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Solid-Phase Extraction Cleanup and Liquid Chromatography with Ultraviolet Detection of Ephedrine Alkaloids in Herbal Products

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A solid-phase extraction (SPE) cleanup and a liquid chromatographic (LC) method with UV detection is presented for analysis of up to 7 ephedrine alkaloids in herbal products. Alkaloids from herbal products are extracted with acidified buffer, isolated on a propylsulfonic acid SPE column, eluted with a high-ionic-strength buffer, and separated by LC with detection at 255 nm. LC separation is performed by isocratic elution on a YMC phenyl column with 0.1M sodium acetate–acetic acid (pH = 4.8) containing triethyl-amine and 2% acetonitrile. Ephedrine alkaloids are completely separated in 15 min. Average recovery of 5 common alkaloids from 3 spiked matrixes is 90%, with an average relative standard deviation (RSD) of 4.4% for alkaloid spikes between 0.5 and 16 mg/g. Average quantitation of ephedrine and pseudoephedrine from 6 herbal products is 97% of declared label claims, and average quantitation of synephrine from an herbal dietary product is 85% of label claim (RSD, 3.2%). Recoveries of synephrine, norephedrine, ephedrine, pseudoephedrine, *N*-methylephedrine, and *N*-methylpseudoephedrine spiked in 4 herbal products averaged 95%. Results of ruggedness testing and of a second laboratory validation of the procedure are also presented.

Ephedrine alkaloids are derivatives of 2-amino-1-phenyl-1-propanol where the amino group is free, methylated, or dimethylated. The 3 pairs of diastereomeric alkaloids include norephedrine (NOR), norpseudoephedrine (NPE), ephedrine (EPH), pseudoephedrine (PSE), methylephedrine (MEP), and methylpseudoephedrine (MPE). These alkaloids are sometimes found in dietary supplements that promote weight loss, body building, and increased energy (1, 2). The main sources of ephedrine alkaloids are raw botanicals and extracts from plants of the genus *Ephedra*. About 100 000 kg *Ephedra* powder and extracts were imported into the United States in 1993 alone (2). The principal ephedrine alkaloids in *ma huang*, a traditional Chinese medicine derived from the dried stem of the plants, are EPH and PSE, with EPH comprising up to 80% of the total alkaloid content (2). Typical *ma huang* is approximately 1% ephedrine alkaloids by weight; however, concentrated extracts frequently contain 4–8% ephedrine alkaloids (2).

Ephedrine alkaloids are chemical stimulants and can affect the cardiovascular and nervous systems of humans. Misuse of these alkaloids is common (2, 3), and since 1993, the U.S. Food and Drug Administration (FDA) has reported more than 800 instances of illnesses and injuries associated with use of products containing or suspected to contain ephedrine alkaloids (1, 2). As a result of these reports, FDA proposed a rule to limit the amount of ephedrine alkaloids in dietary supplements to 8 mg per serving up to 24 mg per day and to limit intake of ephedrine-containing products to 7 days (1, 4).

A quick, reliable, and powerful analytical method is needed to separate and detect these ephedrine alkaloids in herbal matrixes at the levels of concern. However, analysis of herbal products containing ephedrine alkaloids is complicated because of the potential occurrence of up to 7 similar alkaloids, the wide range in concentrations possible, the presence of matrix contaminants, the need to analyze large numbers of samples, and the occurrence of many different matrixes. Several potential multiresidue procedures exist. Liu et al. (5–7) and Flurer (8) used capillary electrophoresis (CE) with UV detection to analyze Chinese herbal products. Betz et al. (9), LeBelle et al. (10), Chiu et al. (11), and Yamasaki et al. (12) analyzed herbal products by gas chromatography (GC) with various detectors. Jian-Sheng et al. (13), Sagara et al. (14), and Price et al. (15) used liquid chromatography (LC) with UV detection to analyze ephedrine alkaloids in plant tissue.

These methods involved very little cleanup. Alkaloids usually were extracted and injected directly into the instrument of choice. Although lack of a cleanup procedure was adequate for CE methods, LC and GC methods suffered from either short column life or from the necessity of derivatization.

Several authors have used cyano, reversed-phase, and mixed-phase, solid-phase extraction (SPE) for cleanup of urine and plasma samples containing ephedrine alkaloids (16–27).

