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Determination of Ephedrine Alkaloids and Synephrine in Dietary Supplements by Column-Switching Cation Exchange High-Performance Liquid Chromatography with Scanning-Wavelength Ultraviolet and Fluorescence Detection

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An HPLC method with on-line cleanup coupled to the separation column is described for determination of (−)-norephedrine, (+)-norpseudoephedrine, (−)-ephedrine, (+)-pseudoephedrine, (−)-*N*-methylephedrine, (+)-*N*-methylpseudoephedrine, and (±)-synephrine in finished dietary supplement products. Test portions were extracted in acidified aqueous acetone. A filtered aliquot was cleaned up on a strong cation exchange (SCX) precolumn that later was automatically coupled to the SCX analytical column. Measurement was by full-scan UV spectra for confirmation of identity by spectral matching and real-time integration of three wavelength signals for multiple quantitation. (±)-Synephrine was also quantitated by native fluorescence. Recovery averaged 95−100%. Determination of the major ingredients (−)-ephedrine, (+)-pseudoephedrine, and (±)-synephrine compared favorably to findings by an independent LC-MS analysis for a set of 25 samples. The results of a survey were reported for total ephedrine alkaloid and synephrine content and were compared to content declaration, for ∼48 finished products.

KEYWORDS: Ephedra; ephedrine alkaloids; synephrine; dietary supplements; cation exchange; HPLC; analysis; determination; UV spectra; fluorescence

INTRODUCTION

Botanical dietary supplements containing sympathomimetic stimulants such as the ephedrine alkaloids (primarily from herbals in the family Ephedraceae) and/or synephrine (from bitter orange) are marketed for weight loss and bodybuilding. Consumption of these supplements, often formulated with caffeine sources such as kola nut, guarana seed, or green leaf tea, and perhaps augmented by drinking caffeinated beverages, has been associated with adverse cardiovascular and central nervous system events, even death, in some persons (*1*). Confronted by a bewildering array of products, the consumer often must rely on label information for safe use and assumes claims of active ingredient content and amounts per serving to be accurate.

Ephedrine alkaloids occur naturally as three diastereomeric pairs grouped by primary, secondary, or tertiary amine function. The *dl*-forms of synephrine both occur in nature, but the *l*-form predominates (*2*). Chemical structures are shown in **Figure 1**. Analytical methods to determine ephedrine alkaloids or synephrine in oriental medicinal and finished dietary products have favored liquid chromatography (LC) with detection by ultraviolet (UV) (*2*−*7*) or mass spectrometry (MS) (*7*−*8*). Phenyl (*5*, *8*) and C-18 (*2*−*4*, *6*, *7*) columns were used; the use of ion-pairing reagents in the mobile phase has been popular with C-18 columns (*3*, *4*, *6*). Most extracts of medicinals and fruits were analyzed without further cleanup. However, extracts of finished products, usually formulated with multiple ingredients that could interfere with the analysis, were cleaned up on strong cation exchange (SCX) disposable solid-phase extraction (SPE) columns (*5*, *8*), but SPE cleanup was unnecessary for an ion-pair HPLC method (*6*). Two of these methods used internal standards not readily available: either an expensive isotopically labeled ephedrine (*8*) or amphetamine sulfate (*6*), a DEA controlled substance. All of the noted methods used fixed-volume injection.

This paper describes a simple, rapid HPLC method that couples on-line cleanup of a dietary supplement extract on a short SCX precolumn and separation of enriched cations on an SCX analytical column. Off-line cleanup was unnecessary. The analytical approach was previously used to determine a nitrogen base (tertiary amine) pesticide in fruits at trace residue levels (*9*). Separated synephrine (SYN) and six ephedrine alkaloids were measured by scanning-wavelength UV and fluorescence detectors in tandem [only (±)-SYN possessed native fluorescence]. The acquisition of full-scan UV spectra permitted postrun confirmation of identity according to numerical fit from a computer match to a user-generated spectral library of standards. Extract volume loaded onto the precolumn was

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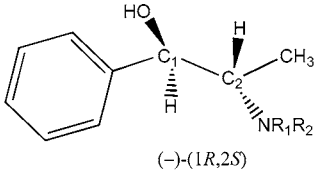
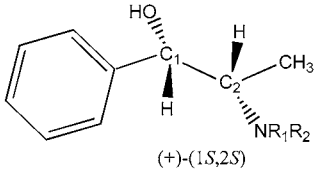
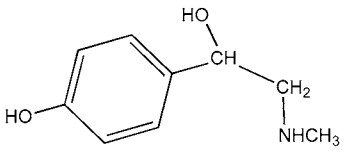


(−)-(1*R*,2*S*)



(+)-(1*S*,2*S*)

