The Ephedra Story: An Automated SPE Procedure and Analysis for the Determination of Ephedra and Ephedra-like Compounds in Dietary Supplements

Application Note 215

Joan Stevens, PhD, Kirby Reed (Gilson, Inc.)

Introduction

Nutraceuticals or "phytoceuticals" can be defined as natural products that are used to supplement the diet by increasing the total dietary intake of important nutrients. This definition includes nutritional supplements such as vitamins, minerals, herbal extracts, antioxidants, amino acids, and proteins. Ephedra is a naturally-occurring substance derived from the Chinese herbal Ma Huang. Its principle active ingredient is ephedrine, which, when chemically synthesized, is regulated as a drug. While products containing natural ephedrine alkaloids have long been used to treat respiratory symptoms in traditional Chinese medicine, in recent years they have been extensively promoted and used to aid weight loss, enhance sports performance and increase energy.

The FDA's concerns about dietary supplements containing ephedra arise in part from ephedra's mechanism of action in the body. Ephedra is an adrenaline-like stimulant that can have potentially dangerous effects on the nervous system and heart. Ephedrine has a chemical structure similar to amphetamines, differing only in that ephedrine is less potent. The FDA proposed to prohibit marketing of dietary supplements containing 8 mg or more of ephedrine per serving; therefore it is imperative to be able to determine the quantity of ephedra found in various foods and dietary supplements.

The purpose of this application is to present the advantages of using an automated solid phase extraction method coupled with HPLC to determine the various levels of ephedra and ephedra-like compounds in dietary supplements versus manual solid phase extraction.

Materials & Methods

Chemicals and Reagents

Pseudoephedrine

Ephedrine

Norephedrine

Methylephedrine

Dietary Supplement

- Yellow Jacket
- Stacker 3
- Stacker 2 Lite

Methanol

Formic acid

Hydrochloric acid

Ammonium hydroxide

Ammonium bicarbonate

0.45 µm filters

Instruments and Accessories

SPE System:

SPE 215, 4 probes, 5-mL syringes, 175-mm Z arm

Water Oasis MCX solid phase extraction columns, 3 cc, 60 mg

HPLC System:

322 pumps, (≤15 mL/min), 215 Liquid Handler, 125 mm Z arm, 819 Injection Module, 7010 Rheodyne valve, 50-µL sample loop

Waters Xterra Column: Phenyl, 4.6 x 150 mm, 3.5 µm

155 UV/VIS Dual-wavelength Detector with analytical flow cell, 5.0-mm path length

735 Sampler Software, version 5.1, Pentium 4, >2 GHz, 512 MB RAM, 80 GB Hard Drive

UniPoint™ System Software Version 3.3, 506C interface

Description of the Procedure

The following stock solutions were prepared for the solid phase extraction procedure:

1% Formic acid

0.1 N HCl

25% MeOH/75% NH_4OH (5% solution)

95% MeOH/5% NH_4OH (5% solution)

10 mM NH_4HCO_3, unadjusted pH 7.9–8.1

10 mM NH_4HCO_3, pH 9.45

80% MeOH/NH_4OH, pH 11.0

Ephedra Alkaloid Extraction and SPE Sample Preparation[(1)]

Sample preparation is equivalent to the FDA LC/MS/MS Method. Weigh 10 grams of sample (dietary supplements) into a 100-mL volumetric flask. Add 20 mL of water and mix, then add 50 mL of MeOH. Sonicate the solution for at least an hour at ambient temperature. Allow the solution to cool, bring to volume with MeOH. Allow the suspended solids to settle. Filter a 3-mL aliquot through a 0.45-µm filter before subjecting the samples to SPE. Use 2 mL of the filtered sample extract for solid phase extraction.

