

Utilisation of a Simple and Fast HPLC-UV Method for Separation and Quantification of Ephedrine Alkaloids in Herb of Different *Ephedra* Species

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ABSTRACT

Ephedra is a plant with a long history of medicinal use, the main indications being asthma, rhinitis and bronchospasm. However, nowadays it is often misused/abused for weight-loss, performance enhancing and doping. The pharmacological effects are ascribed to the ephedrine alkaloids present in the plant. The aim of the study was to quantify Norephedrine (NE), Ephedrine (E), Pseudoephedrine (PE) and Methylephedrine (ME) in herb extracts of nine different *Ephedra* species using high performance liquid chromatography with UV detection (HPLC-UV). The alkaloids were separated on the column Symmetry C18 (5 μ m, 3.9 x 150 mm). The mobile phase was delivered at the flow rate of 1 ml/min. The mobile phase was prepared with 390 ml of acetonitrile, 610 ml of pure water, 0.8 ml of o-phosphoric acid and 4.8 g of sodium lauryl sulfate. The UV detector operated at 208 nm. We found that the utilized HPLC-UV method for separation and quantification of ephedrine alkaloids was very selective, sensitive and simple. The total content of NE, E, PE and ME ranged from 0.4 mg/g dry weight (*Ephedra foeminea*, from Austria) to 71.9 mg/g dry weight (*Ephedra major*, from Morocco). None of the investigated alkaloids were detected in *Ephedra altissima*

INTRODUCTION

The use of herbal medicines and phytonutrients or nutraceuticals continues to expand rapidly across the world with many people now resorting to these products for treatment of various health challenges in different national healthcare settings ^[1]. *Herba Ephedrae*, however, has been used in traditional Chinese medicine for 5000 years for treatment of asthma, cough, cold and mild forms of bronchospasm. In the last century, *Ephedra sinica* became a very popular plant, for its extract was used as a Central Nervous System (CNS) stimulant, mood enhancer as well as a dietary supplement in various health foods in the Western countries ^[2], which are claimed to be effective for weight-loss or as energy booster. In both traditional and more recent popular uses, the pharmacological effects are mainly attributable to six ephedrine-type alkaloids ^[3]. Although *E. sinica* has been the primary source for ephedrine alkaloids, other species of *Ephedra* throughout Eurasia contain the active constituents: *E. equisetina*, *E. intermedia*, *E. gerardiana*, *E. alata*, *E. distachya*, *E. botschantzevii*, *E. fragilis*, *E. major*, *E. minuta*, *E. monosperma*, *E. pachyclada*, *E. likiangensis*, *E. saxatilis*, *E. lomatolepis*, *E. lepidosperma*, *E. przewalskii* and *E. regeliana* ^[4]. The pharmacological studies have shown that ephedrine alkaloids are sympathomimetics, binding to both the α - and β -adrenergic receptors ^[5,6]. The general perception that herbal remedies or drugs are very safe and devoid of adverse effects is not only untrue, but also misleading. Herbs have been shown to be capable of producing a wide range of undesirable or adverse reactions some of which are capable of causing serious injuries, life-threatening conditions, and even death ^[7]. Misuse or abuse of ephedrine-containing