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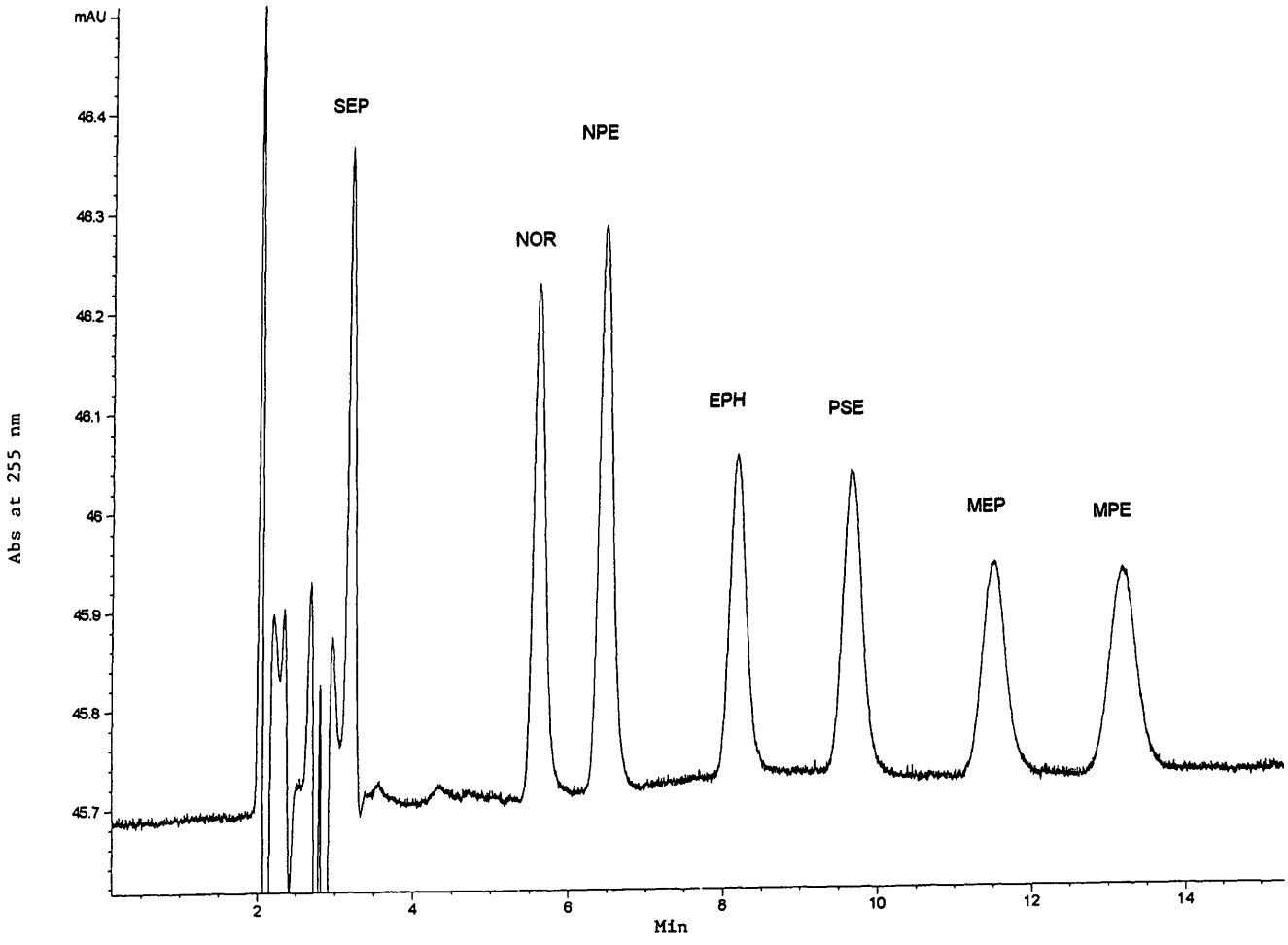

Figure 1. Representative chromatogram of a standard solution containing SEP at 10 μg/mL and NOR, NPE, EPH, PSE, MEP, and MPE, each at 20 μg/mL.

For our preliminary cleanup, separation, and detection method (27), we used a propylsulfonic acid (PRS) SPE column to clean up herbal products containing ephedrine alkaloids. However, ruggedness testing revealed that the method suffered from a wandering internal standard, interfering peaks, plasticizers coming from the SPE column during the organic wash, and low recoveries of late-eluting alkaloids.

On the basis of available equipment, the need for a rugged method, and the desire to keep the procedure simple, we reinvestigated that method (27) and developed a modified procedure using acidic extraction, SPE cation-exchange cleanup, LC separation using an isocratic mobile phase and a YMC phenyl column, and UV detection at 255 nm.

METHOD

Apparatus

(a) *LC systems.*—(*1*) *Laboratory 1.*—Beckman 110B pumps (Fullerton, CA), isocratic flow at 0.80 mL/min, Beckman 420 controller, Rheodyne 7725 injector (20 μL) (Rohnert Park, CA), Beckman 163 UV detector at 255 nm. (*2*) *Laboratory 2.*—Hewlett-Packard 1040 LC (Palo Alto, CA) with a diode array detector at 255 nm.

(b) *pH Meter.*—Orion Model 601A (Cambridge, MA), calibrated at pH 4.00 and 7.00.

(c) *Column.*—(*1*) *Analytical.*—3.0 × 250 mm, S-5 μm, 120A, Phenyl, Cat. No. PH12S052503WT, YMC, Inc. (Wilmington, NC). (*2*) *Guard.*—30 × 4.6 mm, 5 μm, Ultremex Phenyl, Cat. No. 03A-0052-E0, Phenomenex (Torrance, CA).

