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Quantitative Analysis and Enantiomeric Separation of Ephedra Alkaloids in Ma Huang Related Products by HPLC-DAD and UPLC-MS/MS

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Abstract – Ephedra is a genus of the Ephedraceae family and is found in temperate regions, such as Central Asia and Europe. Among the various ephedra species, Ma Huang (*Ephedra* herb) is derived from the aerial parts of *Ephedra sinica* Stapf, *Ephedra equisetina* Bunge, and *Ephedra intermedia* Schrenk & C.A. Mey. Ma Huang contains various ephedra alkaloids, including (−)-ephedrine, (+)-pseudoephedrine, (−)-norephedrine, (+)-norpseudoephedrine, (−)-methylephedrine, and (+)-methylpseudoephedrine, which are found naturally as single enantiomers, although they can be prepared as racemates. Although the use of Ma Huang in foods is prohibited in Korea, products containing Ma Huang can be imported, and so it is necessary to develop a suitable analytical technique for the detection of Ma Huang in foods. Herein, we report the development of analytical methods for the detection of ephedra alkaloids in products containing Ma Huang. Following sample purification by solid phase extraction, quantitative analysis was performed using ultra-performance liquid chromatography-triple quadrupole mass spectrometry (UPLC-MS/MS). Additionally, the enantiomers were successfully separated using HPLC-DAD. We successfully analyzed various food samples, where the ephedra alkaloids were qualitatively and quantitatively determined, and the enantiomers were separated. It is expected that these methods may contribute toward preventing the distribution of illegal products containing Ma Huang.
Keywords – Ma Huang, ephedra alkaloids, UPLC-MS/MS, HPLC-DAD, enantiomeric separation

Introduction

Ephedra, the only genus in the family Ephedraceae, is an evergreen plant with small scaly leaves. Over 60 ephedra species are known, and these are distributed in the temperate regions of Central Asia, North and Central America, Europe, and North Africa. Among the various Ephedra species reported to date, *Ephedra sinica* Stapf, *Ephedra equisetina* Bunge, *Ephedra intermedia* Schrenk & C.A. Mey are the primary species[1]. Ma Huang is derived from the aerial parts of these three species, and is specified in the Korean and European Pharmacopoeia.

Ma Huang has been traditionally used to treat a range of health problems, including coughs, fever, colds, and asthma. It is commonly prescribed in the form of a decoction, either alone or in combination with other herbal medicines[2]. The biological activity of Ma Huang can be attributed to its high contents of ephedra alkaloids, namely (−)-ephedrine, (+)-pseudoephedrine, (−)-norephedrine, (+)-norpseudoephedrine, (−)-methylephedrine, and (+)-methylpseudoephedrine (Fig. 1).[3,4] Among these alkaloids, ephedrine and pseudoephedrine account for approximately 70–99% of the total alkaloid content in ephedra herbs; however, the alkaloid content varies depending on the species, geographical origin, growth conditions, and harvesting conditions.[1] Each of these alkaloids naturally occurs in only one of its two possible enantiomeric forms, although they may be chemically synthesized in the form of racemates. Therefore, the ability to separate the different enantiomers by means of chiral separation can facilitate the determination of the alkaloid source. In addition, chiral separation is important because the different enantiomers may exhibit different pharmacological activities.[5]

Commercial dietary supplements and preparations (i.e., pharmaceuticals or herbal mixtures) containing Ma Huang have been used for weight loss and as performance-enhancing stimulants[6] due to the fact that they produce

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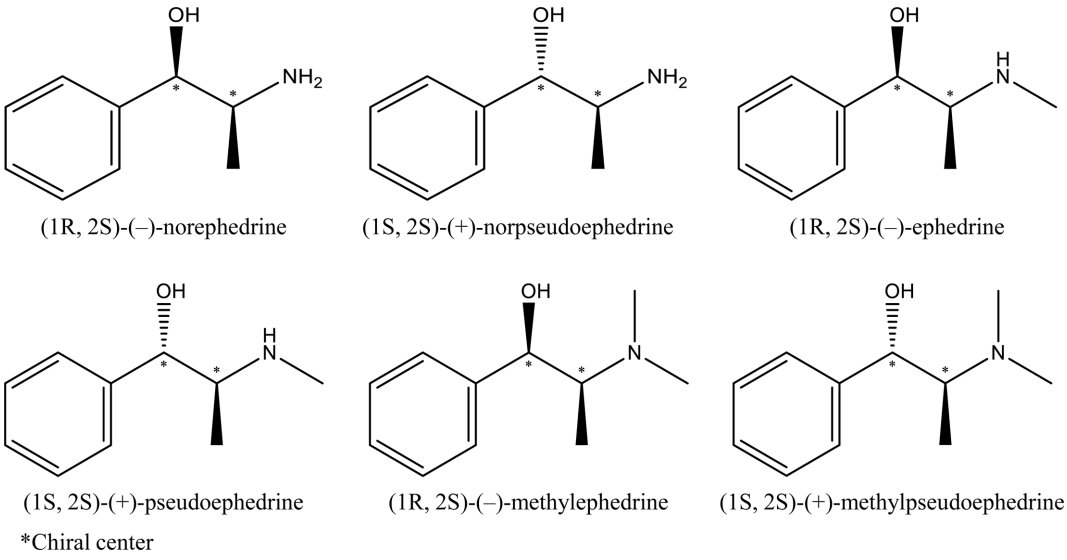

Fig. 1. The chemical structures of active ephedra alkaloids present in Ma Huang.

sympathomimetic, thermogenic, and stimulating effects on the central nervous system, which increase the body heat and the rate of metabolism.[7] However, the intake of dietary supplements containing ephedra alkaloids has been associated with a range of adverse effects, including nausea, seizures, depression, anxiety, and death.[1,8] As a result, the use of Ma Huang and its corresponding ephedra alkaloids in foods and dietary supplements has been prohibited in the US, Canada, Korea, and several European countries (e.g., the United Kingdom, France, Spain, and Sweden).