R_1	R_2	Naturally occurring diastereomers	Abbrev.
H	H	(−)-(1*R*,2*S*)-Norephedrine	(−)-NE
		(+)-(1*S*,2*S*)-Norpseudoephedrine	(+)-NPE
H	CH_3	(−)-(1*R*,2*S*)-Ephedrine	(−)-E
		(+)-(1*S*,2*S*)-Pseudoephedrine	(+)-PE
CH_3	CH_3	(−)-(1*R*,2*S*)-*N*-Methylephedrine	(−)-N-ME
		(+)-(1*S*,2*S*)-*N*-Methylpseudoephedrine	(+)-N-MPE



(±)-Synephrine (±)-SYN

Figure 1. Structures of the ephedrine alkaloids of interest and synephrine.

adjustable over a wide range to easily accommodate diverse concentrations to the calibration range. No internal standard was needed. Determination of (±)-SYN, (−)-ephedrine [(−)-E], and (+)-pseudoephedrine [(+)-PE] in nearly 24 samples was compared to independent analysis by LC-MS (*8*). Accuracy in content claims was assessed by a survey of 48 products.

MATERIALS AND METHODS

Reagents. Only high-purity solvents were used: UV grade acetonitrile (ACN) and residue grade acetone (Honeywell Burdick & Jackson, Muskegon, MI). Water was purified through a Milli-Q cartridge system (Millipore, Bedford, MA). Phosphoric acid (85%; EM Science, Gibbstown, NJ) and hydrochloric acid (Fisher Scientific, Pittsburgh, PA) were of ACS reagent grade; sodium hydroxide solution (50%; J. T. Baker, Phillipsburg, NJ) was of Baker analyzed reagent. The extraction solvent was 4:1 (v/v) acetone/water acidified to 12 mM HCl (pipet 1 mL of concentrated hydrochloric acid into 1 L of aqueous acetone and mix by magnetic stirring). The 0.4 M sodium phosphate (pH 3.00) buffer component of the mobile phase was prepared by adding ∼28 g of sodium hydroxide solution to 46.2 g of phosphoric acid in 950 mL of water while measuring the pH with a standardized (pH 4 and 7 buffers) combination glass electrode (model 91-55, Thermo Orion, Beverly, MA), dispensing the last amount dropwise from a disposable pipet. The buffer was vacuum filtered (Aura unit, Supelco, Bellefonte, PA) through a 0.45-μm membrane (nylon, Alltech, Deerfield, IL). A 4:1 (v/v) water/ACN solution was used to condition the precolumn during loading.

Chemicals and Standard Solutions. (±)-SYN, (+)-PE, and (+)-*N*-methylpseudoephedrine [(+)-N-MPE] were obtained from Sigma (St. Louis, MO); (−)-norephedrine [(−)-NE], (−)-ephedrine−HCl [(−)-E·HCl], and (−)-*N*-methylephedrine [(−)-N-ME] came from Aldrich (Milwaukee, WI); and (+)-norpseudoephedrine−HCl [(+)-NPE·HCl (cathine hydrochloride), a DEA controlled substance] was purchased from Research Biochemicals International (Natick, MA). Bottles were kept in the dark and stored in a freezer except (+)-NPE·HCl was kept in a drawer at room temperature. A seven-component stock solution (25-mL volumetric flask) was prepared in extraction solvent from standards accurately weighed (±0.01 mg) to give component concentrations within 150 ± 15 μg/mL, except (±)-SYN was 80−85 μg/mL. Three calibration standard solutions (10-mL volumetric flasks) were prepared in extraction solvent by 10-fold serial dilution of this stock. A fortification standard mix of (−)-NE, (+)-PE, (−)-N-ME, and (+)-N-MPE nominally 1 mg/mL each in extraction solvent was prepared from 50 ± 0.1 mg of respective solid in a 50-mL volumetric flask. Single-component fortification standards were prepared: 5 mg/mL (−)-E·HCl and 2.5 mg/mL (±)-SYN (prepared in acidified 50:50 acetone/water due to solubility). Stock and fortifying solutions were stored in a −20 °C freezer. Calibration solutions were stored under cover on the bench or in the freezer for long-term storage. After usage, the meniscus was marked on tape affixed to the flask, and solvent was added to replenish evaporative losses, if needed, on subsequent days.

