

# Determination of Caffeine and Sympathomimetic Alkaloids in Weight Loss Supplements by High-Performance Liquid Chromatography

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## Abstract

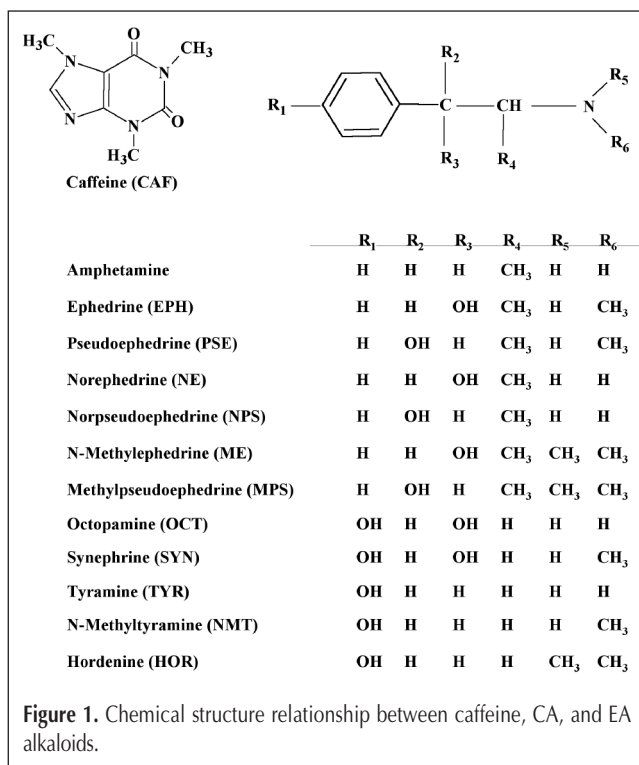
Reversed-phase high-performance liquid chromatography utilizing photodiode array detection is used for the simultaneous determination of caffeine and nine alkaloids from *Citrus aurantium* (CA) and *ephedra* (EA) contained in dietary weight loss products. Since the Food and Drug Administration (FDA) ban of EA, manufacturers have substituted CA in their weight loss formulations, usually combined with high levels of caffeine. The alkaloids contained in CA have some physiological effects similar to those of the EA alkaloids and are, therefore, cause for concern. Caffeine has been shown to potentiate the toxicity of the EA alkaloids. Recently, a federal judge overturned the absolute ban and allowed marketing of low levels (< 10 mg/day) of total EA alkaloids. To support an absolute ban, the FDA is now compelled to perform dose-dependent toxicology studies to determine the toxic dose(s) of EA. The toxicity of the CA compounds is largely unknown, especially in combination with caffeine. The described method enables quantitation over a wide range of product formulations. Recoveries range from 91% to 100% from a variety of fortified plant matrices.

## Introduction

On April 11, 2004, the Food and Drug Administration (FDA) banned the sale of dietary supplements containing *ephedra* (EA) alkaloids due to the unacceptable risk associated with the use of these products (1). On April 14, 2005, a federal judge overturned the absolute ban on procedural grounds and allowed marketing of low levels (< 10 mg/day) of total EA. In the year 2001, products containing EA resulted in almost twice the number of adverse reactions (1178) in the United States as all other herbal products combined (2). Since the ban, many manufacturers of dietary weight loss products have substituted the herb *Citrus aurantium* (CA), otherwise known as bitter orange (3). Through the MedlinePlus and CAERS adverse reaction reporting websites, there are already claims of dozens of adverse reactions reportedly

linked to the use of synephrine (SYN), a major component of CA (4). In addition, in at least one instance, there has been a statistical link made through the Naranjo probability scale between the chronic use of CA and myocardial infarction (5).

