DIETARY SUPPLEMENTS

Determination of Ephedrine Alkaloids in Dietary Supplements and Botanicals by Liquid Chromatography/Tandem Mass Spectrometry: Collaborative Study

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An interlaboratory study was conducted to evaluate the accuracy and precision of a method for ephedrine-type alkaloids [i.e., norephedrine (NE), norpseudoephedrine (NPE), ephedrine (E), pseudoephedrine (PE), methylephedrine (ME), and methylpseudoephedrine (MPE)] in dietary supplements and botanicals. The amount of ephedrine-type alkaloids present was determined using liquid chromatography with tandem mass selective detection. The samples were diluted to reflect a concentration of 0.0200 to 1.00 μg/mL for each alkaloid. An internal standard was added and the alkaloids were separated using a 5 μm phenyl LC column with an ammonium acetate, glacial acetic acid, acetonitrile, and water mobile phase. Eight blind duplicates of dietary supplements or botanicals were analyzed by 10 collaborators. Included was a negative control, *ephedra nevadensis*, and negative controls fortified at 2 different levels with each of the 6 ephedrine-type alkaloids. The spike levels were approximately 100 and 1000 μg/g for NE, 100 and 600 μg/g for NPE, 6500 and 65 000 μg/g for E, 1000 and 10 000 μg/g for PE, 300 and 3000 μg/g for ME, and 100 and 1000 μg/g for MPE. On the basis of the accuracy and precision results for this interlaboratory study, it is recommended that this method be adopted Official First Action for the determination of 6 different individual ephedrine-type alkaloids in dietary supplements and botanicals.

Methodology has been developed and validated for quantification of ephedrine-type alkaloids [i.e., norephedrine (NE), norpseudoephedrine (NPE), ephedrine (E), pseudoephedrine (PE), methylephedrine (ME), and methylpseudoephedrine (MPE)] in dietary supplements and botanicals for use in regulatory compliance and quality control (1). The methodology was based upon a published method originally developed by the U.S. Food and Drug Administration (FDA; 2). These alkaloids are the major active constituents found in *Ephedra*, a genus of shrubs that includes *Ephedra sinica, E. intermedia, E. distachya,* and others. In commerce, the Chinese term ma huang is used in association with ephedra-containing products. Ephedra has been associated with a significant number of adverse health effects. The accurate determination of ephedrine-type alkaloids in dietary supplements and botanicals will facilitate the determination of the amount of ephedrine alkaloids present in individuals reporting such events. An interlaboratory study was designed to evaluate the method's accuracy as well as intra- and interlaboratory performance.

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The recommendation was not approved by the Methods Committee on Dietary Supplements as First Action.
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Interlaboratory Study

Study Design

This study was conducted on 8 materials as blind duplicates. Two of the materials contained known concentrations of 6 fortified ephedrine alkaloids at 2 different levels. One blind duplicate was a negative control, *ephedra nevadensis*. In addition, collaborators were supplied with sufficient quantities of the 6 standards and the internal standard (IS). Random identification numbers were assigned to each of the 8 blind duplicate test samples for each material.

Collaborators

Thirteen laboratories agreed to participate in this study and received interlaboratory study materials. Two declined to participate due to instrumentation unavailability. One additional laboratory was unable to complete this study possibly due to instrument performance. Of the remaining 10, 4 were from the United States, 2 from Canada, 2 from Asia, and 2 from Europe.

Test Sample Preparation

Source of materials.—Test materials, used in this study, were obtained from commercial sources and provided by AOAC INTERNATIONAL. The IS was supplied by the FDA.

Table 1. Validation data results summary

Sample identification	Level found, μg/g	Results, μg/g NE	NPE	E	PE	ME	MPE
Endurance granulation raw material	32400	553	515	23700	5720	1830	100
Ephedra sinica stapf (ephdracae)	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.

Preparation and shipment.—Individual test samples, calibration standards, and IS were provided to each collaborator. Samples were shipped at ambient temperature and the standards were shipped frozen on dry ice with a return receipt document. Collaborators were directed to store samples at ambient temperature and standards frozen. After the study was started, standards were stored at room temperature and the prepared reagents and calibration solutions were stored at refrigerated temperatures of 2–8°C.

The botanical raw material, *ephedra sinica*, and the negative control were ground. Portions of the negative control were spiked directly at low and high levels with the standards and mixed prior to shipment. With the exception of the botanical raw material and the negative control, all test samples remained in the condition received from the suppliers. All of the samples, except for the dietary supplement capsules due to a supply shortage and botanical raw material, were tested either during validation or after preparation by liquid chromatography/tandem mass spectrometry (LC/MS/MS) before they were sent to the collaborators. The laboratories located in the United States received the NPE standard in the amount listed in the method (5 mg); however, all international collaborators received 0.1 mg/mL of this standard. Using the method supplied, the collaborators optimized instrumentation, prepared calibration solutions, weighed and extracted a portion of the test sample contained in each container, analyzed samples, and calculated results. Additional sample sets were prepared and retained at ambient temperature for the duration of the study in case of breakage or loss.

Practice samples.—A set of high- and low-level ephedrine alkaloids and a blank sample were provided. These practice samples were used to optimize instruments and chromatography before proceeding with the study. A Study Director and a technical advisor were available for consultation.

Validated Method Performance (3)

Concentration range.—The calibration curves ranged from approximately 0.0200 to 1.00 μg/mL, which corresponds to approximately <0.500 to 77 100 μg/g individual alkaloids in the various matrixes.