SPE Protocol

Acidify 2 mL of the sample extract with 5 mL of 1% formic acid. Condition the cartridges with 1 mL of MeOH, followed by 1 mL of DI water. Load all of the acidified sample extract into the cartridges. Wash with 1 mL of 0.1N HCl (removes weakly retained cations), then wash with 1 mL of MeOH (removes neutral organics). Equilibrate with 1 mL of DI water. Wash with 1 mL of 25% MeOH/75% NH_4OH (5% solution). Elute ephedra alkaloids with 3.0 mL of 95% MeOH/5% NH_4OH (5% solution). Bring up to 5 mL with 10 mM NH_4HCO_3, unadjusted pH(~7.9–8.1). Analyze as soon as possible (<60 hours), as ephedra degradation is observed at alkaline pH over an extended time.

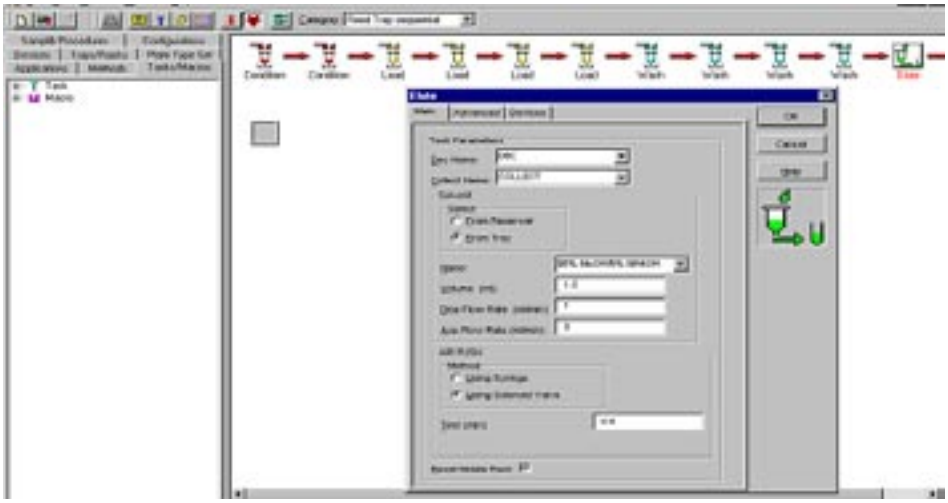

Photo 1: 735 Sampler Software

The 735 Sampler Software Method employed to run the automated solid phase extraction columns on the ephedra extracts, the pictures represents the ease that one can drag and drop tasks into the method and optimize each step as required by the method.

Ephedra Extracted Chromatographic Conditions

System: Gilson 215 Analysis System with 322 Series Pumps and 155 UV/VIS Detector

Column: Waters Xterra Phenyl, 4.6 x 150 mm, 3.5 µm

Mobile Phase: 10% Acetonitrile/90% 10 mM NH_4HCO_3, pH 9.45, isocratic

Column Heater: 50°C

Flow Rate: 1.0 mL/min., back pressure ~2100 psi

Injection Volume: 50 µL

Needle Wash: 80% MeOH/NH_4OH, pH 11

Wavelength: 210 nm, .001 AUFS

Chromatographic Run Time: 25 min.