EA-derived amines (Figure 1) consist of 3 pairs of diastereomeric alkaloids: ephedrine (EPH), pseudoephedrine (PSE), norephedrine (NE), norpseudoephedrine (NPS), *N*-methylephedrine (ME), and *N*-methylpseudoephedrine (MPS). The principal alkaloids in the raw botanical are EPH and PSE, with EPH comprising up to 80% of the total alkaloid content (6). CA contains the alkaloids SYN, octopamine (OCT), tyramine (TYR), hordenine (HOR), and *N*-methyltyramine (NMT), with SYN being the primary component (7). The CA-derived amines differ from those of EA primarily by the presence of a hydroxyl group attached to the aromatic ring (Figure 1). The addition of a ring



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hydroxyl to CA amines results in the class of compounds termed catecholamines, which are present in the human as neurotransmitters in the sympathetic nervous system. Oral activity is greatly accentuated in the noncatechols with the inclusion of a methyl group on the  $\alpha$ -carbon adjacent to the amino group (8). Caffeine (CAF) is a methylxanthine and is structurally unrelated to the CA and EA compounds (Figure 1).

It is the structural similarity to the sympathomimetic catecholamine neurotransmitters norepinephrine and epinephrine that is the basis for the CA and EA amines proposed lipolytic action (7,9,10). Unfortunately, it is this same structural similarity that is the ultimate cause of the adverse reactions seen with the EA compounds. There is evidence of a lipolytic action by the CA compounds resulting from stimulation of the  $\beta_3$ -receptor (10). However, sympathomimetics stimulate to some degree at least two or more of the  $\alpha$  and  $\beta$  subgroups of receptors throughout the sympathetic nervous system. The consequence of receptor stimulation is often cardiovascular in nature (11). In the obese, cardiovascular function is already somewhat compromised with a tendency toward high blood pressure, increased sympathetic tone, and left ventricular mass (12,13). Stress combined with additional stimulation by the consumption of weight loss supplements could have serious effects on these individuals. CAF is a well-known cardiovascular stimulant when used alone, and has been shown to potentiate the toxicity of the EA compounds (14–16). Large quantities of CAF are routinely added to weight loss supplements, which could synergistically magnify the potential danger of these products.

Several analytical methods have been developed for the analysis of the EA alkaloids in a variety of matrices (6,17–22). Of these, only a few have analyzed for EA alkaloids from various complex herbal mixtures (6,20–22). Recently, the analysis of EA alkaloids combined with CAF in dietary supplements has been performed by liquid chromatography tandem mass spectrometry LC–MS–MS (23,24). There have been at least two methods reporting the analysis of EA alkaloids along with SYN utilizing either solid-phase extraction sample cleanup (6) or ion exchange precolumn enrichment (25). Methods found for quantitating the CA alkaloids focused on SYN, OCT, and TYR only, but did not present chromatograms of complex matrices and interferences to analysis (26–28). Only one method has been published which analyzed CAF along with a least one CA and EA alkaloid (29). That method utilized a variant of the methods of Gurley (22) and Okamura (18) in analyzing commercial weight-loss supplements for CAF, SYN, and EPH in addition to forskolin, icariin, and yohimbine. However, the determination of CAF required an additional dilution and injection. The total runtime was 40 min, yet the chromatography did not separate PSE from EPH or quantitate NMT. The separation and quantitation of PSE and EPH individually is important for physiological as well as regulatory reasons. PSE is present in secondary abundance in EA and NMT is one of the more naturally abundant CA alkaloids. These methods were not of use in the analysis of CAF along with all CA and all EA compounds, therefore, another analytical method was required to meet our goals.

A method was needed, capable of quantitating CAF along with all the CA and EA alkaloids due to the potential for product adulteration or legal fortification with one or more of the EA or CA

alkaloids and CAF. Adulteration of herbal products with synthetic compounds has been documented even in oriental commercial sources (30). A method was desired that was cost-effective for the purpose of analyzing large numbers of samples. Also, mobile phase components such as tetrahydrofuran (THF) should be removed to prolong column life and limit possible sample degradation (22). Evaluation of the CA and EA alkaloids relative abundance from the original source herb extracts often used in commercial products was desirable for the purpose of establishing baselines of relative alkaloidal content. Separating, quantitating, and summing the individual EA alkaloids chromatographically is now required for regulatory enforcement of the FDA restriction of less than 10 mg/day EA alkaloids. Due to the high levels of CAF possible from direct addition to dietary supplements combined with indirect sources such as guarana (*Paullinia cupana*), cocoa extract (*Theobroma Cacao*), green tea (*Camellia sinensis*), and yerba mate (*Ilex paraguariensis*), CAF should be monitored as well. Modification of the methods of Gurley (22) and Schaneberg (29) was used in the successful resolution and quantitation of these compounds from a wide variety of commercial weight-loss products.