Validation data.—Validation data, presented in Table 1, showed this method to be effective for dietary supplements and botanicals. The calibration curves for all 6 analytes had correlation coefficients (r) >0.998. The back-calculated values for each calibration curve were within ±15% of theoretical. Three replicates of each matrix were analyzed. The overall precision for each of the 7 matrixes is summarized in Table 2. Two negative control samples ("Red Rose" black tea and instant chocolate, powder-control) were fortified at 3 levels. The relative standard deviation (RSD) for each fortification level was <10% for all analytes for both matrixes. The data, ranging from 0.767 to 3.38% and presented in Table 2, represent the overall precision for fortifications in triplicate at 3 different levels. Overall recoveries were within the target range of 70 to 120%, except PE and ME which were 121% (Table 3).

METHOD

(Method is applicable to the analysis of the ephedrine-type alkaloids NE, NPE, E, PE, ME, and MPE in dietary supplements, raw ephedra herb, ephedra extracts, ephedra capsules, and high-protein drink mix.)

Caution: See Appendix B for laboratory safety. Reference standards may be toxic.

Principle

The ephedrine-type alkaloids are extracted from dietary supplements with methanol–water (80 + 20). The amount of ephedrine-type alkaloids present in dietary supplements is determined by LC/MS/MS.

Table 2. Validation data matrix precision summary

	RSD, %					
Sample identification	NE	NPE	E	PE	ME	MPE
Endurance granulation raw material	7.72	7.84	7.05	6.68	6.61	7.20
Ephedra sinica stapf (ephdracae)	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.

Apparatus

(**a**) *Balances.*—Analytical (readability, 0.0001 g) and top-loading (0.01 g).

(**b**) *Centrifuge tube.*—15 and 50 mL polypropylene with screw-on caps (Becton Dickinson Labware, Franklin Lakes, NJ).

(**c**) *Sonicator.*—Model 5210R-MTH (Branson Ultrasonic Corp., Danbury, CT).

(**d**) *Vortex mixer.*—Cat. No. 099APV6 (Glas-Col, Terre Haute, IN).

(**e**) *Centrifuge.*—IEC Model K, capable of about 3000 rpm (Damon/IEC Division, Needham Heights, MA).

(**f**) *Solid-phase extraction (SPE) columns.*—Isolute, propylsulfonic acid (PRS), 6 mL, 500 mg, P/N 540-0050-C (single source item), International Sorbent Technology Ltd (IST; Hengoed, Mid Glamorgan, UK).

(**g**) *Column.*—YMC phenyl, 5 μm, 250 × 2 mm (Waters Corp., Milford, MA).

(**h**) *Vials.*—LC injection, Target I-D, C4001-2W (National Scientific Co., Scottsdale, AZ).

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

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

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

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

(**m**) *Data system.*—Masslynx Version 3.4 (Waters Corp.).

Note: Equivalent apparatus may be substituted. All glassware is Class A.

Reagents

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

(**b**) *Methanol.*—LC grade, Fisher Chemical.

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

(**d**) *Ammonium acetate.*—Reagent grade, 98%, Acros Organics, Fisher Scientific, (Pittsburgh, PA).

(**e**) *Water.*—Milli-Q®, purification system, Millipore Corp. (Bedford, Massachusetts).

Note: Equivalent reagents may be substituted.

Reference Standards

(**a**) *1R, 2S-(-)-Norephedrine (NE).*—99% (Aldrich Chemical Co., Milwaukee, WI).

(**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 (E).*—99% (Aldrich Chemical Co.).

(**d**) *1S, 2S-(+)-Pseudoephedrine (PE).*—98% (Aldrich Chemical Co.).

(**e**) *1R, 2S-(-)-N-methylephedrine (ME).*—99% (Aldrich Chemical Co.).

(**f**) *1S, 2S-(+)-N-methylpseudoephedrine (MPE).*—99% (Aldrich Chemical Co.).

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

Table 3. Validation data recovery summary

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

Table 4. Calibration standards[a]

Standard level	Concentration NE, NPE, E, 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

[a] *Note*: An additional level at 0.00500 μg/mL is helpful to provide "less than" data.

Note: Equivalent reference material may be substituted. Hydrochloride compounds corrected to free base.

Reference standards may be toxic.

Preparation of Reagents

(**a**) *Mobile phase (MP).*—Add 3.80 g ammonium acetate, 30 mL acetonitrile, and 20 mL glacial acetic acid to 1000 mL volumetric flask containing approximately 500 mL water. Mix and dilute to mark with water. Prepare fresh at least monthly.

(**b**) *Dilute mobile phase (DMP).*—Dilute 50 mL MP to 1 L with water. Prepare fresh at least monthly.

(**c**) *Extraction solvent (ES).*—Place 200 mL water in 1000 mL graduated cylinder. Dilute to the mark with methanol. Prepare fresh at least monthly.

(**d**) *Elution buffer (EB).*—Add 1.20 g ammonium acetate, 0.5 mL glacial acetic acid, and 30 mL acetonitrile to 50 mL water in 100 mL volumetric flask. Mix and dilute to mark with water. Prepare fresh at least monthly.

(**e**) *Dilution solvent (DS).*—Dilute 30 mL acetonitrile to 1 L in volumetric flask with water. Prepare fresh at least monthly.

Preparation of Standard

(**a**) *Internal standard solution.*—Weigh approximately 5 mg ephedrine-d_5 into 10 mL volumetric flask. Dilute to mark with ES. Dilute a portion with ES to make a solution with a concentration of 10 μg/mL. This is the working internal standard (WIS). Store solutions in a refrigerator set to maintain 2–8°C when not in use. Prepare fresh at least monthly.

(**b**) *Stock standard (SS).*—Weigh approximately 50 mg NE, E, PE, ME, and MPE into separate 100 mL volumetric flasks and approximately 5 mg NPE into 10 mL volumetric flask. *Note*: ME should be first dissolved in approximately 1 mL acetone prior to dilution with DS. Dilute to the mark with DS. Store solutions in a refrigerator set to maintain 2–8°C when not in use. Prepare fresh at least monthly.