Results

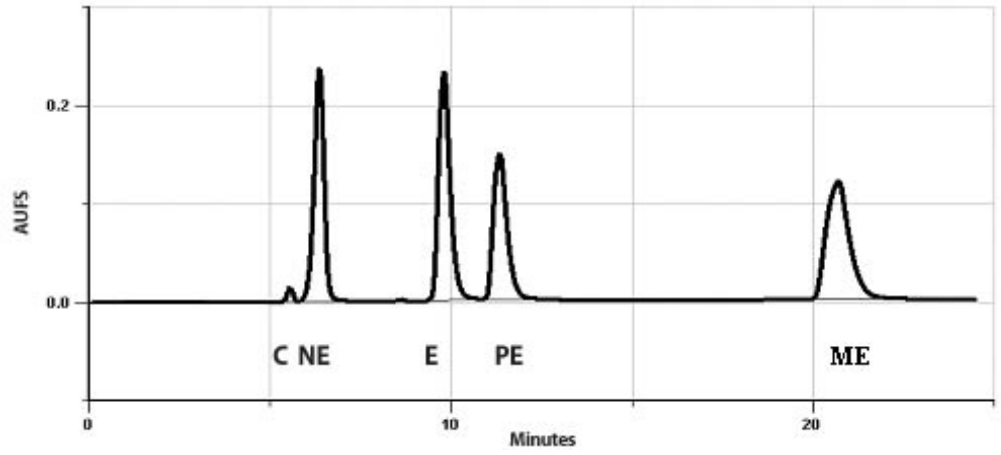

Figure 1: Example Chromatogram Obtained from the Extraction of Herbal Dietary Supplements

The chromatogram from the extraction of a standard solution containing: caffeine (C), norephedrine (NE), ephedrine (E), pseudoephedrine (PE) and methylephedrine (ME). The concentration was 400 µg/mL on the SPE column. Caffeine is a major component of ephedra-containing supplements, usually 5–20 times the quantity of the ephedras.