## Experimental

### Reagents and samples

(1R,2S)-(–)-Ephedrine hydrochloride (99.3% pure), (1S,2S)-(+)-pseudoephedrine hydrochloride (99.3% pure), (+)-norpseudoephedrine hydrochloride (99.3% pure, 1 mg dissolved in methanol), (1R,2S)-(–)-norephedrine (99.6% pure), (1R,2S)-(–)-*N*-methylephedrine (99% pure), and (1S,2S)-(+)-*N*-methylpseudoephedrine (100% pure) were obtained from Chromadex (Santa Ana, CA). Sodium lauryl sulfate (NF/FCC), anhydrous caffeine, ( $\pm$ )-synephrine (min. 99.5% pure), tyramine hydrochloride (min. 99.5% pure), and octopamine hydrochloride (99.55% pure) were obtained from Sigma Chemical Co. (St. Louis, MO). Hordenine sulfate (98.0% pure) was obtained from TCI America (Portland, OR). *N*-Methyltyramine was graciously supplied by Dr. Iklas A. Khan from the National Center for Natural Products Research at the University of Mississippi. Acetonitrile and methanol were HPLC grade and obtained from JT Baker (Phillipsburg, NJ). Sulfuric acid was also obtained from JT Baker. L-Ascorbic acid (99.5% pure) was American Chemical Society grade and obtained from Fisher Scientific Co. (Fair Lawn, NJ). *Citrus aurantium* raw herbal extracts (standardized to 6%, 30%, and 90% SYN) were obtained from Modern Nutrition and Biotech, Inc. (Appleton, WI). Crude and commercial grade *Ephedra sinica* Stapf raw herbal extract was obtained from the Jinke Group USA, Inc. (Diamond Bar, CA). All raw herbal extracts originated in China. Weight loss supplements were obtained from various local retail sources. The EA-containing weight loss supplements were obtained prior to the FDA ban while the remainder were obtained approximately six months afterwards. Any water used was deionized of 18 M $\Omega$ /cm quality. The individual product brand names or manufacturers will not be given here but may be revealed upon written request. Any potential violations of FDA labeling requirements discovered were made available to the regulatory branch of the FDA.

### Prepared reagents and standards

A spiking solution consisting of 10 mg/mL CAF, 2 mg/mL SYN, and 1 mg/mL of each of the following; TYR, HOR, NE, PSE, EPH, and ME was prepared along with L-ascorbic acid (0.05 mol/L) in 30% methanol in water. The concentration of all hydrochloride and sulfate salts used was adjusted so that all concentrations were of the free bases. NMT was not included in the spiking solution due to the insufficient quantity on hand. However, a 100 µg/g solution of NMT in water was prepared and 1-mL aliquots divided and frozen until time of use for stock solution preparation. Standard stock solution concentrations were adjusted so that their detector response was on the linear portion of the concentration vs. response curve. The standard stock solution was prepared in 50:50 mobile phase A (17% acetonitrile in 30 mmol/L sodium lauryl sulfate, pH 2.5) and 30% methanol in water. An antioxidant (0.025 mol/L ascorbic acid of 99.5% purity) was added to aid stability. At the time of use, 2.700 mL of standard stock solution was combined with 0.300 mL of NMT solution to result in final compound concentrations of 2.5 mg/mL CAF, 70 µg/mL EPH, 50 µg/mL of PSE and SYN, 30 µg/mL ME, 10 µg/mL NMT, and 5.0 µg/mL of OCT, TYR, HOR, and NE.