(**c**) *LC calibration standards.*—Prepare a mixed standard by appropriate dilution with EB of the 6 SSs and the WIS to the concentrations listed in Table 4. Store solutions in a refrigerator set to maintain 2–8°C when not in use. Prepare fresh at least weekly. *Note*: An additional level at 0.00500 μg/mL is helpful to provide "less than" data.

Preparation of Sample

Weigh an appropriate amount [0.1–1 g (equivalent to 0.03–8 mg ephedrine-type alkaloid)] of homogenous product into 50 mL screw-capped polypropylene centrifuge tube. Add 20.0 mL ES. (*Note*: If preparing a spiked sample, adjust ES ac-

Table 5. Instrumentation conditions

Column temperature, °C	30			
Flow rate, mL/min	0.230			
Injection volume, μL	10			
Sample introduction mode	Electrospray positive (ES+)			
Mass range	Daughter scan for specific ion, dwell = 0.25 s			
Source temperature, °C	150			
Desolvation temperature, °C	350			
Desolvation gas flow, L/h	600			
Cone gas flow, L/h	40			
Ions to be monitored	Compound	Ions[a]	Cone (V)	Collision (eV)
	NE	117, **134**, 152	10	15
	NPE	117, **134**, 152	10	15
	E	133, **148**, 166	15	20
	PE	133, **148**, 166	15	20
	ME	147, 162, **180**	22	20
	MPE	147, **162**, 180	22	20
	E-d_5	**153**	15	20

[a] Ions in bold are used for quantification, others for confirmation.

Table 6. Interlaboratory results of ephedrine alkaloids in dietary supplements

	Dietary supplements and botanical results, μg/g															
	Botanical raw material		Ephedra powdered extract		Ephedra extract capsules		Dietary supplement capsules		High-protein drink mix		Negative control		Low spike negative control		High spike negative control	
Lab	A_1	A_2	B_1	B2	C_1	C_2	D_1	D_2	E_1	E_2	F_1	F_2	G_1	G_2	H_1	H_2
	Norephedrine															
A	847	785	32.7	43.0	326	309	253	199	<8.00[a]	<8.00[a]	<8.00[a]	<8.00[a]	60.3	82.9	718	475
B	982	1040	72.9	69.4	499	488	267	285	1.16	1.24	2.32	2.12	52.4	63.2	2100	610
C	997	898	76.8	86.6	490	490	269	296	0.742	0.716	1.51	1.51	65.9	41.3	526	244
D	585	731	70.9	69.4	215	233	131	210	1.17	1.37	1.00	0.943	65.3	33.9	401	399
E	931	878	101	96.3	432	502	347	325	0.948	0.708	0.751	0.798	108	41.2	1470	607
F	1060	1090	80.6	85.2	527	498	352	238	<0.500[b]	<0.500[b]	3.11	2.30	75.2	63.2	610	728
G	823	1010	47.1	48.7	399	369	221	245	0.806	0.799	1.92	1.81	47.3	44.9	658	531
H	713	679	48.8	53.0	712	298	324	267	1.15	1.32	4.11[c]	4.22[c]	60.7	42.9	980	378
I	742	782	26.3	31.7	361	461	222	205	0.680	0.630	2.24	2.73	169	65.2	571	1010
J	1450	1470	137	132	698	693	416	391	1.26	1.31	4.12	4.06	108	135	1720	1650
	Norpseudoephedrine															
A	679	709	42.2	53.8	527	486	152	125	<8.00[a]	<8.00[a]	<8.00[a]	<8.00[a]	99.3	101	655	602
B	702	724	59.4	56.9	652	636	120	129	2.08	2.32	<0.500	<0.500	82.8	77.2	200	255
C	887	944	83.6	94.8	806	807	145	166	1.98	1.86	<0.200[d]	<0.200[d]	58.8	95.0	289	595
D	985	1120	143	163	1010	931	234	260	3.48	3.93	<0.500	<0.500	97.1	433	675	1480
E	844	860	106	101	718	832	190	185	2.14	1.76	<0.500	<0.500	88.3	74.1	3300	392
F	902	868	95.4	90.6	802	788	174	108	<0.500[b]	<0.500[b]	<0.500	<0.500	100	80.4	271	328
G	794	823	51.8	52.5	662	620	124	144	1.97	1.78	<0.500	<0.500	70.4	139	1630	786
H	609	754	50.4	51.1	936	469	240	145	2.86	3.78	<0.100[d]	<0.100[d]	141	104	360	385
I	727	677	39.4	38.8	654	750	147	154	1.90	1.73	<0.500	<0.500	101	113	325	1550
J	1370	1420	147	126	1250	1260	253	233	3.35	3.23	<0.500	<0.500	166	135	429	452
	Ephedrine															
A	4970	6160	70200	58800	6600	6900	24100	21100	153	127	10.4	0.390[d]	5090	5990	68700	64200
B	5480	5910	54000	64700	7710	6760	19800	21200	170	172	<0.500	2.20	5470	6950	65800	55500
C	6150	5460	66100	62900	7930	8210	27200	24700	540	329	<0.200[d]	<0.200[d]	5400	6040	64500	60800
D	7940	7450	76500	82000	10100	9840	23500	24600	227	260	0.820	1.32	7100	6970	72000	71600
E	6820	6800	66500	67200	8570	9520	22100	21900	225	184	<0.500	<0.500	6490	6560	67400	67700
F	7010	7080	50800	60500	7250	8530	20700	17600	146	146	<0.500	<0.500	5510	5530	55200	49700
G	6500	6950	62400	46600	8500	8470	21100	21200	185	160	<0.500	<0.500	6180	5830	60000	47400
H	5810	7530	85800	61900	9120	7530	26000	23100	448[c]	331[c]	<0.100[d]	<0.100[d]	7390	5380	69100	78400
I	4460	5140	>29000[b]	>29000[b]	8030	7950	24700	23100	148	140	<0.500	<0.500	9820	14200	>29000[b]	>29000[b]
J	6820	7130	82700	80700	10000	9920	25800	25000	204	192	<0.500	<0.500	7990	7670	58500	59230
	Pseudoephedrine															
A	1110	1430	12400	11800	2490	2670	2520	2190	45.6	39.0	<8.00[a]	<8.00[a]	897	1160	9270	10500
B	1160	1200	7320	9050	2480	2190	1680	1790	44.0	47.2	<0.500	<0.500	661	918	6180	5360
C	1360	1320	10700	10600	2840	2940	4170	3140	163	81.5	<0.200[d]	<0.200[d]	836	787	11100	6970
D	1630	1740	11800	12600	3640	3550	2370	2490	66.3	71.9	<0.500	<0.500	1100	986	9440	12000
E	1560	1610	10400	10700	2860	2140	2420	2380	53.2	46.2	<0.500	<0.500	1120	1010	7420	8980
F	1400	1660	7580	9040	2550	2780	1880	2140	40.9	40.2	<0.500	<0.500	5100	5140	5650	4900
G	1500	1230	8690	7060	2380	2730	2020	1980	52.4	53.9	<0.500	<0.500	792	969	9160	6260