(d) *SPE.*—500 mg, 6 mL PRS, Cat. No. 540-0050-C, Isolute (Mid-Glamorgan, UK); Varian Vac-Elute SPE manifold (Palo Alto, CA).

(e) *Pipettes.*—Calibrated 100–1000 μL Eppendorf (Brinkmann Instruments, Westbury, NY); 4 and 25 mL class A volumetric; 10 mL Mohr.

(f) *Filter paper.*—Whatman No. 1 qualitative, 110 mm filter papers, Cat. No. 1001-110 (Clifton, NJ).

Reagents and Solutions

(a) *Standards.*—(–)-(1*R*,2*S*)-norephedrine, (–)-norpseudoephedrine, (–)-(1*R*,2*S*)-ephedrine, (+)-(1*S*,2*S*)-pseudoephedrine, (–)-(1*R*,2*S*)-*N*-methylephedrine, (+)-(1*S*,2*S*)-*N*-methylpseudoephedrine, and synephrine, all with purity ≥98%, Aldrich Chemical Co. (Milwaukee, WI). HCl salts can be substituted as long as they are appropriately converted to the free

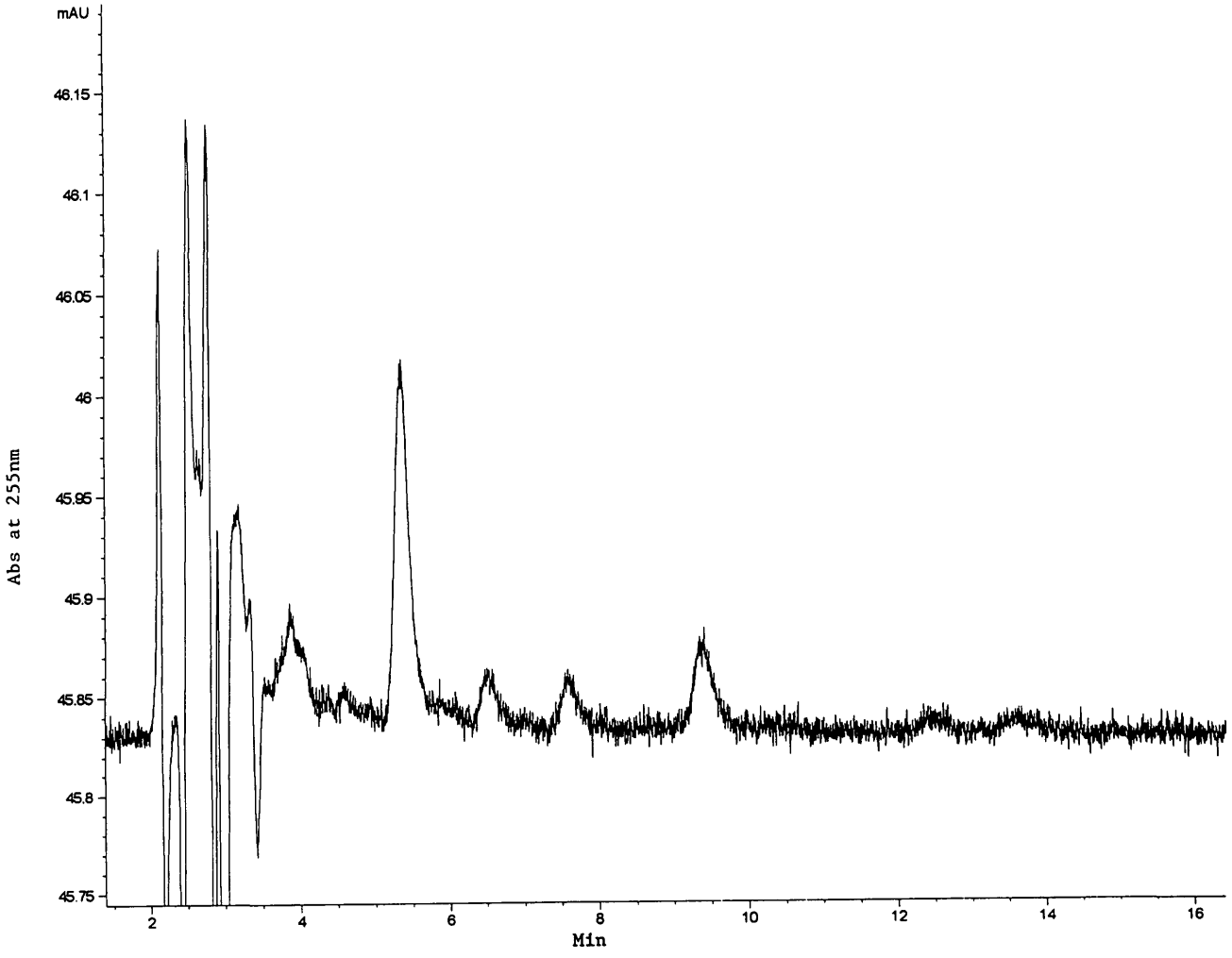

Figure 2. Chromatogram of matrix C blank (1.0 g).

bases. (Norpseudoephedrine is no longer available from Sigma and Aldrich).