## Methods

### Recovery

Triplicate 1.00-g aliquots of either *Citrus aurantium* raw herbal extract (standardized to 6% SYN) or crude *Ephedra sinica* raw herbal extract were placed in identical 50-mL glass polytetrafluoroethylene (PTFE)-lined screw-capped tubes. The raw herbal extracts were fortified with 1.00 mL of spiking solution. Each tube was rolled to disperse the spiking solution throughout the extract and allowed to equilibrate for 15 min prior to extraction. Unfortified control samples of either extract were also prepared. A 30 mL volume of 70% methanol in water was added to each tube and the contents mixed for 1 h on an Eberbach reciprocating shaker (Ann Arbor, MI) set on high speed. Tubes were then centrifuged at 200 × *g* for 10 min. The supernatant was transferred to respective 70-mL glass PTFE-lined screw-capped tubes and a 10 mL volume of 70% methanol in water was added to each remaining extract. Tubes were mixed for 15 min on the reciprocating shaker followed by centrifugation for 10 min. Following the decanting of supernatant, this process was repeated twice more with 10 mL volumes of extraction solvent. The volume of each was adjusted to 60 mL with extraction solvent and a graduated cylinder. After mixing, an aliquot (5 mL) of each was transferred to a 6 mL capacity Bond Elut Reservoir (Varian, Inc., Palo Alto, CA) attached to a 25 mm syringe filter with 0.45 µm nylon membrane (Pall Gelman Laboratory, Ann Arbor, MI). A vacuum chamber was utilized to filter the sample extracts into 13 × 100 mm glass culture tubes (Kimble/Kontes Inc., Vineland, NJ). Spiking standard (1.00 mL) was diluted to 60 mL with extraction solvent and processed in the same manner as the fortified samples were following filtration.

### Dietary supplements and raw herbal extracts

Triplicate 1.00 g amounts of 20 different dietary weight-loss

supplements were weighed and transferred to 50-mL glass PTFE-lined screw-capped tubes. These were extracted with 70% methanol in water in exactly the same manner as the fortified herbal extracts. Some of the commercial supplements were in tablet form, which necessitated pulverization with mortar and pestle. Following filtration, 1.00 mL aliquots from each control extract, fortified sample, standard, or supplement sample were combined with 1.00 mL of HPLC mobile phase A and vortexed. These were transferred into amber autoinjector vials for subsequent analysis. The instrument sample sequence was arranged such that triplicate samples were bracketed by the appropriate standards. Extraction and analysis of the crude and commercial *Ephedra sinica* raw herbal extracts were performed in the same manner as the supplements. Extraction of different *Citrus aurantium* raw herbal extracts (6%, 30%, and 90% SYN) utilized progressively decreasing quantities of herb depending on expected SYN concentration.

### HPLC analysis

HPLC instrumentation used was manufactured by the Waters Corporation (Milford, MA) and included a Model 600E HPLC system controller, 717 plus autoinjector and 996 photodiode array detector. The PDA wavelength region from 200 nm to 300 nm was acquired. Extracted detector wavelengths used for quantitation were 206 nm for EA alkaloids, 222 nm for CA alkaloids, and 297 nm for CAF. Other than for CAF, these wavelengths represented the UVmax for EA and CA alkaloids. The wavelength chosen for CAF minimized interferences while placing the peak area for all samples well within the linear portion of the CAF standard curve. Injections of samples or standards (50 µL) were passed through a Synergi Hydro-RP 250 mm × 4.60 mm (4 µm particle) polar end-capped C18 HPLC column (Phenomenex Corp., Torrance, CA). The column temperature was held at 30°C with a column temperature controller (Thermasphere TS-130, Phenomenex Corp., Torrance, CA). Mobile phase A consisted of 17% acetonitrile in 30 mmol/L sodium lauryl sulfate adjusted to pH 2.5 with concentrated sulfuric acid. Mobile phase B consisted of 43% acetonitrile in 30mM sodium lauryl sulfate, also acidified. Mobile phase A was maintained at 1.1 mL/min for 2.5 min prior to a step change to B. Mobile phase B was maintained at the same flow rate for 30.5 min prior to switching back to mobile phase A for an 8 min column re-equilibration prior to the next injection. At the time of preparation, both mobile phases were passed through a 0.2 micron Teflon filter and sparged with helium prior to use.