Table 6. (*continued*)

	Dietary supplements and botanical results, μg/g															
	Botanical raw material		Ephedra powdered extract		Ephedra extract capsules		Dietary supplement capsules		High-protein drink mix		Negative control		Low spike negative control		High spike negative control	
Lab	A_1	A_2	B_1	B2	C_1	C_2	D_1	D_2	E_1	E_2	F_1	F_2	G_1	G_2	H_1	H_2
H	1230	2300	7800	8600	2810	3210	3500	2010	114[c]	81.7[c]	<0.100[d]	<0.100[d]	1400	1380	9840	12800
I	1070	950	7190	7980	1960	1910	1320	1300	36.3	27.7	<0.500	<0.500	1120	950	7050	6840
J	1850	1810	12100	11800	3710	3830	2870	2770	58.1	61.2	<0.500	<0.500	1330	1370	10600	11200
	Methylephedrine															
A	262	262	121	132	575	450	887	726	<8.00[a]	<8.00[a]	<8.00[a]	<8.00[a]	200	179	1400	4080
B	238	240	152	154	575	575	748	773	0.980	0.998	<0.500	<0.500	295	260	1750	1100
C	389	365	238	257	869	887	1040	1190	1.02	0.835	<0.200[d]	<0.200[d]	228	296	4700	3680
D	509	561	454	434	1170	1130	1120	1130	1.93	1.72	<0.500	<0.500	439	363	4640	8630
E	325	326	256	255	707	842	900	896	1.01	0.889	<0.500	<0.500	531	238	1980	2460
F	307	260	185	167	694	650	832	614	<0.500[b]	<0.500[b]	<0.500	<0.500	192	212	1200	1430
G	277	286	118	122	579	593	723	744	0.752	0.638	<0.500	<0.500	166	332	1300	1830
H	212	659[c]	152	226	740	769	1050	1070	1.33	1.60	<0.100[d]	<0.100[d]	376	307	3610	2990
I	182	173	90.5	93.2	560	579	571	572	<0.500[b]	<0.500[b]	<0.500	<0.500	218	238	1750	2930
J	1070	980	783	750	1260	1280	3120	2970	1.47	1.52	<0.500	<0.500	803	833	6530	6780
	Methylpseudoephedrine															
A	6.34[c]	13.9[c]	130	161	20.6	8.97[c]	36.0	27.4[c]	<8.00[a]	<8.00[a]	<8.00[a]	<8.00[a]	84.4	92.1	1791	1300
B	7.88	8.58	192	186	18.7	18.4	30.6	29.5	0.962	1.03	<0.500	<0.500	76.8	76.8	1120	960
C	6.53	7.70	282	296	22.6	22.3	29.7	34.2	1.05	0.862	<0.200[d]	<0.200[d]	88.6	119	1840	1840
D	32.3	21.3	504	434	42.1	41.8	63.8	65.9	1.88	2.15	<0.500	<0.500	355	145	1640	1380
E	13.0	12.0	204	306	31.1	36.3	46.8	44.8	0.995	0.884	<0.500	<0.500	108	107	1010	1080
F	<0.500[b]	<0.500[b]	198	199	<0.500[b]	<0.500[b]	<0.500[b]	<0.500[b]	<0.500[b]	<0.500[b]	<0.500	<0.500	93.3	87.9	644	765
G	8.14	9.56	135	150	21.5	20.6	27.9	30.8	0.881	0.764	<0.500	<0.500	68.1	72.9	951	957
H	10.2	15.2	164	215	38.5	41.6[c]	33.6	39.3	1.51	1.71	<0.100[d]	<0.100[d]	157	140	1050	968
I	9.29	8.14	150	150	24.1	27.0	31.0	30.2	2.54	2.44	<0.500	<0.500	138	117	914	1190
J	167	154	960	900	32.4	36.4	132	117	1.53	1.45	<0.500	<0.500	530	540	3490	3660

[a] Less than value above the limit of detection (<0.500 μg/g); data not used.
[b] Less than or greater than values; data not used.
[c] Extrapolated value; data not used.
[d] Values reported below the required limit of detection (<0.500 μg/g), used as <0.500 μg/g.