dietary supplements has resulted in various adverse effects. The adverse effects are hypothesized to be related to coronary artery constriction, vasospasm, shortening of cardiac refractory periods allowing re-entrant cardiac arrhythmias, hypertension-induced subarachnoid hemorrhage, cerebral artery vasoconstriction and sympathomimetic-induced platelet activation^[8]. Therefore, U.S.A. and many Western countries have imposed regulations or banned the sale of ephedrine-containing dietary supplements since 1994. Several case reports have also linked the use of *Ephedra sinica* and *Ephedra*-containing dietary supplements to adverse events such as hepatotoxicity^[9,10], and transient blindness^[11]. Because of the health and legal implications associated with the use of products containing ephedrine alkaloids, it is desirable to have a chromatographic method that can accurately and reproducibly quantify E and PE in botanical products and dietary supplements. Ephedrine alkaloids present a number of challenges in their analysis. All 6 ephedrine alkaloids are structurally very similar. They are very hydrophilic amine compounds that have poor retention on traditional reversed-phase High-performance Liquid Chromatography (HPLC) systems. Their basic nature often leads to excessively broad peaks and peak tailing on chromatographic systems. Lastly, they have poor UV absorption above about 210 nm^[12]. Various methods have been used for the quantification of the ephedrine alkaloids present in some *Ephedra* species: Gas Chromatography with Mass Spectrometry detection (GC-MS), Capillary Electrophoresis (CE), HPLC-UV^[13]. In our work we used HPLC-UV to separate and quantify NE, E, PE and ME in herb of different *Ephedra* sp.

MATERIALS AND METHODS

Plant Materials

Dried aerial parts of different Ephedra species were used to determine the content of NE, E, PE and ME by HPLC-UV

Ephedra distachya subsp. *helvetica* (C.A. Mey.) Asch. & Graebn. (Botanical Garden of the University of Vienna, Austria, IPEN number: XX-O-WU-EPH130012, garden reference number 22015).

Ephedra fragilis Desf. (Botanical Garden of the University of Vienna, Austria, IPEN number: XX-O-WU-EPH130013, garden reference number 22102; Botanical Garden of the University of Hamburg, Germany, identified by Finckhi Staudinger, garden reference number: 500146, 1999; project Biota Maroc 01LL0601A).

Ephedra foeminea Forssk. (Botanical Garden of the University of Vienna, Austria, identified by I. Racz and F. Lauria, IPEN number: XX-O-WU-EPHE120010, garden reference number 23028; Botanical garden "Jevermovac" of the Faculty of Biology, University of Belgrade, Serbia, identified by P. Marin, garden reference number: BEOU 17072).

Ephedra major Host (Botanical Garden of the University of Vienna, Austria, identified by I. Racz and F. Lauria, IPEN number: XX-O-WU-EPH120009, garden reference number 23026; Botanical Garden of the University of Hamburg, Germany, identified by Finckhi and Oldeland, garden reference number not assigned, 2015, project Biota Maroc 01LL0601A).

E. sinica Stapf (purchased from a Chinese Herbal Shop in London and produced by Hefei Shenlu Double-Crane JiuHua Pharmaceutical Co. Ltd, China).

Ephedra monosperma J.G. Gmel. ex C.A. Mey. (Botanical Garden of the University of Vienna, Austria, IPEN number: XX-O-WU-EPH1300014, garden reference number 22104).

Ephedra alata Decne. (Botanical Garden of the University of Hamburg, Germany, identified by Finckhi Staudinger, garden reference number: 500392, 2002, project Biota Maroc 01LL0601A).

Ephedra altissima Desf. (Botanical Garden of the University of Hamburg, Germany, identified by Finckhi Staudinger, garden reference number: 500044, 2001, project Biota Maroc 01LL0601A).

Ephedra foliata Boiss. ex C.A. Mey. (Botanical Garden of the University of Hamburg, Germany; identified by Finckhi Staudinger, garden reference number: 500882, 2002, project Biota Maroc 01LL0601A).

Sample Preparation

The extracting solution consisted of 0.716 g sodium lauryl sulfate, 58.2 ml acetonitrile, 119 µl phosphoric acid and 91 ml water (HPLC grade). Dry and finely grounded plant material (100 mg) was suspended in 5 ml of that solution, allowed to stand for 20 min at room temperature and then sonicated for 25 min. The extracts were centrifuged, 10000 rpm for 5 min, supernatants filtrated (membrane filter, 0.25 µm) and stored at -20 °C before analysis.