To identify the ephedra alkaloids, several analytical approaches have been reported, which generally involve sample extraction and purification, followed by detection or quantification using high-performance liquid chromatography-ultraviolet detection (HPLC-UV)[2,9], liquid chromatography-tandem mass spectrometry (LC-MS/MS),[6,8,10-15] gas chromatography-mass spectrometry (GC-MS)[16,17] and nuclear magnetic resonance (NMR) spectroscopy.[18-19] For example, Roman et al. employed an HPLC-UV approach to determine the ephedrine alkaloids present in botanical reference materials and in dietary supplements containing ephedra ground herb or ephedra extract.[9] For sample preparation, they extracted the ephedra alkaloids with a potassium phosphate buffer and utilized a strong-cation exchange cartridge for the purification step. In addition, Zhang et al. developed a quantification method for five ephedra alkaloids in three typical dietary supplement matrices (i.e., solid, liquid, and oil) using LC-MS/MS, wherein liquid-liquid extraction was found to result in a satisfactory separation.[14] In terms of the chiral separation of enantiomers, Wang et al. developed an enantioselective method for quantifying chiral amphetamine-type illicit drugs in water.[15] Using LC-MS/MS, they employed a CHIRALPAK CBH column to separate (−)-ephedrine, (+)-pseudoephedrine and (±)-norephedrine, and this method was successfully adopted to determine the target compounds in aqueous samples.

Thus, the aim of the current study was to develop an analytical protocol for the quantitation and enantiomeric separation of the ephedra alkaloids to help prevent the illegal distribution and adulteration of products containing Ma Huang or its corresponding alkaloid components. For this purpose, not only a solid-phase extraction (SPE) method was optimized for the purification by comparing four types of cartridges (i.e., Oasis HLB, Oasis PRiME HLB, Oasis MCX, and Sep-Pak C18), but also the UPLC-MS/MS method for quantitative analysis of analytes was optimized and validated. Furthermore, an analytical method for chiral separation was developed using HPLC-DAD. Finally, these methods were tested for applicability to botanical reference materials and commercial products.

Experimental

General experimental procedures – (1*R*,2*S*)-(−)-Ephedrine, (1*S*,2*R*)-(+)-ephedrine, (1*R*,2*R*)-(−)-pseudoephedrine, (1*S*,2*S*)-(+)-pseudoephedrine, (*R*/*S*)-(±)-norephedrine, and (1*S*,2*S*)-(+)-norpseudoephedrine were purchased from Lipomed AG (Arlesheim, Switzerland). (*R*/*S*)-(±)-Methylephedrine was purchased from Santa Cruz Biotechnology (Texas, USA), and (1*S*,2*S*)-(+)-methylpseudoephedrine was

purchased from Toronto Research Chemicals (Toronto, Canada). Methanol, acetonitrile, ethanol, 2-propanol, phosphoric acid, ammonium formate (HPLC grade), and water (LC-MS grade) were purchased from Sigma-Aldrich (St Louis, MO, USA), while formic acid (LC-MS grade) was purchased from Thermo Fisher Scientific (MA, USA), and ammonium hydroxide (NH_4OH) was purchased from Merck (Darmstadt, Germany). Distilled water (DW) was obtained (18.2 MΩ·cm) using a Milli-Q system (Millipore, USA). The Oasis HLB (6 cc, 500 mg), Oasis PRiME HLB (6 cc, 500 mg), Oasis MCX (6 cc, 500 mg), and Sep-Pak Vac C18 (6 cc, 500 mg) SPE cartridges were purchased from Waters (Milford, MA, USA).

Preparation of the standard solutions – Stock solutions of the ephedra alkaloids were prepared at a concentration of 1 mg/mL in methanol. Working solutions were prepared by diluting each stock solution with methanol and these working solutions were employed to obtain the calibration curves and for method validation of UPLC-MS/MS. All solutions were stored at 4 ºC until use.

Blank samples – For development and validation of the proposed method, two types of blank samples, which are not expected to contain ephedra alkaloids, were purchased from an online market, and their details are as follows: a protein supplement (solid, USA) and black cohosh extract (liquid, USA).

Sample preparations – For analysis of the analytes present in complex matrices of botanical reference materials and commercial products, the solid or liquid sample (1.0 g) was added to a 50 mL conical tube with methanol: water (50:50, v/v) (20 mL) as the extraction solvent. Each sample solution was ultrasonically treated for 30 min and then centrifuged at 4,000 rpm for 30 min. The supernatant was put into a 20 mL volumetric flask, and 50% methanol was added to obtain a final volume of 20 mL.

Following preconditioning of the desired SPE cartridge using methanol (4 mL) and water (4 mL), the sample extract (0.2 mL) was loaded onto the cartridge. The cartridges were washed with 5% 2-propanol (2 mL), and then the analytes were eluted into a 20 mL conical tube using an eluent composed of 5% NH_4OH in methanol:water (95:5, v/v) (4 mL). After subsequent evaporation of the eluate under a stream of nitrogen at 45 ºC, methanol (0.5 mL) was added, and the tube was shaken using a vortex mixer. After reconstitution, the solution was transferred to a 1 mL volumetric flask, and methanol was added to obtain a final volume of 1 mL. The obtained solution then adequately diluted and injected into the HPLC-DAD and UPLC-MS/MS systems for chiral analysis and quantitative analysis, respectively.

HPLC-DAD conditions – Enantiomeric separation of the ephedra alkaloids was performed using an Agilent 1260 infinity HPLC equipped with a DAD (HPLC-DAD) (Agilent Technologies, USA). An Agilent InfinityLab Poroshell Chiral-CD column (100 mm × 2.1 mm, 2.7 μm) was used and the column temperature was maintained at 23 ºC. Mobile phase consisted of 2 mM ammonium formate in DW (pH 3.7) (A) and methanol:acetonitrile (70:30, v/v) (B), and isocratic elution conditions of 97% A and 3% B were used. The flow rate was 0.2 mL/min and the injection volume was 1.0 μL. All detections were performed at 210 nm.