cordingly. Spike at level approximately equal to the inherent amount.) Cap the centrifuge tube, and sonicate at room temperature for at least 20 min. Mix on a Vortex mixer for at least 1 min. Centrifuge approximately 2200 × *g* (about 3000 rpm) for at least 20 min. Dilute a portion of the supernatant with ES to 10 mL. The concentration of each alkaloid should fall in the range of 0.0100 to 0.500 μg/mL. This can usually be accomplished with 2 dilutions: one for the NE, NPE, ME, and MPE, another for the E and PE. Keep in mind the 2× concentration occurring with the SPE column cleanup step below, which should bring the dilution within the standard curve range of 0.0200 to 1.00 μg/mL. Add 50 μL WIS to each of the dilutions. Prepare an SPE column by passing successive 2 mL portions of methanol, water, and DMP through the SPE column, using a flow rate of approximately 5–10 mL/min. The 10 mL of diluted test solution is then added to the SPE column followed by two 3 mL portions of DMP. The SPE column is then dried for 5 min by evacuating the reservoir apparatus. Wet the SPE column with 2 mL methanol and discard all effluent. Elute the SPE column with 4 mL EB into 15 mL tube and dilute to 5 mL with EB.

Determination

Chromatographic conditions.—Analyze standards and test samples according to instrumental conditions in Table 5.

Inject the LC calibration standards during the run. Inject at least one standard at the beginning of the run and one at the end of the run.

Table 7. Correlation coefficients (r) from interlaboratory results for ephedrine alkaloids in dietary supplements

	Correlation coefficient (r)					
Lab	NE	NPE	E	PE	ME	MPE
A	0.9911	0.9967	0.9994	0.9975	0.9992	0.9991
	0.9988	0.9997	0.9999	0.9997	0.9980	0.9990
B	0.99985	0.99963	0.99980	0.99898	0.99954	0.99994
C	0.99096	0.98788	0.99812	0.99728	0.99949	0.99953
D	0.99959	0.99989	0.99933	0.99806	0.99540	0.99874
	0.99991	0.99993	0.99797	0.99674	0.99544	0.99607
	0.99984	0.99992	0.99823	0.99587	0.99305	0.99805
	0.99990	0.99992	0.99826	0.99625	0.99585	0.99857
E	0.99991	0.99982	0.99932	0.99959	0.99988	0.99937
	0.99984	0.99981	0.99857	0.99912	0.99981	0.99961
F	0.99910	0.99935	0.99955	0.99935	0.99875	0.99955
G	0.9991	0.9992	0.9996	0.9985	0.9996	0.9996
H	0.99357	0.99950	0.99276	0.99698	0.99654	0.99946
	0.99625	0.99963	0.99949	0.99664	0.99657	0.99979
I	0.98846	0.99179	0.96558	0.95161	0.95754	0.95678
	0.98950	0.99322	0.96551	0.95091	0.95614	0.95556
	0.98391	0.99312	0.94417	0.92146	0.90215	0.92564
	0.98391	0.99312	0.94417	0.92146	0.90215	0.92564
J	0.99980	0.99979	0.99993	0.99982	0.99987	0.99973

System optimization.—Inherent variability between instruments necessitates the optimization of the cone and collision voltages, along with the collision cell gas flow for each analyte. Prior to analysis of test samples, optimize the system by infusing each analyte (dissolved in mobile phase) and acquire spectra that show sufficient fragmentation of the parent molecules. The ratio of each confirmation ion to quantification ion should be greater than 1:10. In some instances, a ratio of 1:2 can be achieved.

Quality assurance.—Fortify one test sample and run one test sample in duplicate with each analytical run consisting of approximately 20 test samples.

Calculation

Quantification.—Generate a standard calibration curve by using the ratio of the quantification ion area vs the quantification ion area of the IS for each concentration level. Prepare a calibration curve for each analyte. Weighting ($1/x$) may be necessary to obtain acceptable percent deviation at lower standard concentrations.

$$y = mx + b$$

where y = relative peak area (area of analyte/area of IS); m = slope of the line generated by a standard curve; x = concentration of analyte found (µg/mL); b = y-intercept of the line generated by the standard curve.

The amount of analyte found in test sample (µg/g) is calculated as follows:

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

where A = µg/g of ephedrine-type alkaloid found in test sample; C = concentration (µg/mL) of ephedrine-type alkaloid found in test samples from the standard curve; V_f = final volume of extracts (5 mL); D = dilution (20 mL for extraction; include any other dilutions performed); W = test portion weight (g, wet or dry weight).

Confirmation.—Divide the peak area detected in the standards for each confirmation ion by the peak area of the quantification ion and average for all standards. The sample ratio of confirmation to quantification ion should be ±10% (arithmetic difference, not relative difference) of the averaged standard ratio. For example, if an average ratio for the standards is 50%, the window for sample ratio would be 40–60% (not 45–55%).

Notes: While no interferences were observed, inject a sufficient number of injections (3–5) of test sample or standard to equilibrate the LC/MS/MS system. In the case of a high-protein drink mix, the reproducibility of replicate injections was increased with the addition of an equilibration injection of test sample just prior to actual test sample injection, and an EB blank after the high-protein drink mix test samples.

Table 8. Statistical analysis of interlaboratory results for ephedrine alkaloids in dietary supplements