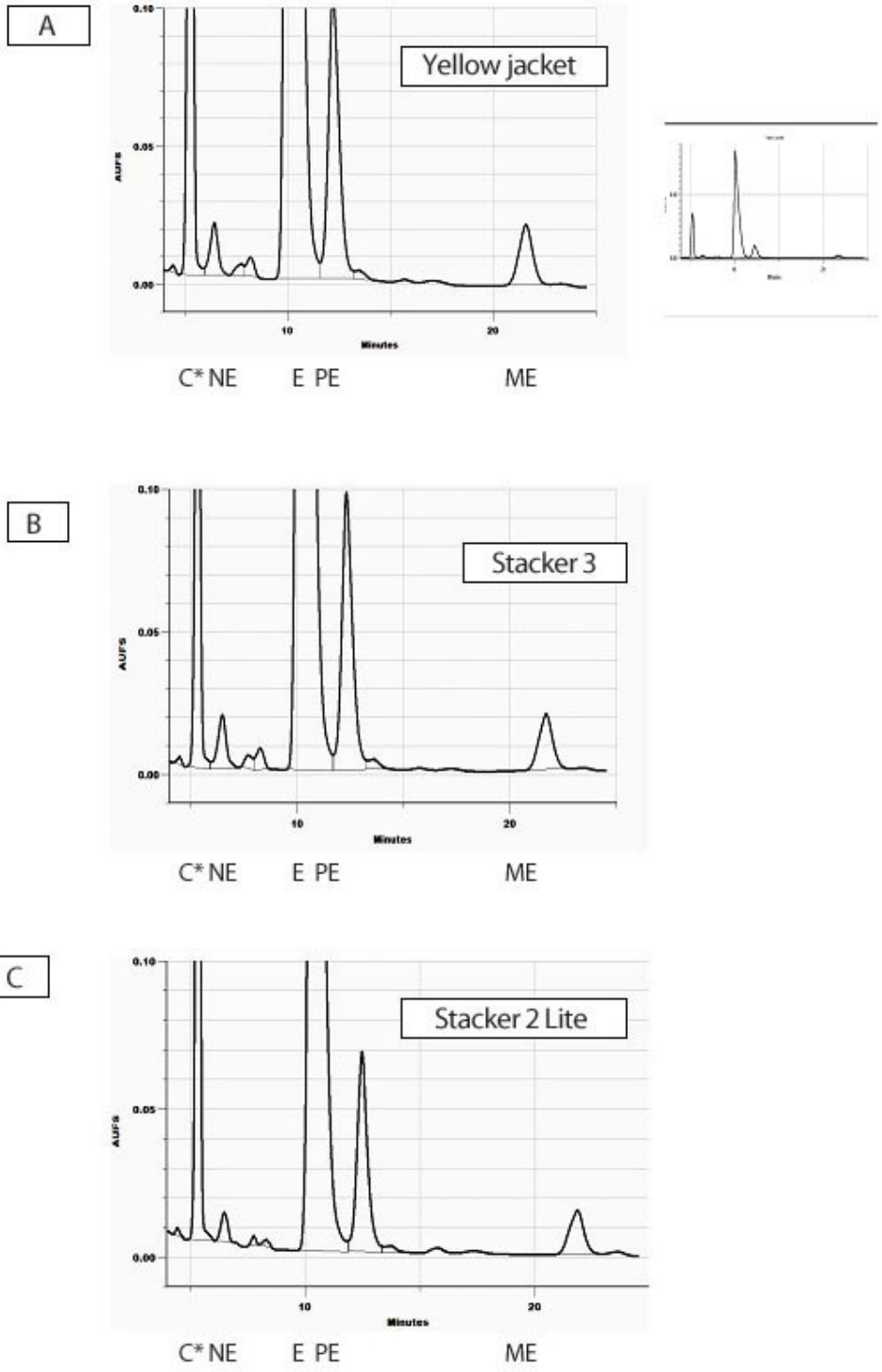

Figure 2, 3, 4: Chromatograms Obtained from the Extraction of Over-the-Counter Herbal Diet Supplements*

Peaks: C = caffeine, NE = norephedrine, E = ephedrine, PE = pseudoephedrine, ME = methylephedrine.

A: **Yellow Jacket**, proprietary blend (661 mg): ephedra extract/sida cordifolia, supplying 25 mg ephedrine alkaloids; kola nut extract, supplying 300 mg caffeine alkaloids; citrus aurantium, supplying 6 mg synephrine.

B: **Stacker 3**, proprietary blend (550 mg): ephedra extract, supplying 25 mg ephedrine alkaloids; kola nut extract, supplying 250 mg caffeine alkaloids.

C: **Stacker Lite**, proprietary blend (260 mg): ephedra extract, supplying 12.5 mg ephedrine alkaloids; kola nut extract, supplying 100 mg caffeine alkaloids; white willow bark, chromium picolinate.

*The chromatograms are magnified in order to view the trace amounts of the nor-, pseudo-, and methylephedrine. All peaks are on scale (see inset above in chromatogram A).

Expected Ephedra Concentrations for Botanicals, Extracts, and Dietary Supplements (based on the *AOAC Ephedra Validation Task Group: preliminary data)*

in µg/g

Norephedrine = 240

Norpseudoephedrine = 400

Ephedrine = 20,064

Pseudoephedrine = 4,955

Methylephedrine = 702

Methylpseudoephedrine = 198

in µg/mL

Norephedrine = 0.96

Norpseudoephedrine = 1.6

Ephedrine = 80.3

Pseudoephedrine = 19.8

Methylephedrine = 2.8

Methylpseudoephedrine = 0.8

For a 1 g sample extracted with 100 mL of 80% MeOH and SPE diluted 2 mL into 5 mL dilution solution.

Ephedra Product	Amount (µg/mL)	Amount (µg/mL)	Amount (µg/mL)	Amount (µg/mL)
n = 8	**Norephedrine**	**Ephedrine**	**Pseudoephedrine**	**Methylephedrine**
Yellow Jacket	5.2	339.6	44.9	11.0
Stacker 3	5.0	266.3	43.1	10.6
Stacker 2 Lite	3.2	235.8	37.1	10.4

Table 1: Standard Curve for Each Ephedra-like Component

The amounts shown above were derived from a standard curve for each ephedra-like component (10 g extracted). The amount is an average from two different automated SPE assays within a two week period. The ephedra samples from the initial extracts were used in both assays.