UPLC-MS/MS conditions – UPLC-MS/MS analysis was performed on a Waters ACQUITY UPLC system consisting of a binary solvent manager, a column heater, a vacuum degasser, and an autosampler coupled with a Xevo TQ-XS mass spectrometer equipped with an electrospray source (Milford, USA). The separation was performed using an ACQUITY UPLC HSS PFP column (100 mm × 2.1 mm, 1.8 μm), and the column and autosampler temperatures were maintained at 40 and 10 ºC, respectively. Mobile phase consisted of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B), and isocratic elution conditions of 95% A and 5% B were used. The flow rate was 0.25 mL/min and the injection volume was 2.0 μL. The mass spectrometer was operated with electrospray ionization in the positive mode (ESI+). The optimized parameters of the mass spectrometer were as follows: capillary voltage = 0.5 kV, cone voltage = 30 V, nebulizer and desolvation gases = high purity nitrogen, desolvation gas flow rate = 800 L/h, cone gas flow rate = 150 L/h, source temperature = 150 ºC, and desolvation temperature = 650 ºC. The multiple reaction monitoring (MRM) mode was employed for quantification of analytes, and conditions of the MRM transitions were optimized by the direct infusion of individual standard solutions (100 ng/mL) into the mass spectrometer. For each analyte, the protonated precursor ion $[M+H]^+$ and four product ions were obtained and used for the four MRM transitions. Instrument control and data acquisition were performed using MasslynxTM V4.2 software (Waters, Milford, MA, USA).

Method validation – To establish a quantification method for UPLC-MS/MS, method validation was performed on two types of blank samples (solid and liquid). More specifically, validation was performed based on specificity, linearity, the limit of detection (LOD), limit of quantitation (LOQ), precision, accuracy and recovery according to the International Council for Harmonization and the US Food and Drug Administration guidelines.[20,21]

Specificity – The specificity was evaluated by comparing the chromatograms obtained from the blank samples that did not contain the analytes and the blank samples spiked with the analytes at a concentration of 10 ng/mL.

LOD and LOQ – The LOD and LOQ were evaluated based on the standard deviation (σ) of the response and the slope (S) of calibration curves. The σ was estimated by analyzing seven replicates of the blank samples spiked with lowest concentration expected to be close at detection limit. The slope, S, was estimated from the calibration curves obtained by analyzing blank samples spiked with the concentrations in the range expected to be detection limit, including the lowest concentration. The LOD and LOQ were calculated as follows: LOD = 3.3 × σ / S; LOQ = 3 × LOD.

Linearity – The linearity was evaluated using matrix-matched calibration curves to minimize the influence of the matrices. Considering the LOQ value obtained for each analyte, calibration curves were obtained by plotting the peak areas versus the six individual concentrations (2, 5, 10, 20, 40, and 80 ng/mL for norephedrine and norpseudoephedrine; 0.5, 1.25, 2.5, 5.0, 10, and 20 ng/mL for ephedrine, pseudoephedrine, methylephedrine, and methylpseudoephedrine).

Accuracy and precision – The accuracy and precision were evaluated at three concentrations: low (near LOQ), medium (approximately 10-times the low value), and high (approximately 40-times the low value). More specifically, The accuracy and precision were determined by analyzing the blank samples spiked with low (1.25 ng/mL for ephedrine, pseudoephedrine, and methylephedrine; 20 ng/mL for norephedrine and norpseudoephedrine), medium (5 ng/mL for ephedrine, pseudoephedrine and methylephedrine; 20 ng/mL for norephedrine and norpseudoephedrine), and high (20 ng/mL for ephedrine, pseudoephedrine, and methylephedrine; 80 ng/mL for norephedrine and norpseudoephedrine) concentrations on the same day (intraday) and over three separate days (interday).

Recovery – The recovery was defined as the percentage ratio of the peak areas of the spiked blank samples to the peak areas of the standard solutions. The two types of blank samples (i.e., the solid and liquid) spiked with the three (low, medium and high) concentrations of analytes were examined in triplicate.

Result and Discussion

We optimized the sample preparation process, including the extraction and purification steps, based on the extraction efficiency, which was expressed in terms of the recovery (%). Considering the complexity of the large variety of matrices that could be required for analysis, such as plants, powders, tablets (solids), and aqueous solutions (liquids), representative solid and liquid blank samples were selected. To select an appropriate extraction solvent, two blank samples spiked with the ephedra alkaloids were extracted using 50% methanol, 50% ethanol, 50% acetonitrile, and 100% methanol. With the exception of norephedrine, the ephedra alkaloids exhibited a good extraction efficiency in all the extraction solvents, with 50% methanol resulting in the highest recovery (i.e., 86.0–105.3%). Thus, using 50% methanol as the extraction solvent, SPE was performed to remove any interfering compounds in the samples that may affect the analyte signals. Among the four SPE cartridges evaluated (i.e., Oasis HLB, Oasis PRiME HLB, Oasis MCX, and Sep-Pak C18), the highest recovery of the six ephedra alkaloids was obtained using the Oasis PRiME HLB column.

As mentioned above, ephedra alkaloids are present naturally as a single enantiomer, although it is possible to obtain their opposite enantiomers or racemic mixtures by means of chemical synthesis.[5] Thus, the origin of the ephedra alkaloids in botanical reference materials or products can be confirmed by analysis of the enantiomers that are present. Several reports have described stationary phases and analytical techniques for enantioselective analysis of illicit drugs or chiral pharmaceuticals containing ephedra alkaloids. Wang et al.[15] and Kasprzyk-Hordern et al.[24] used the CHIRALPAK CBH column to separate (−)-ephedrine, (+)-pseudoephedrine and (±)-norephedrine. In this study, we tried to separate four pairs of enantiomers (i.e., *R/S*-(±)-norephedrine, *R/S*-(±)-ephedrine, *R/S*-(±)-pseudoephedrine, and *R/S*-(±)-methylephedrine) using the Agilent InfinityLab Poroshell 120 Chiral-V and Chiral-CD columns.[22] The Chiral-V column successfully separated the *R/S*-(±)-methylephedrine enantiomers, but did not separate *R/S*-(±)-norephedrine, *R/S*-(±)-ephedrine, or *R/S*-(±)-pseudoephedrine, which were observed as single peaks. In contrast, the Chiral-CD column adequately separated the enantiomers of *R/S*-(±)-ephedrine, *R/S*-(±)-pseudoephedrine, and *R/S*-(±)-methylephedrine, but not separated *R/S*-(±)-norephedrine (Fig. 2a). Hence, subsequent separation of the enantiomers was carried out using the Chiral-CD column, which is packed with hydroxypropylated beta-cyclodextrin as a chiral selector, for *R/S*-(±)-ephedrine, *R/S*-(±)-pseudoephedrine and *R/S*-(±)-methylephedrine. Compared to conventional methods,[15,24] our analytical method has separated more paired enantiomers, enabling accurate identification of the origin of ephedra alkaloids in Ma huang-related samples. Furthermore, a good sepa-