Ephedra alkaloid	Added, µg/g	Average, µg/g	S_r	RSD_r, %	S_R	RSD_R, %	No. of outlier labs[a]	HORRAT	Recovery, %	No. of labs[b]
Norephedrine										
Botanical raw material	NA[c]	925	63.3	6.85	235	25.4	0	4.44	NA	10
Ephedra powdered extract	NA	70.5	4.09	5.80	31.5	44.7	0	5.30	NA	10
Ephedra extract capsules	NA	444	31.1	7.00	135	30.4	1 (H)	4.76	NA	10
Dietary supplement capsules	NA	273	37.7	13.8	71.8	26.3	0	3.82	NA	10
High-protein drink mix	NA	1.00	0.0931	9.30	0.278	27.8	0	1.74	NA	8
Negative control	0	2.08	0.245	11.8	1.08	51.9	0	3.62	NA	8
Low spike negative control	114	59.3	21.1	35.7	21.1	35.7	2 (I, J)	4.12	52.0	10
High spike negative control	1450	819	430	52.5	519	63.3	0	10.9	56.5	10
Norpseudoephedrine										
Botanical raw material	NA	812	52.0	6.41	132	16.3	1 (J)	2.78	NA	10
Ephedra powdered extract	NA	82.3	7.60	9.24	39.9	48.5	0	5.89	NA	10
Ephedra extract capsules	NA	788	42.5	5.39	220	27.9	1 (H)	4.77	NA	10
Dietary supplement capsules	NA	171	28.4	16.6	48.9	28.5	0	3.86	NA	10
High-protein drink mix	NA	2.51	0.290	11.6	0.813	32.4	0	2.33	NA	8
Negative control	0	<0.500	—[d]	—	—	—	—	—	NA	9
Low spike negative control	103	101	22.5	22.2	28.5	28.1	1 (D)	3.52	98.5	10
High spike negative control	614	626	406	64.8	460	73.6	1 (E)	12.1	102	10
Ephedrine										
Botanical raw material	NA	6380	550	8.62	956	15.0	0	3.50	NA	10
Ephedra powdered extract	NA	66700	8180	12.3	11400	17.1	0	5.68	NA	9
Ephedra extract capsules	NA	8370	558	6.66	1120	13.4	0	3.26	NA	10
Dietary supplement capsules	NA	22900	1410	6.14	2430	10.6	0	3.01	NA	10
High-protein drink mix	NA	177	16.4	9.22	37.9	21.3	1 (C)	2.91	NA	9
Negative control	0	<0.500[e]	—	—	—		3 (A, B, D)	—	NA	7
Low spike negative control	6830	6310	654	10.4	885	14.0	1 (I)	3.27	92.4	10
High spike negative control	66700	63100	4810	7.62	8200	13.0	0	4.29	94.6	9
Pseudoephedrine										
Botanical raw material	NA	1420	124	8.72	273	19.2	1 (H)	3.58	NA	10
Ephedra powdered extract	NA	9760	715	7.33	1980	20.3	0	5.05	NA	10
Ephedra extract capsules	NA	2780	224	8.05	580	20.8	0	4.30	NA	10
Dietary supplement capsules	NA	2350	418	17.8	713	30.4	0	6.11	NA	10
High-protein drink mix	NA	49.0	3.71	7.57	12.0	24.4	1 (C)	2.74	NA	9
Negative control	0	<0.500	—	—	—	—	—	—	NA	9
Low spike negative control	926	1040	112	10.7	226	21.7	1 (F)	3.86	113	10
High spike negative control	8840	8580	1520	17.8	2410	28.1	0	6.86	97.0	10
Methylephedrine										
Botanical raw material	NA	310	18.8	6.06	109	35.1	1 (J)	5.20	NA	9
Ephedra powdered extract	NA	202	8.78	4.35	114	56.6	2 (H, J)	7.87	NA	10
Ephedra extract capsules	NA	774	44.5	5.74	259	33.4	0	5.69	NA	10
Dietary supplement capsules	NA	866	73.6	8.50	203	23.4	1 (J)	4.05	NA	10
High-protein drink mix	NA	1.19	0.114	9.55	0.413	34.6	0	2.22	NA	7
Negative control	0	<0.500	—	—	—	—	—	—	NA	9
Low spike negative control	316	282	85.3	30.3	97.9	34.8	1 (J)	5.08	89.1	10

Table 8. (*continued*)

Ephedra alkaloid	Added, µg/g	Average, µg/g	S_r	RSD_r, %	S_R	RSD_R, %	No. of outlier labs[a]	HORRAT	Recovery, %	No. of labs[b]
High spike negative control	4990	3240	1160	35.9	2160	66.8	0	14.1	64.9	10
					Methylpseudoephedrine					
Botanical raw material	NA	12.1	3.30	27.2	7.20	59.4	1 (J)	5.40	NA	8
Ephedra powdered extract	NA	195	30.0	15.4	57.2	29.4	2 (D, J)	4.06	NA	10
Ephedra extract capsules	NA	28.2	1.94	6.86	8.69	30.8	0	3.18	NA	7
Dietary supplement capsules	NA	38.4	2.26	5.88	13.1	34.1	1 (J)	3.69	NA	8
High-protein drink mix	NA	1.41	0.111	7.81	0.605	42.8	0	2.82	NA	8
Negative control	0	<0.500	—	—	—	—	—	—	NA	9
Low spike negative control	95.4	102	10.5	10.3	27.2	26.8	2 (D, J)	3.35	107	10
High spike negative control	1360	1190	156	13.1	377	31.7	1 (J)	5.75	87.4	10

[a] Laboratories identified A–J.

[b] Includes number of laboratories used before outliers removed.

[c] NA = Not applicable.

[d] — = Statistical parameters not calculated; levels were below limits of detection.

[e] For calculation, <0.500 µg/g values were used as 0.500 µg/g.

Furthermore, a decrease of retention times for all analytes occurred after many injections and over time. While no adverse separation problems were observed, the retention time of the last analyte (MPE) may decrease from 22.7 to 18.4 min over the course of 4–5 months of system and column use.

Refs.: *J. AOAC Int.* **84**, 761–769(2001); **86**, 471–475(2003)

CAS 492-41-1 (1R,2S-(-)-Norephedrine)
CAS 492-39-7 ((+)-Norpseudoephedrine hydrochloride)
CAS 50-98-6 (1R, 2S-(-)-Ephedrine hydrochloride)
CAS 552-79-4 (1R, 2S-(-)-N-methylephedrine)
CAS 51018-28-1 (1S, 2S-(+)-N-methylpseudoephedrine)
CAS 90-82-4 (1S, 2S-(+)-Pseudoephedrine)

Results and Discussion

Interlaboratory Study Results

Ten collaborators participated in the study. The complete set of data submitted for dietary supplement are presented in Table 6. The table is subdivided, presenting individual results for NE, NPE, E, PE, ME, and MPE. The data are shown as individual pairs of results for each laboratory (A–J).