## Results and Discussion

The PDA responses for a series of SYN standards ranging from 0.01 µg/mL (0.6 µg/g) to 250 µg/mL (15.0 mg/g) were linear ( $R^2 = 0.998$ ). Over a similar HOR and OCT standard range, the regression analysis resulted in  $R^2$  of 1.00 and 0.999, respectively. The PDA responses for a series of EPH standards ranging from 0.01 µg/mL to 500.0 µg/mL (0.6 µg/g to 30.0 mg/g) were linear ( $R^2 = 0.985$ ) as were the standard responses to CAF from 0.004 µg/mL (0.24 µg/g) up to 200.0 µg/mL (12.0 mg/g) with a  $R^2 =$

0.996. The identity of each alkaloid peak was tentatively confirmed by comparison of the retention time to that of the standard. In addition, identity was checked by comparing the acquired PDA spectra from 200 nm to 300 nm for each alkaloid peak to that of a standard. Tabulated results are expressed as the mean ( $n = 3$ ) of the measurements and all values resulted in % relative standard deviations (%RSD) < 10% (Tables I and II). Repeatability and reproducibility of the method was determined for SYN and CAF using Supplements #17 and #18. Repeatability (within-day) %RSD averaged 3.4% and 3.1% for SYN and CAF,

respectively. Reproducibility (between-day) %RSD averaged 8.6% and 2.9% for SYN and CAF, respectively.

Recovery of caffeine and CA alkaloids from fortified crude *Ephedra sinica* raw herbal extract ranged from 91 to 100% of the fortified amount. Recovery of the CA alkaloids ranged from 91% for OCT to 95% for SYN. The recovery of the EA alkaloids from fortified *Citrus aurantium* (labeled 6% SYN) raw herbal extract was similarly quantitative (> 95%). Crude *Ephedra sinica* raw herbal extract, *Citrus aurantium* raw herbal extract and Metabolite tablets (without CAF or CA alkaloids) were used to estimate control backgrounds for each alkaloid. However, because background levels vary depending on the particular supplement being analyzed, the limit of detection (LOD) for each compound was determined utilizing the Root Mean Square Error (RMSE) method (31). From these data, the limit of quantitation (LOQ) was estimated as 3.33 times the LOD. For CAF, the LOD was 9.33  $\mu\text{g/g}$  (LOQ 31.1  $\mu\text{g/g}$ ) while for OCT the LOD was 38.9  $\mu\text{g/g}$  (LOQ 129  $\mu\text{g/g}$ ). Both TYR and SYN had an LOD of 6.97  $\mu\text{g/g}$  (LOQ 23.2  $\mu\text{g/g}$ ) while HOR and NMT had LOD of 23.3  $\mu\text{g/g}$  (LOQ 77.6  $\mu\text{g/g}$ ). EPH was determined to have an LOD of 5.57  $\mu\text{g/g}$  (LOQ 18.5  $\mu\text{g/g}$ ) and represented all the EA alkaloids due to their structural similarity and the extremely low background levels.

Analysis of the supplements shows a wide range of CAF and individual alkaloid levels (Table I and II). In Supplement formulations # 3, 6, 8, 10, 14, 16, 17, 18, and 20, up to 167 mg/g CAF was found which was derived totally from plant sources such as yerba mate, green tea, cocoa, and guarana. In other formulations (Supp. # 4, 13, and 19), additional anhydrous CAF was added to that already present in the plant material resulting in extremely high levels in some. CAF levels ranged from 36.7 mg/g in Supplement # 11 to 362 mg/g in Supplement # 4.

Chromatograms showed HOR < LOQ for all 20 supplements, however, HOR was quantitated in the *Citrus aurantium* raw herbal extracts (standardized to 30% and 90% SYN) at 1.77 and 0.500 mg/g, respectively. Supplement concentrations of OCT ranged from < LOQ to the 187 mg/g found in Supplement #15 (Figure 2). Pharmaceutical grade OCT was used legally to fortify this particular supplement to obtain such a high level. Calculation of the manufacturer's recommended maximum total daily dose (MRDD) from the container labeling information further exhibited the disparity of concentrations between supplements. Supplement #7 had a MRDD of 125 mg caffeine but contained only trace amounts of SYN (even though the manufacturer claimed it contained 19 mg CA), while the MRDD for Supplement #15 was 517 mg CAF combined with 867 mg CA compounds (Table I).