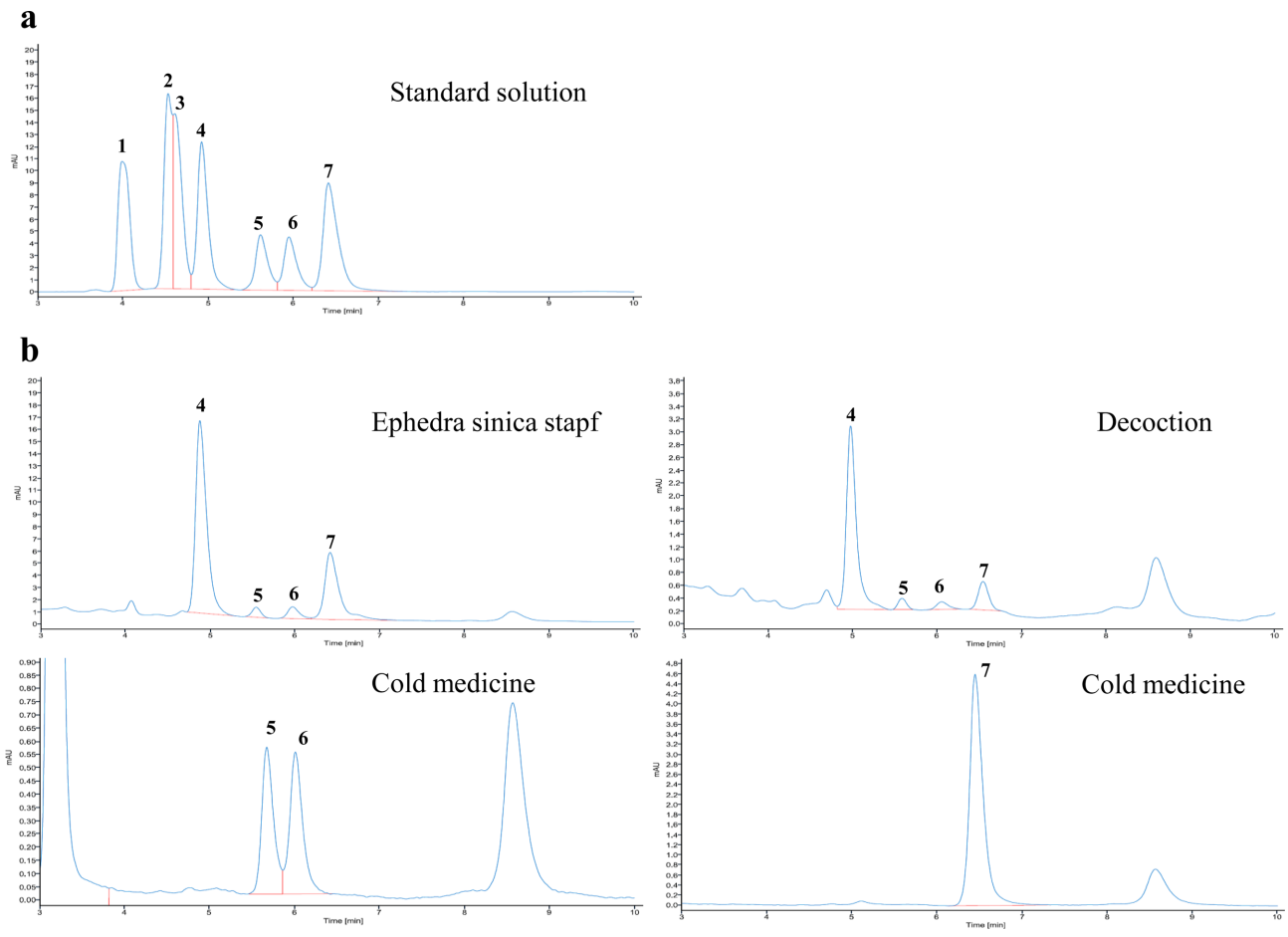

Fig. 2. HPLC-DAD chromatograms for the enantiomeric separation of the ephedra alkaloids on the Chiral-CD column (100 mm × 2.1 mm, 1.8 μm): (a) Mixture of the standard solution of the ephedra alkaloids at a concentration of 10 μg/mL, (b) the four representative samples (botanical reference materials and products). Peak numbering: 1 = (±)-norephedrine, 2 = (−)-pseudoephedrine, 3 = (+)-ephedrine, 4 = (−)-ephedrine, 5 = (+)-methylephedrine, 6 = (−)-methylephedrine, and 7 = (+)-pseudoephedrine.

ration of the ephedra alkaloids was achieved in less than 10 minutes using a mobile phase condition consisting of 97% of solvent A (2 mM ammonium formate in DW) and 3% of solvent B (acetonitrile:methanol=70:30, *v/v*), which was selected as the optimized solvent combination.