(**b**) *Chemicals.*—Anhydrous sodium acetate, Mallinckrodt, AR grade (Paris, KY), Cat. No. 7372, or equivalent; acetonitrile, Baker Analyzed LC Grade, Cat. No. 9255-03 (J.T. Baker, Phillipsburg, NJ), or equivalent; methanol, J.T. Baker, LC grade, Cat. No. 9093-33, or equivalent; glacial acetic acid, Baker Analyzed, Cat. No. 9508-01, or equivalent; 99% triethylamine (TEA), Sigma (St. Louis, MO), Cat. No. T-0886, or equivalent. *Note*: If TEA is yellow, then distill.

(**c**) *Mobile phase (MP).*—Dissolve 16.4 g sodium acetate in 1.94 L LC grade water and add 16 mL acetic acid, 6.0 mL TEA, and 40 mL acetonitrile; pH should be 4.8. Filter through 0.45 μm filter and store at 4°C when not in use.

(**d**) *Diluted mobile phase (DMP).*—Dilute 100 mL MP to 500 mL with LC grade water. Store at 4°C when not in use.

(**e**) *Elution buffer (EB).*—Dissolve 16.4 g sodium acetate in 970 mL LC grade water and add 8.0 mL acetic acid, 3.0 mL TEA, and 20 mL acetonitrile; pH should be 4.8. Store at 4°C.

(**f**) *Standard solutions.*—Prepare individual stock standards of SEP, NOR, NPE, EPH, PSE, MEP, and MPE at 4.0 mg/mL each in DMP. Prepare a combination stock standard of these alkaloids at 4.0 mg/mL each in DMP. Sonication is necessary for dissolution. Prepare working standards by diluting stock standard with DMP. All standards are stable for >1 month if stored at 4°C. MEP and MPE solids are hygroscopic and must be protected from moisture.

Calibration Curve

(**a**) *Retention times.*—Prepare individual 20 μg/mL standards of each ephedrine alkaloid in DMP from the 4.0 mg/mL stock standards. Inject each into the LC system and determine retention times.

(**b**) *Calibration curve.*—Prepare 5 combination working standards in DMP with ephedrine concentrations ranging from 4.0 to 150 μg/mL. Each standard will contain all of the ephedrine alkaloids. Inject each of the 5 combination standards into the LC system and obtain retention times and peak areas. Prepare calibration curves for each ephedrine alkaloid by plotting peak area vs the alkaloid concentration (μg/mL). Correlation coefficients (r^2) should all be ≥0.999. Resolution of all adjacent ephedrine peaks should be >2; the tailing factors for all peaks should be <1.4; and the maximum retention time should be <15 min for flow rates greater than 0.7 mL/min.

Table 1. Recoveries[a] of ephedrine alkaloids from spiked matrixes

Spike, mg/g	Matrix[b]	Recovery, % (RSD, %)				
		NOR	EPH	PSE	MEP	MPE
0.48	A	98.3 (4.2)	99.7 (6.9)	101 (5.8)	91.0 (8.8)	88.6 (8.0)
2.0	A	88.3 (5.3)	102 (2.1)	89.4 (5.3)	88.5 (4.7)	87.8 (5.0)
4.0	A	82.1 (3.8)	93.5 (2.5)	90.4 (2.4)	85.7 (3.7)	82.2 (2.9)
8.0	A	97.8 (2.5)	99.3 (2.8)	88.3 (2.0)	95.0 (3.6)	91.7 (4.3)
4.0	B	90.2 (3.6)	96.8 (6.8)	94.8 (1.9)	89.8 (2.0)	87.2 (2.5)
8.0	B	85.5 (4.4)	96.4 (4.6)	98.0 (3.3)	82.6 (6.1)	90.2 (7.2)
2.0	C	84.9 (3.7)	83.1 (2.9)	84.1 (2.7)	81.4 (6.1)	71.5 (2.6)
4.0	C	88.3 (1.4)	89.1 (1.8)	87.8 (4.9)	79.3 (6.2)	72.3 (7.4)
8.0	C	81.7 (6.6)	88.3 (2.0)	89.0 (11)	80.0 (3.3)	80.0 (7.2)
16	C	94.6 (4.7)	93.7 (3.0)	96.1 (2.7)	88.7 (1.0)	83.4 (2.6)
2.0	C[c]	88.7 (5.4)	95.5 (3.7)	98.6 (7.5)	86.4 (4.0)	79.9 (5.5)
4.0	C[c]	91.4 (5.8)	99.6 (4.7)	91.7 (7.1)	90.0 (7.0)	82.0 (3.2)
8.0	C[c]	97.1 (5.8)	102 (3.2)	94.0 (2.4)	94.7 (4.6)	89.3 (5.0)

[a] Results are based on analyses of 5 separate samples.
[b] Matrix consisting of green tea, kola nut, and black tea at ratios of 2:1:0 (A), 1:2:0 (B), and 1:1:1 (C).
[c] Samples were analyzed in laboratory 2 by a different analyst using different sets of reagents and a different LC system.