**Table I. Concentrations (mg/g) of Caffeine and CA Alkaloids in Weight Loss Supplements. LOQ (CAF) = 31.1  $\mu\text{g/g}$ , LOQ (NMT & HOR) = 77.6  $\mu\text{g/g}$ , LOQ (SYN & TYR) = 23.2  $\mu\text{g/g}$ , LOQ (OCT) = 129  $\mu\text{g/g}$ . (-) = < LOQ**

Supplement	CAF	OCT	SYN	TYR	NMT	MRDD (caf)	MRDD (ca)
1	-	-	-	-	-	-	-
2	152	-	6.27	-	0.509	340	15.5
3	167	-	3.34	-	0.157	401	8.54
4	362	-	9.41	0.071	0.544	706	19.7
5	94.7	-	1.77	-	0.137	438	8.96
6	126	-	12.8	0.089	0.623	605	65.3
7	59.5	-	0.079	-	-	125	0.170
8	55.5	-	8.06	0.092	0.290	253	39.1
9	119	0.129	6.87	-	0.190	486	36.9
10	119	-	12.4	0.113	0.516	536	58.5
11	36.7	-	5.32	-	0.138	339	50.6
12	171	-	-	-	-	728	-
13	336	-	3.78	-	0.474	585	7.41
14	77.9	-	6.79	-	0.420	427	39.9
15	118	187	9.98	0.069	0.394	517	867
16	57.8	-	2.32	-	0.163	194	8.53
17	124	-	3.26	-	0.135	317	8.78
18	143	-	6.32	0.054	0.179	440	20.3
19	334	-	-	-	-	387	-
20	162	-	-	-	-	492	-
CA Extract (6% SYN)	-	1.02	72.5	0.860	6.31	n/a	n/a
CA Extract (30% SYN)	-	2.67	348	0.368	7.49	n/a	n/a
CA Extract (90% SYN)	-	3.88	925	0.162	4.70	n/a	n/a

**Table II. Concentrations (mg/g) of EA Alkaloids in Weight Loss Supplements. LOQ = 18.5  $\mu\text{g/g}$**

Supplement	NPS	NE	PSE	EPH	ME	MRDD(ea)
1	n/a	0.059	0.64	6.26	0.190	73.2
2	n/a	0.296	1.55	12.4	0.180	32.5
3	n/a	0.054	3.10	4.23	0.651	18.1
4	n/a	0.356	6.10	25.2	4.45	70.6
5	n/a	0.084	1.67	6.61	0.200	39.7
Crude <i>Ephedra sinica</i>	0.580	0.600	9.60	32.5	2.20	n/a
Commercial <i>Ephedra sinica</i>	0.450	0.570	9.07	77.5	1.94	n/a

Powder and protein supplements with high levels of cocoa flavoring were not analyzed due to the interferent phenylethylamine. Among the EA-containing supplements tested, EPH content varied from 4.23 mg/g to 25.2 mg/g and the MRDD from 18.1 mg/day to 73.2 mg/day total EA alkaloids (Table II). Such disparity in supplement EA content has been documented previously (24,32). Also, of the five EA containing supplements analyzed, the MRDD of commercial raw products ranged from 2.20 to 10.2 g. Similar differences were seen across all products examined. Due to this extreme variability, calculation of the MRDD of commercial product is required on an individual basis. Another name for NE or norephedrine is phenylpropanolamine (PPA). This adrenergic amine has been removed by order of the FDA from all prescription and over-the-counter decongestants due to the excess risk of fatal hemorrhagic stroke. In the five EA-containing supplements, the highest MRDD of NE is only 0.694 mg obtained with Supplement # 4. The chromatography resolved NPS from the other EA alkaloids; however, NPS was not quantitated due to the unavailability of a suitable quantity of standard material. NPS (cathine) is a DEA schedule IV drug in pure form and is difficult to obtain and maintain the required paperwork.