A UPLC-MS/MS method was then designed to quantitatively analyze the six ephedra alkaloids (i.e., (−)-ephedrine, (+)-pseudoephedrine, (−)-norephedrine, (+)-norpseudoephedrine, (−)-methylephedrine, and (+)-methylpseudoephedrine) present in the botanical reference materials and products of interest. Initially, several columns were tested for their ability to separate the six analytes, including Acquity UPLC BEH C18 (100 mm × 2.1 mm, 1.7 μm), Acquity UPLC HSS T3 (100 mm × 2.1 mm, 1.8 μm), and Acquity UPLC HSS PFP (100 mm × 2.1 mm, 1.8 μm) columns. The BEH C18 and HSS T3 columns gave poor peak shapes with tailing and splitting, while the HSS PFP column, which consists of a fluorophenyl stationary phase, showed good peak shapes and sensitivities for the analytes. Thus, the HSS PFP column was selected for quantitative analysis, and a good separation of the ephedra alkaloids was achieved using a mobile phase condition consisting of 95% of solvent A (0.1% formic acid in water) and 5% of solvent B (0.1% formic acid in acetonitrile). The six ephedra alkaloids were then detected by four MRM transitions (precursor ion → product ions) in the ESI+ ion mode. The protonated precursor ions $[M+H]^+$ were observed at *m/z* 152.09 for (−)-norephedrine and (+)-norpseudoephedrine, *m/z* 166.23 for (−)-ephedrine and (+)-pseudoephedrine, and *m/z* 180.15 for (−)-methylephedrine and (+)-methylpseudoephedrine. The most abundant product ion of the ephedra alkaloids corresponded to a loss of water ($[M+H-H_2O]^+$), and was observed at *m/z* 134.07 for (−)-norephedrine and (+)-norpseudoephedrine, *m/z* 148.17 for (−)-ephedrine and (+)-pseudoephedrine, and *m/z* 162.11 for (−)-methyle-

Table 1. Optimized parameters for MRM transitions of six ephedra alkaloids in UPLC-MS/MS

Compounds	Chemical formula	Ion mode	Precursor ion (m/z)	Product ion (m/z)	Cone voltage (V)	Collision energy (eV)
(–)-norephedrine	$C_9H_{13}NO$	+	152.09 $[M+H]^+$	134.07[a]	30	10
				117.05		15
				115.04		20
				91.05		25
(+)-norpseudoephedrine	$C_9H_{13}NO$	+	152.09 $[M+H]^+$	134.07[a]	30	10
				117.05		15
				115.04		20
				91.05		25
(–)-ephedrine	$C_{10}H_{15}NO$	+	166.23 $[M+H]^+$	148.17[a]	30	10
				133.16		20
				117.17		20
				115.15		25
(+)-pseudoephedrine	$C_{10}H_{15}NO$	+	166.23 $[M+H]^+$	148.17[a]	30	10
				133.16		20
				117.17		20
				115.15		25
(–)-methylephedrine	$C_{11}H_{17}NO$	+	180.27 $[M+H]^+$	162.18[a]	30	15
				147.19		20
				135.17		15
				117.18		20
(+)-methylpseudoephedrine	$C_{11}H_{17}NO$	+	180.27 $[M+H]^+$	162.18[a]	30	15
				147.19		20
				117.18		20
				115.03		25

[a]Quantitative ion

Table 2. Linearity, limits of detection (LOD) and limits of quantification (LOQ) of six ephedra alkaloids in two types of sample analyzed by UPLC-MS/MS

Compounds	Sample types	Linear range (ng/mL)	R^2	LOD (ng/mL)	LOQ (ng/mL)
(–)-norephedrine	Solid	2–80	0.9961	0.68	2.03
	Liquid	2–80	0.9983	0.72	2.15
(+)-norpseudoephedrine	Solid	2–80	0.9995	0.67	2.00
	Liquid	2–80	0.9983	0.67	2.00
(–)-ephedrine	Solid	0.5–20	0.9993	0.24	0.72
	Liquid	0.5–20	0.9992	0.17	0.52
(+)-pseudoephedrine	Solid	0.5–20	0.9999	0.17	0.50
	Liquid	0.5–20	0.9997	0.22	0.67
(–)-methylephedrine	Solid	0.5–20	0.9971	0.19	0.57
	Liquid	0.5–20	0.9989	0.21	0.62
(+)-methylpseudoephedrine	Solid	0.5–20	0.9999	0.23	0.68
	Liquid	0.5–20	0.9987	0.23	0.70

phedrine and (+)-methylpseudoephedrine. Therefore, this product ion was selected as the quantitation ion, while the remaining three product ions were chosen as the qualification ions. Further information related to the precursor and product ions is summarized in Table 1.

The UPLC-MS/MS method for the determination of ephedra alkaloids in foods was validated with respect to parameters including specificity, linearity, LOD, LOQ,