Prior to sending study materials, sample identifications were coded and randomized to ensure the samples were analyzed in a random manner. When the summary results were received, the sample identifications were decoded and the names of the participating laboratories were coded for presentation in the tables. Individual values of each of the 6 ephedrine-type alkaloids were reported for each test sample (i.e., 8 test samples × 2 blind duplicates × 6 analytes) for a total of 96 data points from each laboratory. Two of the blind duplicate test samples were negative controls. Collaborators supplied the correlation coefficient (r) for the calibration curves generated. These data are presented in Table 7.

The dietary supplement results, found in Table 6, were used to generate the statistics found in Table 8. For Collaborator A, the duplicate results for botanical raw material, ephedra extract capsules, and dietary supplement capsules were not used for statistical purposes for MPE because either one or both values were extrapolated values. In addition, the duplicate results for high-protein drink mix for NE, NPE, ME, and MPE were not used from this collaborator due to the fact that they were less than values above the required limit of detection (LOD). Also, all of the duplicate results for the negative control were not used because they were either less than values above the required LOD or values reported below the LOD. For Laboratory C, 5 of the 6 negative control duplicate values were reported below the required LOD and were used as <0.500 for statistical analysis. For Collaborator F, 4 of the 6 high-protein drink mix duplicates were reported as less than the LOD and were not used. Also, the botanical raw material, the ephedra extract capsule, and the dietary supplement capsule duplicate values were reported as less than the LOD and were not used. For Laboratory H, 5 of the 6 duplicates for the negative control were reported as values below the required LOD; however, they were used as <0.500 for statistical analysis. This laboratory also reported extrapolated values for the high-protein drink mix duplicates for E and PE, one of the botanical raw material duplicates for ME, and one of the ephedra extract capsules duplicates for MPE. For Collaborator I, the duplicate ephedrine results for both ephedra powdered extract and the high spike negative control were reported as a greater than value which were not used for statistical purposes. Also, this laboratory reported ME duplicates for high-protein drink mix as less than the LOD and were not used.

The data from the individual alkaloids were compiled to give total alkaloid summaries for dietary supplements in Ta-

Table 9. Interlaboratory results of total ephedrine alkaloids in dietary supplements

	Dietary supplements and botanical results, μg/g															
	Botanical raw material		Ephedra powdered extracts		Ephedra extract capsules		Dietary supplement capsules		High-protein drink mix		Negative control		Low spike negative control		High spike negative control	
Lab ID	A_1	A_2	B_1	B_2	C_1	C_2	D_1	D_2	E_1	E_2	F_1	F_2	G_1	G_2	H_1	H_2
A	—[a]	—	82900	71000	—	—	—	—	—	—	—	—	6430	7600	82500	81200
B	8570	9120	61880	74200	11900	10700	22600	24200	219	225	—	—	6640	8350	77200	63800
C	9790	8990	77500	74200	13000	13400	32900	29500	—	—	—	—	6680	7380	83000	74100
D	11700	11600	—	—	16200	15700	27400	28800	302	341	—	—	—	—	88800	95500
E	10500	10500	77600	78700	13300	13900	26000	25700	283	234	—	—	8450	8030	—	—
F	—	—	58900	70100	—	—	—	—	—	—	—	—	—	—	63600	57900
G	9900	10300	71400	54000	12500	12800	24200	24300	242	218	—	—	7320	7390	73700	57800
H	—	—	—	—	—	—	31100	26600	—	—	—	—	9520	7350	84900	95900
I	7190	7730	—	—	11600	11700	27000	25400	—	—	—	—	—	—	—	—
J	—	—	—	—	17000	17000	—	—	270	261	—	—	—	—	—	—

[a] — = Not applicable, laboratories with previously identified outliers, data not used, or less than values are not included.

ble 9. In generating the total alkaloid summaries, the data was compiled if a laboratory had complete duplicate results for each alkaloid. For any collaborator that did not have acceptable data for an alkaloid or was determined to be an outlier, no total summaries were made. Statistical analysis was performed on the total data and is shown in Table 10.

Precision statistical analysis was performed using the AOAC Interlaboratory Statistical Program 2001 for Blind Replicates (3). Accuracy was evaluated through determining percent spike recovery, by dividing the average observed amount of each analyte by the fortified amount and multiplying by 100. Tables 8 and 10 describe the analyte, average analyte concentration, standard deviations for repeatability (S_r) and reproducibility (S_R), relative standard deviations for repeatability (RSD_r) and reproducibility (RSD_R), number of statistical outlier laboratories, HORRAT value (RSD_R/predicted RSD_R), and percent recovery after removal of outliers. Cochran's and Grubbs' tests, as part of the statistical package, were used to remove outliers. The Horwitz predicted value in the statistics package was calculated from the simplified Horwitz equation $RSD_R = 2C^{-0.15}$ where C is the measured concentration of the analyte expressed as a decimal mass fraction (e.g., 1 g/100 g = 0.01; 4).