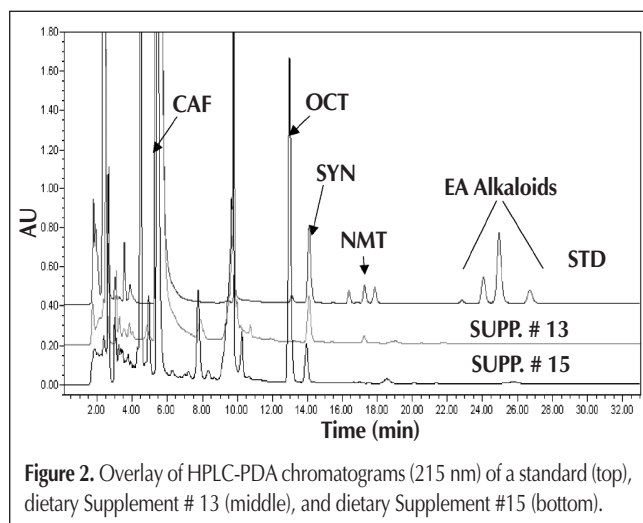


Figure 2. Overlay of HPLC-PDA chromatograms (215 nm) of a standard (top), dietary Supplement # 13 (middle), and dietary Supplement #15 (bottom).

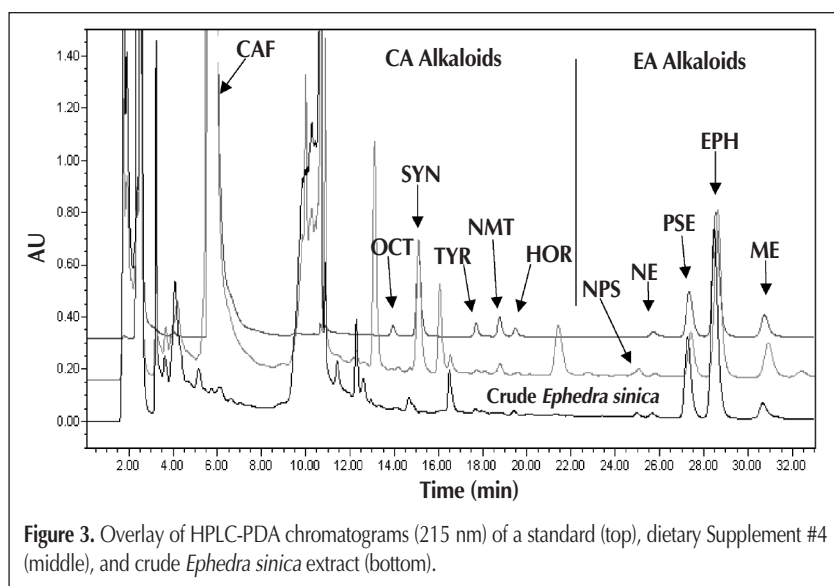


Figure 3. Overlay of HPLC-PDA chromatograms (215 nm) of a standard (top), dietary Supplement #4 (middle), and crude *Ephedra sinica* extract (bottom).

Because NPS is present in such small amounts in both the crude and commercial *Ephedra sinica* raw herbal extract, it was decided that quantitation was not necessary (Figure 3).

Analysis of the raw herbal extracts revealed alkaloidal selectivity in the manufacturer's herbal extraction and purification ability. The *Citrus aurantium* raw herbal extracts had a progressively higher SYN content (from 6% to 90% standardized to SYN) as expected; however, the concentration of the other alkaloids showed little variation between extracts (Table I). The same was true of the crude and commercial *Ephedra sinica* raw herbal extracts. The EPH was much higher in the commercial *Ephedra sinica* raw herbal preparation, accompanied by no significant increase in the other EA alkaloids (Table II). It has been shown that EA alkaloidal concentrations vary widely between various *Ephedra* species (33). The manufacturer may legally use any of more than 31 *Ephedra* species or any one of several "purified" commercial extracts of a single species in dietary weight loss formulations.