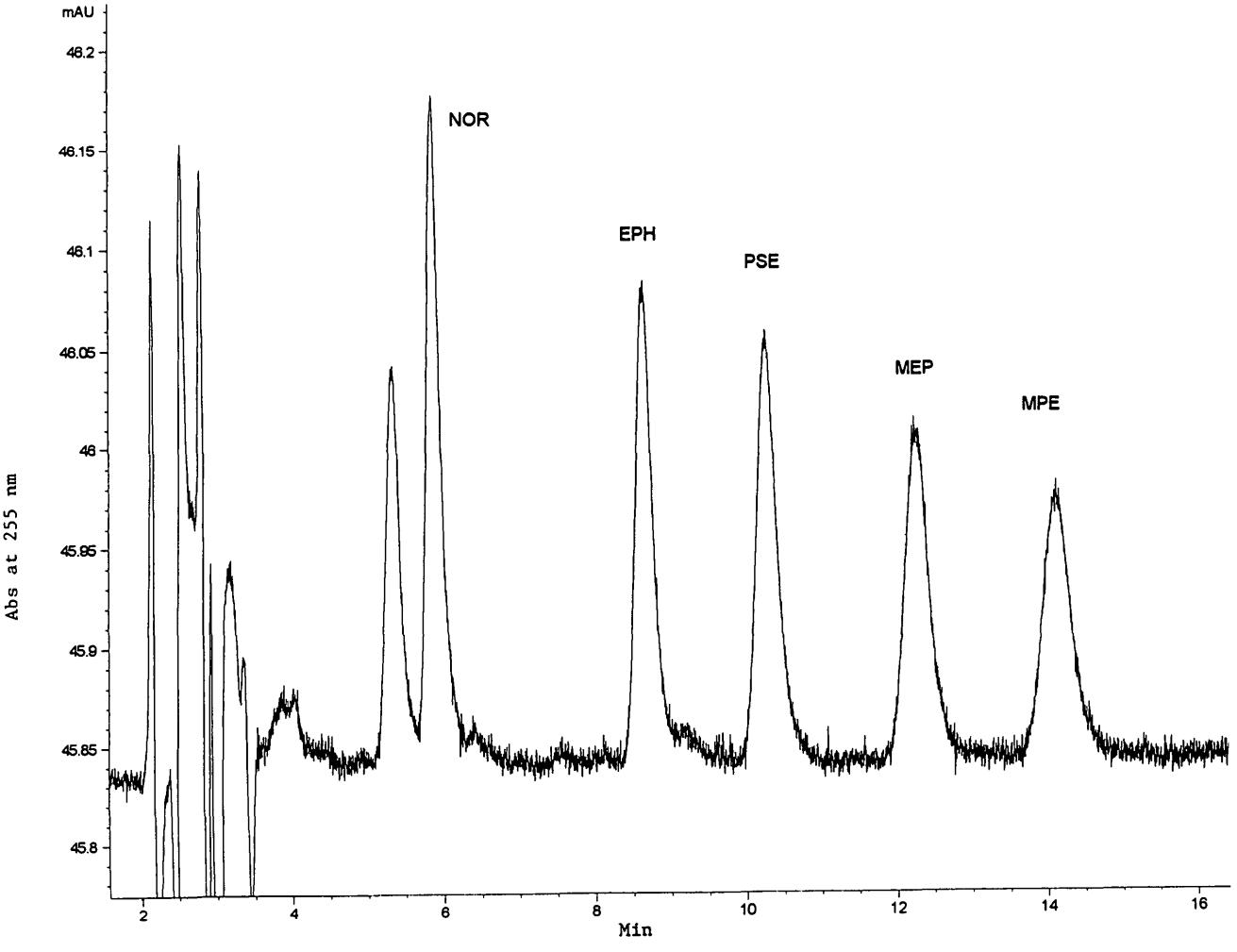

Figure 3. Representative chromatogram of matrix C (1.0 g) spiked with NOR, EPH, PSE, MEP, and MPE, each at 2.0 mg/g.

Sample Procedure

(a) *Step 1.*—Accurately weigh 0.5 g herbal product into a 50 mL Erlenmeyer flask. If desired, a wet spike of the 4.0 mg/mL combination standard can be added at this time.

(b) *Step 2.*—Add a magnetic stir bar and 25.0 mL DMP. Cover flask with Parafilm and stir for 20 min at room temperature on a magnetic stir plate.

(c) *Step 3.*—Gravity filter extract through 11 cm Whatman qualitative filter in a 60° glass funnel, and catch several milliliters of extract in a dry 125 mL Erlenmeyer flask. Use 1.00 mL filtered extract in step 5 if alkaloid content is ≥8 mg/g, and use 2.00 mL if alkaloid content is <8 mg/g.