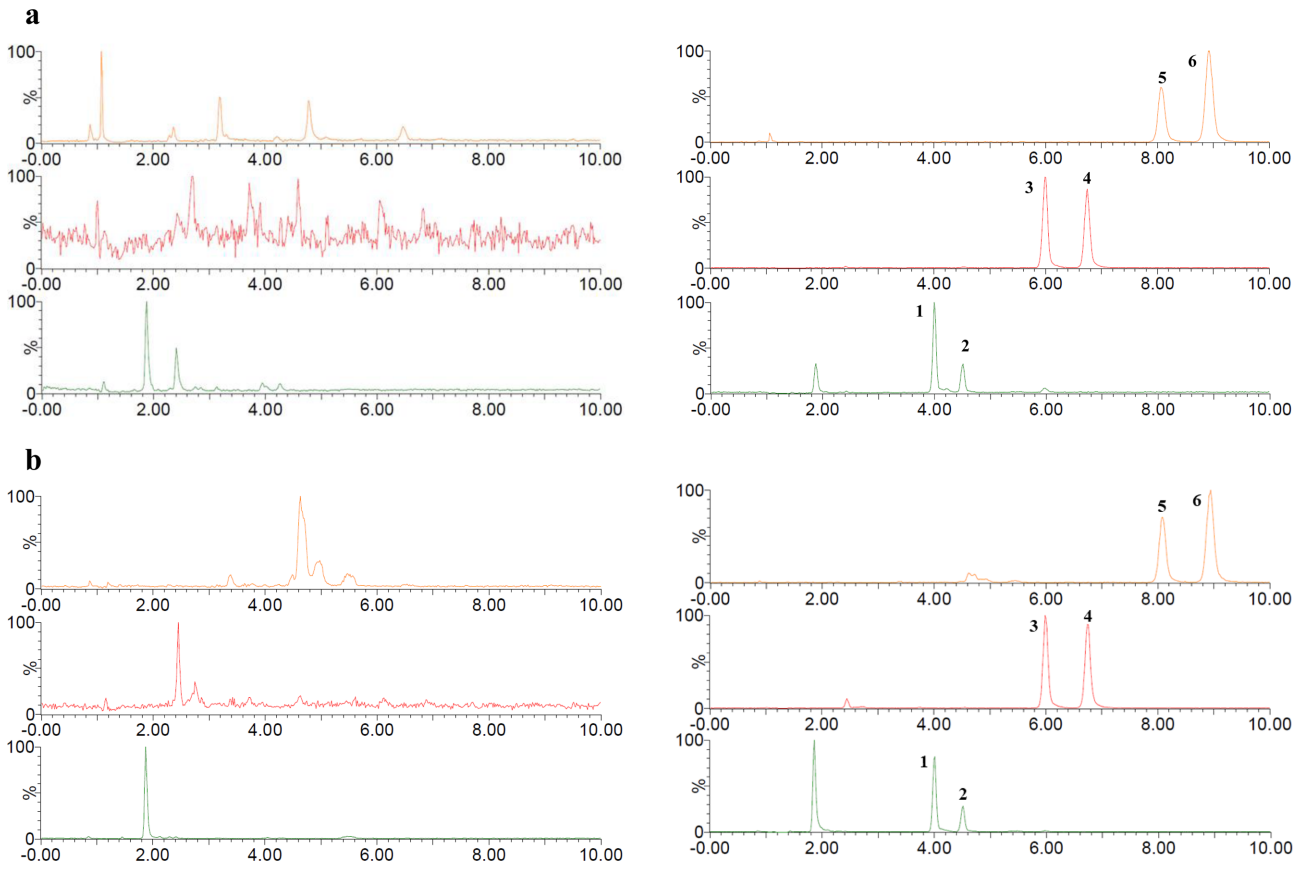

Fig. 3. Total ion chromatograms (TICs) for six ephedra alkaloids in blank samples by UPLC-MS/MS: (a) blank sample (solid, left) and spiked blank sample (solid, right, 10 ng/mL) (b) blank sample (liquid, left) and spiked blank sample (liquid, right, 10 ng/mL). Peak numbering: 1 = (−)-norephedrine, 2 = (+)-norpseudoephedrine, 3 = (−)-ephedrine, 4 = (+)-pseudoephedrine, 5 = (−)-methylephedrine, and 6 = (+)-methylpseudoephedrine.

precision, accuracy and recovery. The specificity indicate that no peaks corresponding to the interfering compounds were observed near the retention times of the analytes in the blank samples (Fig. 3). The calibration curves for the six analytes show high correlation coefficients in the range of 0.9964–0.9997 (Table 2). The LODs and LOQs for the solid sample ranged from 0.17 to 0.68 ng/mL and from 0.50 to 2.03 ng/mL, respectively, while those for the liquid sample ranged from 0.17 to 0.72 ng/mL and from 0.52 to 2.15 ng/mL, respectively (Table 2). For the solid sample, the accuracy values ranged from 94.7 to 113.6% (intraday) and from 94.6 to 108.1% (interday), and the precision values were ≤ 9.4%. For the liquid sample, the accuracy values ranged from 90.3 to 111.1% (intraday) and from 92.0 to 111.2% (interday), and the precision values were ≤ 9.4% (Table 3). The recoveries were in the range of 84.4–107.0% for the solid samples and 81.2–108.7% for the liquid samples (Table 4).

The developed methods were employed to analyze 44 samples (14 botanical reference materials and 30 commercial products) purchased from pharmacies and oriental medicine clinics, purchased online, or obtained from related institutions (Herbal Resource Bank of Traditional Korean Medicine and Herbal Medicine Research Division of the Ministry of Food and Drug Safety). The botanical reference materials consisted of Ma Huang (five *E. sinica* Stapf, one *E. equisetina* Bunge, and two *E. intermedia* Schrenk & C.A. Mey), *Ephedra* species (one *E. viridis* and one *E. torreyana*), and other plants known to contain ephedra alkaloids[1,23] (three *Pinellia ternata* and one *Taxus baccata*). The products consisted of nine decoctions, six cold medicines, seven oriental medicines, and eight dietary supplements. The cold medicines contain either (±)-methylephedrine or (+)-pseudoephedrine. The various products examined were mainly sold to treat coughs, colds, and asthma or for weight loss purposes.

The 44 samples were quantitatively analyzed using UPLC-MS/MS, and ephedra alkaloids were detected in the 32 samples. Among the 32 samples, six ephedra alkaloids were quantified in 26 samples (eight botanical

Table 3. Intra-day and inter-day precision and accuracy of 6 ephedra alkaloids at three concentrations (low, medium and high) in two types of sample analyzed by UPLC-MS/MS