Chemicals

Acetonitril Chromasolv® (for HPLC), methanol (HPLC grade), methylephedrine and norephedrine standards were purchased from Sigma Aldrich, o-phosphoric acid (85%, p.a.) and sodium lauryl sulfate (Ph Eur) from Merck KGaA, ephedrine-hydrochloride and pseudoephedrine-hydrochloride standards from BASF, water (Chromasolv® plus, for HPLC, Sigma Aldrich).

Instrumentation and Chromatographic Conditions

The HPLC-UV analysis was performed using and modifying the parameters published by Wang et al. A component Shimadzu HPLC system consisted of a pump, vacuum degasser, reservoir of the mobile phase, a thermostated autosampler, a thermostated

column compartment, UV absorbance detector operated at 208 nm. A C-18 column (Symmetry C18, 100Å, 5 µm, 3.9 x 150 mm, Waters, USA) was operated with a mobile phase consisting of 390 ml acetonitrile, 610 ml water, 0.8 ml o-phosphoric acid and 4.8 g sodium lauryl sulfate. The mobile phase was delivered at a flow-rate of 1.0 ml/min. Column temperature was maintained at 37 °C, the injection volume was 10 µl and the run time was 15 min. Detector output was recorded and chromatograms were analysed by the software Shimadzu Class-VPTM.

Standard Preparation

Ephedrine hydrochloride standard (6.74 mg that equal to 5.53 mg ephedrine standard) was dissolved in 50 ml methanol. From that solution, 10 ml were taken and diluted with methanol up to 20 ml. Norpehedrine (6,46 mg), methylephedrine (6.52 mg) and pseudoephedrine hydrochloride was weighed (7.31 mg that equal to 5.99 mg pseudoephedrine standard) and prepared in the same way.

Validation of Quantification

The contents of NE, E, PE and ME were calculated using the peakarea of their standards. Analyte concentrations were expressed as mg/g dry weight. The retention times (tR) were 9.8 min for NE, 10.4 min for PE, 11.3 min for E and 12.1 min for ME. The limit of detection in the injection volume (10 µl) was 4.2 ng for NE, 4.8 ng for PE, 4.7 ng for E and 5.3 ng for ME.

RESULTS AND DISCUSSION

The contents of NE, E, PE and ME were calculated using the peak area and known concentration of their standards (**Figure 1**). The results are presented in **Table 1**.