(d) *Step 4.*—Prepare 500 mg, 6 mL PRS SPE column by washing with 2 to 5 mL each of methanol, followed by water, and followed by DMP. Discard all washes. Do not let column dry. Use vacuum to pull washes through column.

(e) *Step 5.*—Pipet either 1.00 or 2.00 mL filtered extract (step 3) onto top of prepared SPE column and allow extract to soak into column. A mild vacuum may be needed to start the flow. Do not allow column to dry.

(f) *Step 6.*—Wash column with 4.0 mL DMP followed by 5 to 6 mL methanol. With vacuum, pull air through column for 1 to 2 min. Discard all washes.

(g) *Step 7.*—Place 10 mL volumetric flask under column to collect alkaloids. Add 4.0 mL EB to column. Allow 4 mL EB to sink into column, apply vacuum, and elute at 0.5 to 1 mL/min. Pull air through for 1 to 2 min. Bring collected eluate up to 10.0 mL with water.

(h) *Step 8.*—Inject 20 μL of 10 mL extract from step 7 into LC system and obtain retention times and peak areas.

(i) *Step 9.*—Inject appropriate working standard containing ephedrine alkaloids 3 times during analysis. Obtain retention times and average peak areas for each alkaloid. Calculate concentration of each alkaloid in sample.

Results and Discussion

This rapid and reliable procedure for separating and detecting several ephedrine alkaloids in herbal matrixes resulted from ruggedness testing of an earlier procedure we developed (27). This earlier procedure involved use of 4 items that caused problems: a (1 + 1) ethyl acetate–acetone wash, phentermine as internal standard, Phenomenex phenyl column, and 0.15M sodium acetate EB. The ethyl acetate–acetone wash extracted plasticizers from the SPE column, resulting in 2–4 interfering peaks in the chromatogram. Switching to a methanol wash resulted in no detectable plasticizers and cleaner chromatograms. Use of phentermine as an internal standard lengthened and complicated the procedure because of its rapidly changing retention times when used with the Phenomenex column: Resolution of phentermine and PSE peaks varied from 1.2 to 2.1 within 20 injections. Elimination of the internal standard and use of external calibration curves resulted in a reliable and more rapid procedure. Interfering peaks from herbal products were sometimes observed with the Phenomenex column. Switching to the YMC phenyl column eliminated this problem by moving interfering peaks away from the NOR and EPH peaks. A small peak sometimes interfering with <0.5 mg/g PSE was observed. Finally, low recoveries of the late-eluting MEP and MPE alkaloids were traced to retention on the SPE column when EB was 0.15M sodium acetate. Use of 0.20M sodium acetate corrected this.

Standards containing SEP, NOR, EPH, PSE, MEP, and MPE were run through the method, and several variables were examined. Varying the DMP wash volume between 3 and 6 mL and the methanol wash volume between 4 and 8 mL had less than a 5% effect on recoveries on 5 alkaloids. However, up to 10% of early-eluting SEP was washed off with volumes of DMP > 4 mL. The volume of the 0.2M sodium acetate EB was also investigated: A plot of alkaloid recoveries versus EB volumes levels off at 3.5 mL; 4.0 mL is adequate to recover >90% of NOR, EPH, and PSE and >80% of SEP, MEP, and MPE

Table 2. Quantitation[a] of EPH and PSE in various herbal products

Herbal product[b]	Laboratory	Label claim, mg EPH/g	Recovery, mg/g		Recovery, % of label claim[c]	RSD, %
			EPH	PSE		
Product 1, finished product	1	15	14.8	0.4	98.5	4.7
Product 1, finished product	2	15	15.2	<0.5	101	5.2
Product 2, raw product	1	60	58.9	<0.4	98.2	6.3
Product 3, raw product[d]	1	60	56.9	—[e]	94.8	15
Product 3, raw product	2	60	59.1	2.8	98.5	5.6
Product 4, finished product	1	20	20.8	6.10	104	6.2
Product 5, finished product	1	25	20.9	<0.4	83.7	4.5
Product 6, finished product	1	10.6[f]	7.78	2.54	97.4	1.5
Product 6, finished product	2	10.6[f]	7.93	2.53	98.7	5.0

[a] Results are based on analyses of 5 separate samples.
[b] Finished products were on-shelf products; raw products were *ma huang* extracts.
[c] Recoveries were based on label claims for EPH concentration, except those for product 6, which were based on label claims for total ephedrine alkaloid concentration.
[d] PSE peaks were present but not integrated.
[e] —, PSE analysis was not performed due to poor baseline from a noisy deuterium lamp.
[f] Unit of label claim is mg alkaloids/g.