Compound	Conc. (ng/mL)	Solid				Liquid			
		Intra-day		Inter-day		Intra-day		Inter-day	
		Precision (RSD, %)	Accuracy (%)	Precision (RSD, %)	Accuracy (%)	Precision (RSD, %)	Accuracy (%)	Precision (RSD, %)	Accuracy (%)
(–)-norephedrine	5	6.8	113.6	5.2	107.2	4.2	94.9	1.9	92.9
	20	0.6	110.6	4.9	106.2	0.6	106.6	0.8	106.3
	80	0.8	100.3	4.1	98.7	1.7	98.6	1.0	98.3
(+)-norpseudoephedrine	5	9.4	106.8	9.4	96.9	4.3	90.3	1.6	92.0
	20	5.2	112.9	5.7	106.2	2.6	107.5	1.5	105.8
	80	7.3	109.7	5.3	103.4	1.6	99.1	1.9	100.5
(–)-ephedrine	1.25	4.9	101.0	1.8	103.0	2.2	97.2	9.4	108.9
	5	4.6	94.7	3.7	98.3	4.0	93.7	4.9	99.1
	20	7.3	100.0	5.4	94.6	2.8	99.3	0.7	98.8
(+)-pseudoephedrine	1.25	3.3	100.2	0.4	99.9	5.1	103.0	5.7	92.9
	5	6.2	103.4	3.7	101.2	8.2	97.2	3.9	106.3
	20	5.8	100.2	0.9	99.8	1.3	98.3	2.3	98.3
(–)-methylephedrine	1.25	3.1	107.7	2.3	108.1	3.1	108.8	4.5	111.2
	5	0.7	100.1	0.6	99.6	8.6	99.0	3.2	102.1
	20	9.0	101.8	3.2	98.2	1.2	100.1	2.9	96.9
(+)-methylpseudoephedrine	1.25	3.7	99.6	1.1	100.6	4.5	111.1	1.5	109.4
	5	3.0	98.2	3.2	101.1	9.5	91.8	1.5	93.4
	20	8.7	100.2	3.3	96.6	1.9	100.4	0.9	99.7

Table 4. Recovery (extraction efficiency) of six ephedra alkaloids at three concentrations (low, medium and high) in two types of Sample analyzed by UPLC-MS/MS

Compound	Conc. (ng/mL)	Solid		Liquid	
		Mean,%	RSD, %	Mean,%	RSD, %
(–)-norephedrine	5	84.3	2.7	102.8	2.3
	20	84.9	3.1	95.4	2.7
	80	86.9	1.0	91.6	4.2
(+)-norpseudoephedrine	5	97.2	1.8	108.7	45
	20	93.6	5.3	106.8	5.3
	80	96.8	5.2	105.4	4.0
(–)-ephedrine	1.25	86.8	5.6	85.2	2.2
	5	84.4	5.3	91.8	2.4
	20	94.4	5.6	96.1	5.3
(+)-pseudoephedrine	1.25	87.2	5.4	85.1	6.0
	5	90.4	1.7	91.9	8.2
	20	89.8	4.5	95.0	0.4
(–)-methylephedrine	1.25	88.6	2.1	82.6	5.6
	5	84.5	3.0	81.2	6.7
	20	85.3	6.1	92.1	3.1
(+)-methylpseudoephedrine	1.25	99.0	1.5	88.5	4.9
	5	107.0	6.1	95.5	1.0
	20	99.2	4.2	101.0	6.1

reference materials and 18 commercial products), and a single ephedra alkaloid ((±)-methylephedrine or (+)-pseudoephedrine) was quantified in the six cold medicines (Table 5). After the quantitative analysis, chiral separation

Table 5. The quantitative results of six ephedra alkaloids in Ma Huang-related samples

No.	Sample type	Sample formulation	(−)-NE[a] (μg/g)	(+)-NP[a] (μg/g)	(−)-EP[a] (μg/g)	(+)-PS[a] (μg/g)	(−)-ME[a] (μg/g)	(+)-MP[a] (μg/g)
Sample-01	*Ephedra sinica* Stapf	Raw material	273.2	1971.5	430.8	6542.3	34.8	74.2
Sample-02	*Ephedra equisetina* Bunge	Raw material	490.8	530.5	6781.1	2981.6	641.7	28.2
Sample-03	*Ephedra sinica* Stapf	Raw material	335.3	3616.6	2217.4	8873.3	123.1	48.7
Sample-04	*Ephedra sinica* Stapf	Raw material	1.7	5.8	54.3	31.0	5.9	0.4
Sample-05	*Ephedra sinica* Stapf	Raw material	12.3	17.7	265.3	96.8	17.9	0.9
Sample-06	*Ephedra intermedia* Schrenk & C.A. Mey	Raw material	213.5	799.6	8853.4	1846.5	919.9	2.0
Sample-07	*Ephedra sinica* Stapf	Raw material	594.8	1426.5	9306.7	4232.1	692.4	37.1
Sample-08	*Ephedra intermedia* Schrenk & C.A. Mey	Raw material	221.7	877.6	8138.5	1762.3	718.7	1.8
Sample-09	*Ephedra viridis*	Raw material	N.D.[b]	N.D.	N.D.	N.D.	N.D.	N.D.
Sample-10	*Ephedra torreyana*	Raw material	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Sample-11	*Pinellia ternate*	Raw material	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Sample-12	*Pinellia ternate*	Raw material	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Sample-13	*Pinellia ternate*	Raw material	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Sample-14	*Taxus baccata*	Raw material	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Sample-15	Dietary supplement (containing Ma Huang)	Tablet	9.4	19.1	323.2	210.6	48.1	3.5
Sample-16	Dietary supplement (containing Ma Huang)	Tablet	18.8	25.5	426.1	136.7	39.2	1.1
Sample-17	Dietary supplement (Ephedra free)	Capsule	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Sample-18	Dietary supplement	Tea	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Sample-19	Dietary supplement	Tea bag	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.

[a]NE, norephedrine; NP, norpseudoephedrine; EP, ephedrine; PS, pseudoephedrine; ME, methylephedrine; MP, methylpseudoephedrine; [b]N.D., not detection

