.

The validation process showed the precision and accuracy required for determination of ephedra alkaloids in dietary supplements. The collaborative study protocol for the method has been approved by AOAC INTERNATIONAL, and a collaborative study is currently in progress.