Collaborators' Comments

Laboratory E suggested to make up the stock solutions with elution buffer rather than dilution solvent. Concerns over stability of stock solutions in elution buffer prevent this suggested change. The recommended storage time of the mixed standard in elution buffer is now decreased from 1 month to 1 week maximum in the method. Laboratory E also reported that some ion ratios did not meet the ±10% specification for confirmation for some samples for NE, NPE, and once for ME. Because this method was to be used for confirmation as well as quantification, it would be necessary to overcome this difficulty through re-injection or injection of a more concentrated solution. This same participant also reported that automated processing of data was not possible for some ion to ion transitions (e.g., 152 to 152). The solution is to offset the transition slightly (e.g., 152.00 to 152.01). This same participant found the preparation of samples for analysis to be much more time-consuming than the protocol suggested. Many participants verbally indicated the same experience and the Study Director concurs. This participant also pointed out that the IS used in the mixed standard was not treated the same as in the samples. The mixed standard, which included the IS, was not passed through an SPE cartridge. Although this aspect was not investigated prior to the start of the study, this part of the method was developed to reflect the same procedure as the original FDA method. Laboratory D found that their ratios between confirmation ions and quantification ions were very good. They indicated that the optimum collision voltage for each daughter ion should be an important part of instrument optimization rather than using a universal collision energy. The Study Director concurs and the method states that optimization is necessary. This may be an additional solution to Laboratory E's difficulty. One laboratory, having agreed to participate, was not able to complete this study, even after an extensive amount of trouble shooting. A speculative conclusion was that the elution buffer caused a large sensitivity decrease in instrument performance. No other laboratories experienced this difficulty with the method.

Performance Characteristics of Method

Recoveries for dietary supplements ranged from 52.0 to 113% for the individual alkaloid fortified samples and 90.6 to

Table 10. Statistical analysis of interlaboratory results for total ephedrine alkaloids in dietary supplements

Ephedra alkaloid	Added, µg/g	Average, µg/g	S_r	RSD_r, %	S_R	RSD_R, %	No. of outlier labs[a]	HORRAT	Recovery, %	No. of labs[b]
				Total						
Botanical raw material	NA[c]	9660	342	3.54	1460	15.2	0	3.77	NA	6
Ephedra powdered extract	NA	71000	7830	11.0	8760	12.3	0	4.14	NA	6
Ephedra extract capsules	NA	13600	406	2.98	2150	15.8	0	4.13	NA	7
Dietary supplement capsules	NA	26800	1670	6.22	2970	11.1	0	3.21	NA	7
High-protein drink mix	NA	260	21.5	8.28	42.1	16.2	0	2.34	NA	5
Negative control	0	—[d]	—	—	—	—	—	—	NA	NA
Low spike negative control	8380	7600	898	11.8	898	11.8	0	2.84	90.6	6
High spike negative control	84000	77100	7130	9.24	13000	16.9	0	5.75	91.8	7

[a] Laboratories identified A–J.

[b] Includes number of laboratories used before outliers removed.

[c] NA = Not applicable.

[d] — = Statistical parameters not calculated; levels were below limits of detection.

91.8% for total alkaloid fortified samples. For the analytical range covered, these recoveries are near the recommended guidelines for recovery of about 75–110% (5) except for NE, the low spike PE, and the high spike ME. The low recoveries of NE and high spike MPE from the negative control are a concern and may need further investigation. This is an implication of possible lack of method scope and applicability for analysis of raw ephedra herb. Based on results, the RSD_r for dietary supplements ranged from 4.35 to 64.8% for each individual alkaloid. The overall RSD_r for individual alkaloids shows good repeatability. The RSD_R ranged from 10.6 to 73.6% for each individual alkaloid. The overall RSD_R for individual alkaloids shows questionable reproducibility. However, the individual alkaloids E and PE show better repeatability with RSD_r values ranging from 6.14 to 12.3% and 7.33 to 17.8%, respectively. Also, these 2 alkaloids show better reproducibility with RSD_R ranging from 10.6 to 21.3% and 19.2 to 30.4%, respectively. These observations may indicate that the method is better suited for the determination of the high level E and PE alkaloids as compared to the lower level minor alkaloids.

The RSD_r for dietary supplements ranged from 2.98 to 11.8% for total ephedrine-type alkaloids. The RSD_R ranged from 11.1 to 16.9% for total ephedrine-type alkaloids. The overall RSD_r and RSD_R for total ephedrine-type alkaloids show good precision. The total ephedrine-type alkaloid statistics show good RSD_r, RSD_R, and recovery; but the HORRAT values appear to be inadequate. Acceptable HORRAT values range from 0.5 to 2 (5). The method did not show acceptable precision for dietary supplements, according to HORRAT values that ranged from 1.74 to 14.1, for each individual alkaloid and from 2.34 to 5.75 for total ephedrine-type alkaloids. In addition, the dynamic range of each alkaloid in various matrixes is extreme (i.e., approximately 4 orders of magnitude amongst most samples tested.) From these observations, HORRAT values may not be applicable to the method or instrumentation used in this study.

Seventy of the 114 reported correlation coefficients met or exceeded the target validation value of 0.998. Those not meeting this value ranged from 0.90215 to 0.99728 with the majority of them reported from Laboratory I.

Study Clarifications

For this interlaboratory study, the IS, ephedrine-d_5, was supplied by the FDA. In order to obtain the specified amount of the IS, as listed in the method, contractual agreements will have to be arranged prior to future use. The NPE standard is a regulated substance and the international laboratories received 0.1 mg/mL solution instead of 5 mg due to shipping regulations. All outliers for NPE were from international laboratories, which may indicate a lack of ruggedness in the NPE standard preparation.

Due to the interlaboratory study design, samples were not supplied to enable quality assurance checks as specified by the method; however, system optimization was accomplished with the use of practice samples and calibration standards interspersed throughout the analytical run.

Recommendations

On the basis of the accuracy and precision results for this interlaboratory study, it is recommended that this method be adopted Official First Action for the determination of 6 different individual ephedrine-type alkaloids in dietary supplements and botanicals. For practical use of this method, a supplier of the IS needs to be identified and a consistent and adequate supply of NPE needs to be available for shipment worldwide. Further study and method modification may be needed to improve interlaboratory precision and HORRAT values.

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