Table 5. continued

No.	Sample type	Sample formulation	(−)-NE[a] (μg/g)	(+)-NP[a] (μg/g)	(−)-EP[a] (μg/g)	(+)-PS[a] (μg/g)	(−)-ME[a] (μg/g)	(+)-MP[a] (μg/g)
Sample-20	Dietary supplement	Tea	474.1	1162.0	7026.8	3326.0	493.4	27.9
Sample-21	Dietary supplement	Capsule	N.D. [b)]	N.D.	N.D.	N.D.	N.D.	N.D.
Sample-22	Dietary supplement	Tablet	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Sample-23	Decoction (containing Ma Huang)	Granule	15.1	45.4	839.2	125.4	58.9	0.2
Sample-24	Decoction (containing Ma Huang)	Granule	22.4	88.3	1304.3	260.5	87.7	0.6
Sample-25	Decoction (containing Ma Huang)	Granule	22.5	77.4	1610.5	228.9	115.6	0.5
Sample-26	Decoction (containing Ma Huang)	Granule	4.3	6.3	969.4	112.6	79.2	0.3
Sample-27	Decoction (containing Ma Huang)	Liquid	2.1	5.8	105.7	11.7	10.0	0.0
Sample-28	Decoction (containing Ma Huang)	Liquid	0.5	1.4	28.2	4.9	5.0	Trace[c]
Sample-29	Cold medicine (containing (±)-methylephedrine)	Liquid	N. D.	N. D.	N. D.	N. D.	625.2	N. D.
Sample-30	Cold medicine (containing (±)-methylephedrine)	Liquid	N. D.	N. D.	N. D.	N. D.	915.8	N. D.
Sample-31	Cold medicine (containing (±)-methylephedrine)	Liquid	N. D.	N. D.	N. D.	N. D.	762.2	N. D.
Sample-32	Cold medicine (containing (±)-methylephedrine)	Liquid	N. D.	N. D.	N. D.	N. D.	956.9	N. D.
Sample-33	Cold medicine (containing (+)-pseudoephedrine)	Liquid	N. D.	N. D.	N. D.	2440.4	N. D.	N. D.
Sample-34	Cold medicine (containing (+)-pseudoephedrine)	Liquid	N. D.	N. D.	N. D.	2354.8	N. D.	N. D.
Sample-35	Decoction (containing Ma Huang)	Granule	70.0	185.5	1763.4	769.2	154.3	7.0
Sample-36	Decoction (containing Ma Huang)	Liquid	3.8	26.9	311.8	100.3	36.9	2.7
Sample-37	Decoction (containing Ma Huang)	Tablet	53.8	149.8	1478.2	575.1	118.9	4.8

[a]NE, norephedrine; NP, norpseudoephedrine; EP, ephedrine; PS, pseudoephedrine; ME, methylephedrine; MP, methylpseudoephedrine; [b]N.D., not detection; [c]Trace: <LOQ

Table 5. continued

No.	Sample type	Sample formulation	(−)-NE[a] (μg/g)	(+)-NP[a] (μg/g)	(−)-EP[a] (μg/g)	(+)-PS[a] (μg/g)	(−)-ME[a] (μg/g)	(+)-MP[a] (μg/g)
Sample-38	Oriental medicine	Granule	251.4	493.0	5774.7	2777.7	511.7	26.4
Sample-39	Oriental medicine	Tablet	211.5	577.6	8816.0	1356.3	559.7	1.4
Sample-40	Oriental medicine	Capsule	261.1	508.8	14909.5	2262.9	958.6	2.5
Sample-41	Oriental medicine	Capsule	270.8	562.1	15093.4	2259.2	940.5	2.5
Sample-42	Oriental medicine	Capsule	N. D. [b)]	N. D.	N. D.	N. D.	N. D.	N. D.
Sample-43	Oriental medicine	Liquid	3.7	8.6	85.4	407.0	5.0	0.5
Sample-44	Oriental medicine	Granule	155.2	485.1	6716.2	894.5	405.8	0.9

[a]NE, norephedrine; NP, norpseudoephedrine; EP, ephedrine; PS, pseudoephedrine; ME, methylephedrine; MP, methylpseudoephedrine; [b]N.D., not detection

for the *R/S*-(±)-ephedrine, *R/S*-(±)-pseudoephedrine and *R/S*-(±)-methylephedrine present in the 32 samples was performed by HPLC-DAD. In the mixture of standard solution, although two peaks attributed to (1*R*,2*R*)-(−)-pseudoephedrine and (1*S*,2*R*)-(+)-ephedrine overlapped, neither compound was not found in any samples (Fig. 2b). In addition, both compounds are known not to be found in nature, so overlapped peaks are not a problem. Indeed, only (1*R*,2*S*)-(−)-ephedrine and (1*S*,2*S*)-(+)-pseudoephedrine were detected in the 32 samples, which means that both components present in positive samples are of natural origin. In the case of the samples in which methylephedrine was detected, at least one of (1*R*,2*S*)-(−)-methylephedrine and (1*S*,2*R*)-(+)-methylephedrine were detected. These results were confirmed based on the retention times, UV spectra (HPLC-DAD), and ion ratios (UPLC-MS/MS) of the standard ephedra alkaloid samples.

In conclusion, we developed UPLC-MS/MS and HPLC-DAD analytical methods for the quantitative analysis and enantiomeric separation of ephedra alkaloids. Optimization studies showed that columns with a phenyl stationary phase gave a better peak shape and separation than C18 columns. In addition, among the four types of SPE cartridges evaluated for the purification step, the Oasis PRiME HLB cartridge showed the best analyte recovery. Furthermore, method validation was conducted for the UPLC-MS/MS method in terms of specificity, LOD, LOQ, linearity, precision, accuracy and recovery, which confirmed the reliability of the quantitative method. The established methods were applied to analyze 44 samples consisting of botanical reference materials and commercial products that could potentially contain Ma Huang or its corresponding alkaloids. As a result, ephedra alkaloids were detected and quantified in 32 samples, as well as chiral separation was successfully performed, which are the meaningful results that the combined method of HPLC-DAD and UPLC-MS/MS can successfully identify the presence of ephedra alkaloids in various Ma Huang-related samples. We note that this study was limited to the number of botanical reference materials for ephedra species and three species of Ma Huang due to the difficulty of obtaining samples, and the separation of norephedrine enantiomers was not successful using our method. However, we obtained consistent results for quantitative analysis of six ephedra alkaloids, and enantiomeric separation of compounds other than norephedrine was successful, thereby providing strong evidence for the presence of Ma Huang in foods. Therefore, we expect that our combined methodology will be applicable in various food samples, thereby contributing toward preventing the illegal distribution and adulteration of products containing Ma Huang or its corresponding alkaloids.

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