There was insufficient evidence on the labels to ascertain the disparities observed between supplements in alkaloidal content or daily alkaloidal consumption. The true content of CAF is often much greater than that shown on labels, due to CAF being present in many herbs included in the formulation. These disparities may be inherently dangerous considering the pharmacology of these compounds and the already compromised cardiovascular function found in the obese (13). An additional high risk group is those who take weight loss supplements for enhancement of athletic performance due to the amphetamine-like stimulants they contain. A recent survey found that 30% of responders took these supplements strictly to aid performance (34). It would not be surprising to find that many exceed the MRDD on a routine basis. Many with an already enhanced cardiovascular system resulting from the stress of exercise may expect an additive effect on blood pressure and heart rate when consuming large quantities of weight loss supplements containing CAF, CA, and EA (8,15). Compounding the risk is the possibility that the consumer who switched commercial products could unknowingly be taking ten times or more total CAF, CA and EA alkaloids daily than he or she had taken previously.

In addition to their sympathomimetic stimulation capabilities, the CA alkaloids may be linked to thrombosis and stroke. OCT and SYN can be taken up by platelets and may be involved to some extent with platelet activation and deactivation (35). Also, it has been observed that circulating trace amines, OCT, TYR, and SYN levels are increased in patients with cluster and/or migraine headaches (36). Recent work has shown that the risk of stroke is increased in persons suffering from migraine headaches (37).

The described method is the only method found capable of simultaneously separating and quantitating CAF along with all pertinent CA and EA alkaloids from complex dietary supplement matrices. The wide range of concentrations determined for these alkaloids are a testament to the utility of the method. This method eliminated

THF, triethylamine, and the antioxidant butylhydroxytoluene (BHT) from the mobile phase because they can degrade easily and have a deleterious effect on chromatography (6,22, 38). Also, the UV-cutoff wavelength for THF is 220 nm and because extracted wavelengths of 206 nm and 222 nm were being used for quantitation, some degree of quenching by THF would occur. At 206 nm, the UV absorption of mobile phase containing 5% v/v THF was triple that of the mobile phases used here without UV absorbing composition. By increasing the concentration of sodium lauryl sulfate in the mobile phase aqueous portion to 30 mM and acidifying to pH 2.5 with sulfuric acid, ionization of all alkaloids was assured, resulting in complete ion-pairing and excellent resolution (18,22,29). The chromatogram degraded at sodium lauryl sulfate concentrations nearer 40mM. Good resolution was not observed at 5mM to 10mM sodium lauryl sulfate concentrations. The Synergi-Hydro RP analytical column contains a C18 bonded phase endcapped with a unique proprietary polar group to enable retention of both polar and hydrophobic compounds over a greater range of organic to aqueous mobile phase compositions. Compared to conventional C18 columns, this column resulted in shorter run and reequilibration times.

The described method is simple and the necessary instrumentation and chemicals are relatively affordable compared to many other methods reviewed. The ion-pairing agent sodium lauryl sulfate is roughly ten times cheaper to use than the next lowest cost long-chain sulfonic acid ion-pairing agent. The HPLC instrumentation is common compared to LC-MS-MS with less maintenance and operator time required. The relatively short runtime of 41 min per sample (analysis plus reequilibration time) enables somewhat rapid analysis of a large number of samples. THF was successfully eliminated from the mobile phases. The same analytical column was utilized for over six months without fouling or degradation while analyzing hundreds of extracted samples in support of ongoing toxicology studies.

## Conclusion

A simple and cost effective yet accurate and reliable method of analysis for the determination of caffeine, CA, and EA alkaloids in dietary weight loss supplements has been described. The method is applicable to the determination of total EA alkaloids in monitoring compliance with a recent judicial ruling allowing the commercial availability of 10 mg/day or less. The method is also applicable to the determination of caffeine in conjunction with many of the CA alkaloids in the monitoring of nutrient content claims by the manufacturer. This method was utilized to assist in establishing the biogenic amine content of proposed bitter orange reference materials at the request of the National Institute of Standards and Technology (NIST).

## Acknowledgments

This work was supported by Interagency Agreement 224-93-0001 between the National Institute for Environmental Health Sciences and the United States Food and Drug Administration.

The views represented in this article do not necessarily represent those of the FDA.

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Manuscript received March 29 2006;  
revision received May 30, 2007.