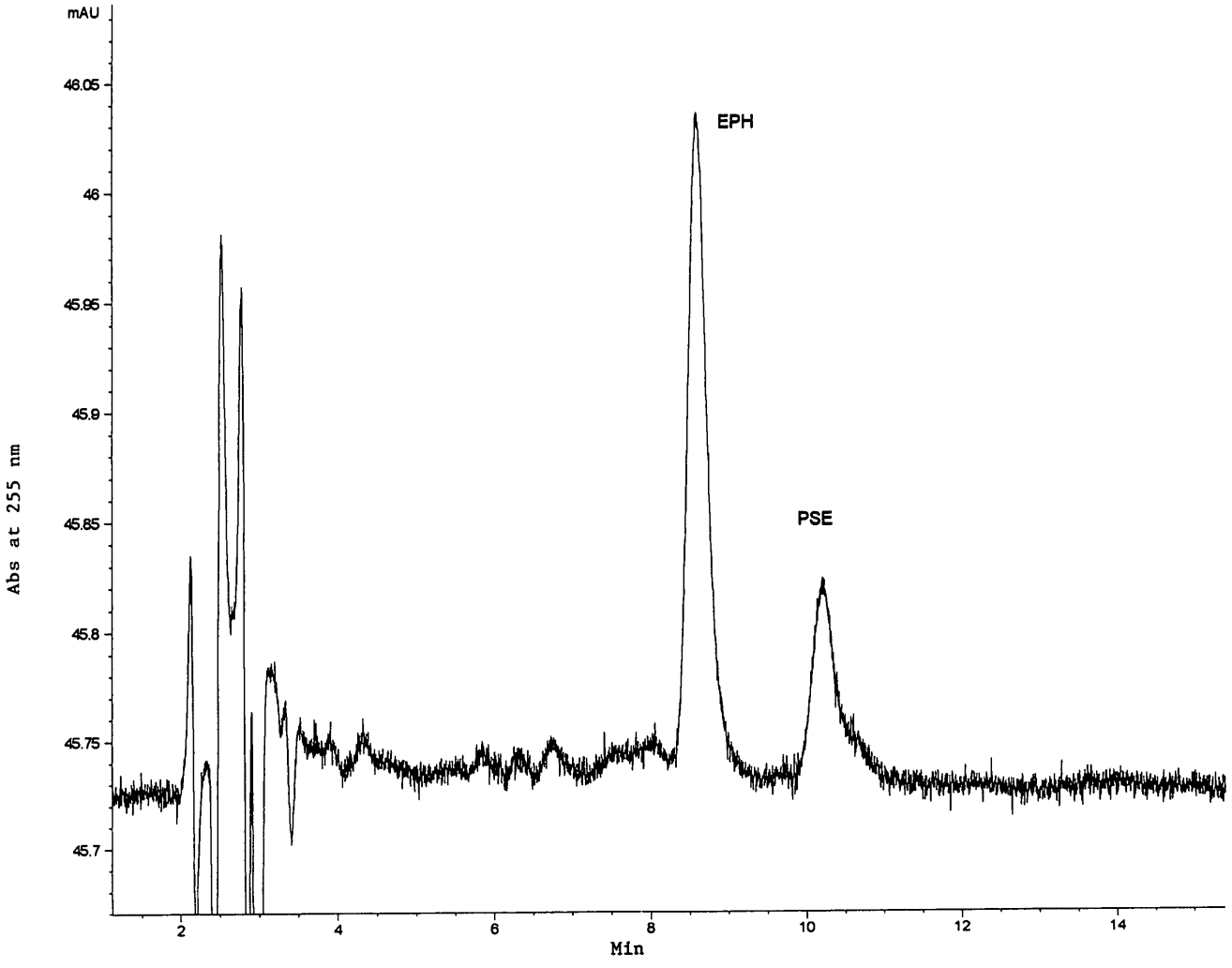

Figure 4. Chromatogram of herbal product (0.62 g) containing 8.8 mg EPH/g and 3.2 mg PSE/g.

(step 7). Flow rates through the SPE column can vary between 0.5 and 2 mL/min with no change in results. Four lots of Isolute SPE columns and 3 YMC analytical columns gave identical results. However, different brands of both PRS SPE and phenyl analytical columns require changes in the procedure in order to obtain good recoveries. Changes in column temperatures (15° to 40°C), flow rates (0.5 to 1.2 mL/min), LC pump brands (Beckman, Hewlett-Packard, and Perkin Elmer), detector brands (Beckman, Hewlett-Packard, and Perkin Elmer), and acetonitrile concentration (1.0 to 4.0%) did not significantly influence results. Retention times typically varied less than ± 0.4% during a full day of manual injections; however, variations of up to 5% were experienced from one day to another, and these changes were traced to changes in the actual flow rate from bubbles in the pump heads. Over a 6-month period, retention times and column pressure on the YMC column did not change. Freshly prepared or 3-month-old stock standards and freshly prepared or 2-week-old working standards gave similar results when stored at 4°C; no protection from light was needed. The 500 mg SPE columns could retain up to 4 mg total ephedrine alkaloid standard with less than 10% breakthrough. However, when herbal matrix was used, then maximum alkaloid load on the SPE column was 1 mg. Finally, both recoveries and precision (relative standard deviation, RSD) were within a few percent among several analysts. Both experienced analytical chemists and junior-level chemistry students quickly mastered the method with standards and actual samples. NPE was not used because it is not readily available at present.