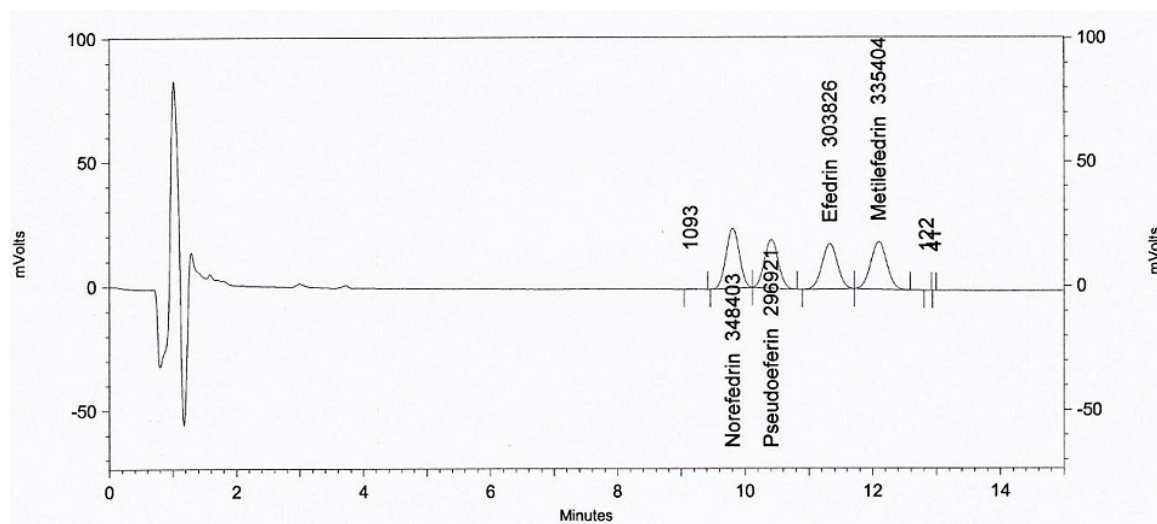


Figure 1. Chromatogram of standards for NE (646 ng /10 µl), PE (599 ng /10 µl), E (553 ng /10 µl) and ME (652 ng /10 µl) with the following retention times, respectively: 9.8 min, 10.4 min, 11.3 min and 12.1 min.

Table 1. The content of NE, PE, E and ME (expressed in mg/g dry weight) in the analyzed *Ephedra* sp.

Ephedra sp.	NE (mg/g dry weight)	PE (mg/g dry weight)	E (mg/g dry weight)	ME (mg/g dry weight)	Total (mg/g dry weight): NE+PE+E+ME
<i>Ephedra distachya</i> subsp. <i>helvetica</i> (Austria)	0.1	10.6	8.7	0.7	20.1
<i>Ephedra fragilis</i> (Austria)	0.2	1.5	3.4	0.2	5.3
<i>Ephedra fragilis</i> (Morocco)	0.2	/	0.4	0.2	0.7
<i>Ephedra foeminea</i> (Austria)	0.1	0.3	/	/	0.4
<i>Ephedra foeminea</i> (Serbia)	0.1	0.5	0.1	/	0.7
<i>Ephedra major</i> (Austria)	0.1	8.8	6.9	0.8	16.5
<i>Ephedra major</i> (Morocco)	7.3	/	63.4	1.2	71.9
<i>Ephedra sinica</i> (China)	0.9	16.6	28.3	3.3	49.1
<i>Ephedra monosperma</i> (Austria)	0.2	7.0	2.2	0.3	9.6
<i>Ephedra alata</i> (Morocco)	0.2	1.5	6.2	1.0	8.8
<i>Ephedra foliata</i> (Morocco)	1.5	/	14.7	0.3	16.5
<i>Ephedra altissima</i> (Morocco)	n.d.				

The content of alkaloids was first determined in East Asian *Ephedra* species. The ephedrines are present in most Eurasian *Ephedra* species, but are more abundant in the Chinese species [14]. American species are believed to be devoid of them [4]. Review of literature shows different content of total alkaloids in Ephedrae herba with a range of 0.5-49.9 mg/g [15], where E and PE account together for 70-99%. Our study showed that *E. major* (Morocco) had the highest total content of NE, E, PE and ME, as

opposed to *E. fragilis* and *E. foeminea*. In *E. altissima*, there was no ephedrine alkaloids detected, which is in line with literature reports [4]. The collected species originate from different geographical regions [16]. The total content of alkaloids in the herb of *E. major* that originates from Morocco is four times higher than in the same *Ephedra* sp. from Austria. Such a difference may be due to different growth conditions, soil characteristics, amount of rainfall and most probably due to the freshness of the plant material. Our results confirm that the relative proportion of the individual alkaloids is also variable, and that E and PE are the dominant alkaloids in all analysed *Ephedra* sp., except for in *E. altissima* which is devoid of ephedrine alkaloids.

CONCLUSIONS

Quantitative analyses have become crucial and most common approaches in quality control of herbal preparations and plant ground materials. The content of NE, E, PE and ME was determined in nine different *Ephedra* species using HPLC-UV. Our results showed that *E. major* (Morocco) had the highest total content of NE, E, PE and ME. *Ephedra foeminea* had a very low total content of the alkaloids, while they were not detected in *E. altissima* at all. The relative proportion of the individual alkaloids was also variable. The utilized method proved to be very sensitive, selective and simple. It can be a valuable tool in clinical and forensic toxicology where quick analyses are required, as ephedrine is often misused/abused for weight-loss and doping.

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