Ephedra Product	Norephedrine		Ephedrine		Pseudoephedrine		Methylephedrine	
	A	M	A	M	A	M	A	M
	CV (%)		CV (%)		CV (%)		CV (%)	
Yellow Jacket	7.4	10.6	6.1	11.3	7.6	9.6	6.8	10.6
Stacker 3	7.1	16.7	6.2	16.5	5.8	16.9	7.7	18.7
Stacker 2 Lite	5.0	12.3	4.0	7.6	3.8	7.1	4.7	7.1

Table 2: Variance Determined for the Ephedra Extracts: Automated and Manual SPE Methods

A = Automated, M = Manual, CV = Coefficient of Variance.

Summary

- The application represents a novel automated method for the analysis of ephedra alkaloids.
- Automation of the SPE procedure allows for a high degree of optimization not available in a manual procedure.
- Retention of the highly water soluble amines occurs at an alkaline pH.
- Use of a transparent buffer enhances analyte confirmation and allows for the use of MS detection.
- Detection at 210 nm is required in order to quantitate the trace nor- and methyl- analogs.
- Caffeine (common ingredient of dietary supplements) can be 5 to 20 times the ephedra alkaloid concentration. Caffeine elutes before the ephedra alkaloids at alkaline pH and does not interfere with their identification.
- Some interferences will influence integration and precision associated with the analysis of the ephedra alkaloids extracts; therefore, solid phase extraction is required.

Conclusion

The extraction of ephedra alkaloids from dietary and food supplements is presented in this application. Natural products are a complex matrix, such that interfering peaks may compromise the identification and quantitation of components and trace components. The automated solid phase extraction procedure presented in this application is required in order to remove much of the interfering matrix that would otherwise interfere with analysis at 210 nm—the necessary wavelength for detection of ephedra alkaloids.

Using a standard solution (1g/100 mL extraction), the ephedrine response approached 2 AUFS and showed a non-linear response. A less concentrated sample places ephedrine within the linear range; however, the lower concentrations make the analysis of the trace components difficult. As shown in Figure 1, ephedrine and pseudoephedrine are >90% of all the present ephedra alkaloids found within the supplements. The method is compatible with MS for on-line analysis, since 10% AcCN/10 mM NH_4HCO_3, pH 9.4 is a volatile buffer. Caffeine is a major component of these dietary supplements, and although it may be more than 5 times the ephedra alkaloid quantity, it elutes prior to norephedrine and does not interfere with the analysis of the ephedra alkaloids.

The automated SPE 215 allows for adjustments in the addition rates of each solution and sample extract to the SPE columns. Optimization by the automated SPE 215 enhances recovery, which is imperative for natural products—where many components may be present in trace amounts and where the sample may contain particulates.

This degree of control is not possible with a manual method; therefore, the automated SPE 215 plays an integral part in the sample preparation of natural products. (In the case of the ephedra extracts, the ephedrine and pseudoephedrine are at high concentrations relative to the trace nor- and methyl-analogs.) This is apparent when comparing the CVs for the automated versus manual SPE method. The CVs for the ephedra-like compounds from the extracts were in the 4–8% range for the automated SPE 215 method, whereas the CVs were twice as high for the manual SPE procedure (8–19%). The automated SPE 215 also showed a higher extraction rate (relative recovery) versus the manual SPE method, (a 2–40% increase in extraction for the compounds relative to the sample extract). This is attributable to the fact that all rates and gas pushes of the solvents/sample through the columns are adjustable through the software.

Reference

(1) J. Krol, "The Ephedra Story: Analysis of Ephedra Alkaloids Using LC/UV, Part 2: The Sample Preparation Validation," Waters Corp. (Milford, MA, 2002).

Gilson, Inc. World Headquarters
3000 W. Beltline Hwy., P.O. Box 620027, Middleton, WI 53562-0027 USA
Telephone: 800-445-7661 or 608-836-1551• Fax: 608-831-4451

Gilson S.A.S.
19, avenue des Entrepreneurs, BP 145, F-95400 VILLIERS LE BEL France

www.gilson.com

sales@gilson.com, service@gilson.com, training@gilson.com