Synephrine (SEP) can be isolated and detected with this procedure, even though it elutes close to the solvent front. It is a biologically active ephedrine-like compound (28) that is found in *Citrus aurantium*. SEP recently has been found in herbal diet products, and it is apparently used in place of ephedrine. Analysis of a commercial dietary product containing 13 mg SEP/g and advertised as "ephedrine free" gave an average recovery of 85% based on the label claim (RSD for 5 runs was 3.2%). Recoveries of spikes adding 4 mg SEP/g averaged 102%.

Calibration curves for NOR, EPH, PSE, MEP, and MPE were linear for each alkaloid at concentrations between 4 and 300 μg/mL. Peak areas produced better linear relationships than peak heights. Slopes ranged from 0.14 area units/ppm for MEP to 0.31 area units/ppm for PSE. Y intercepts ranged from 0.051 for MPE to 0.27 for EPH. Correlation coefficients (r^2) were all greater than 0.9999. A typical chromatogram of a standard containing 10–20 μg/g each of SEP, NOR, NPE, EPH, PSE, MEP, and MPE is shown in Figure 1.

Three herbal matrix blanks were used to test the new procedure. The matrixes were prepared from commercially available green tea, kola nut, and black tea because these 3 are common herbal dilutants. Matrix A was a 2 + 1 mixture of green tea and kola nut; matrix B was a 1 + 2 mixture of green tea and kola nut; and matrix C was a 1 + 1 + 1 mixture of green tea, black tea, and kola nut. Blanks containing 0.5 or 1.0 g of each of these matrixes produced no interfering peaks that caused >5% error at an ephedrine level of 2 mg/g. Matrix C gave the most complicated blank and was used for the study by the second laboratory (Figure 2). These 3 matrixes were spiked with 3–4 levels of NOR, EPH, PSE, MEP, and MPE and then analyzed. Spike levels corresponded to individual ephedrine concentrations between 0.5 and 16 mg/g. Average recovery (Table 1) based on 325 determinations of 5 alkaloids was 89.9%, and average RSD was 4.4%. Average recoveries of MEP and MPE (87.2 and 83.5%) could be increased to >90% by increasing the volume of EB from 4 to 5 mL. Average recoveries of SEP (not shown in Table 1) and NOR (85.0 and 89.9%) could be increased a few percent by decreasing the volume of DMP during SPE wash from 6 to 4 mL. Figure 3 shows a chromatogram of matrix C spiked with 5 ephedrine alkaloids each at 2 mg/g.

Six commercial products containing ephedrine alkaloids and with quantitative label claims were analyzed. These products contained various amounts of EPH and PSE along with caffeine, *ma huang* extract, green tea, black tea, St. John's wort extract, guaifenesin, and kola nut. Average recovery based on label claims and on 45 runs of 6 commercial products was 97.2% of label claim, and average RSD was 6.0% (Table 2). Figure 4 shows a chromatogram of a typical herbal dietary product with a label claim of 10.6 mg total ephedrine alkaloids (EPH and PSE)/g. Four herbal products were also spiked with an additional 2 mg of each of the ephedrine alkaloids per 0.5 g and run through the procedure in triplicate. Recoveries of spiked SEP, NOR, EPH, PSE, MEP, and MPE varied from 76 to 112%; average recovery was 95%.

A second analyst in a second laboratory analyzed the experimental matrixes and herbal products, using different sets of standards, different reagents, and different equipment. Average recovery of 2, 4, and 8 mg/g spikes of matrix C was 92.1%, and average RSD was 5.0%. By contrast, laboratory 1 obtained an average recovery of 85%, with an RSD of 4.4%. Average recovery from 3 commercial herbal products by laboratory 2 was 99.4% of label declaration, with an average RSD of 5.3%. By contrast, analysis by laboratory 1 gave an average of 96.9% of label declaration, with an RSD of 7.0% (Table 2).

The procedure is rapid, is consistent from laboratory to laboratory, produces good recoveries, yields acceptable RSD values, and produces very little hazardous waste. Recoveries from spiked herbal matrixes averaged 90%, and analysis of commercial herbal products yielded results that were 97% of label claims. Recoveries of SEP, NOR, EPH, PSE, MEP, and MPE spiked in commercial products averaged 95%. Two laboratories and 3 analysts obtained recoveries and RSD values that were within a few percent of each other. Few modifications are required for samples containing 2–20 mg ephedrine alkaloids/g, alkaloid concentrations as low as 0.5 mg/g can be detected, and analysis time is short: A complete analysis of 5 samples can be performed in less than 5 h. We are continuing this work by developing a GC/MS confirmation based on double derivatization of the ephedrine alkaloids, as suggested by Clouette (17).

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