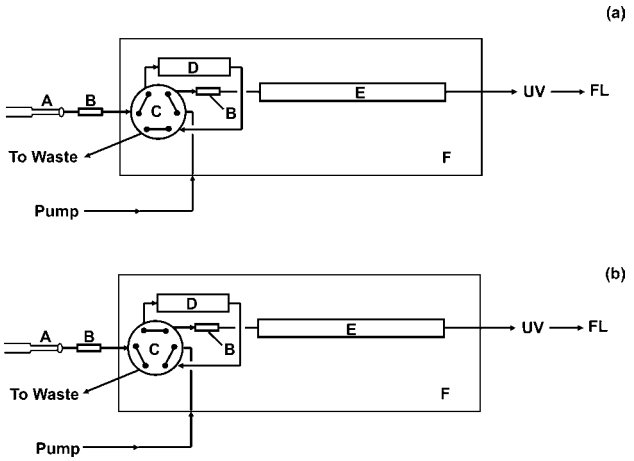


Figure 2. Schematic of column-switching LC system showing valve C in the (a) load position and (b) inject position. Other components: (A) syringe and syringe adapter; (B) in-line filter; (D) precolumn; (E) analytical column; (F) convective column oven.

Instrumentation and Apparatus. The LC system (**Figure 2**) included a ternary solvent pump (model 8800 with sapphire pistons and back seals in contact with 90:10 water/methanol static flush solution), a scanning-wavelength UV detector (FOCUS, 10-mm cell path), a programmable fluorescence detector (model FL2000, 3-μL cell), chromatography data system software (PC1000 ver. 3.0.1), and spectral analysis software (Spectacle)—all from Spectra Physics (subsequently acquired by Thermo Finnigan, San Jose, CA). The electrically activated injection valve (Valco, Houston, TX) and HPLC columns were thermostated at 40 °C inside a convective column oven compartment (old DuPont Instruments unit). A 4.6 mm i.d. × 3 cm, 10-μm, Whatman Partisil SCX precolumn (guard column, PN 635-030-26, Thermo Hypersil-Keystone, Bellefonte, PA) was installed in place of the injection loop. The analytical column was a 4.6 mm i.d. × 25 cm, 5-μm, Zorbax 300-SCX (Agilent Technologies, Wilmington, DE). Filters with replaceable 0.5-μm frits (Upchurch Scientific, Oak Harbor, WA) were installed ahead of the injection valve filling port and the analytical column. An assortment of gastight syringes (25-, 100-, 250-, 500-, and 1000-μL; Hamilton, Reno, NV) was used.

The pump dynamically proportioned (A/B/C) a 50:35:15 isocratic mobile phase where A, B, and C were, respectively, 0.4 M sodium phosphate buffer (pH 3.00), water, and ACN. The flow rate was 1.0 mL/min. [For Ripped Fuel protein drink mixes (various flavors), use a 75:15:10 or 50:40:10 mobile phase, as required, to separate an unknown matrix component sandwiched between elution of (−)-E and (+)-PE.] Before analyses, the coupled columns were conditioned at 2.0 mL/min with the following unoptimized mobile phase program: 0:50:50 for 10 min, 5 min gradient to 0:100:0 and hold for 5 min, 5 min gradient to 100:0:0 and hold for 10 min, 5 min gradient to 50:25:25 and hold for 20 min. The flow rate was reduced to 1.0 mL/min, the mobile phase was stepped to 50:35:15, and analysis was started 10 min later. The scanning-wavelength UV detector collected spectra in the 200−300

nm region scanned at 5 nm intervals and simultaneously integrated signals at 205, 210, and 225 nm for quantitation. Real-time spectral data, after background removal, were compared by a correlation algorithm against a user-generated Spectacle library of standards. Fluorescence was excited at 270 nm (20-nm slit width), and emission was monitored at 304 nm (8-nm slit width) for quantitation; other settings included 100 Hz pulsed xenon lamp, 600 V PMT, 20 range, and 1 V full-scale analogue output to data system. To prepare for chromatography, the following precolumn loading sequence was performed with the valve in the load position: 0.5 mL of aqueous ACN conditioning solution, 20−500 μL of test solution, 0.5 mL of ACN, and 0.5 mL of conditioning solution. Switching the valve to the inject position sent a start signal to all units to begin chromatography and data acquisition.

Calibration. A five-point linear regression calibration of peak area versus micrograms of standard was performed periodically. Freshly prepared calibration standards were aged for 2 days. [(+)-PE signal increased markedly the day after preparation but stabilized by the third day. Fortunately, extracts required no aging unless synthetic (+)-PE had been added to the product test portion initially for recovery determination as discussed later.] Loadings of 200 μL of the lowest concentrations, 60 and 200 μL of the middle concentrations, and 60 and 200 μL of the highest concentrations were chosen to calibrate detector responses in these ranges (micrograms): ephedrine alkaloids from 0.030 ± 0.003 to 3.00 ± 0.30 and (±)-SYN from approximately 0.016 to 1.60. The regression coefficients, slope m and intercept b, were used to calculate Q (micrograms) from peak area A: $Q = (A - b)/m$. A normalization factor (NF), the average ratio of found quantity to true value, was calculated daily for each analyte from chromatography of the middle and highest concentration calibration standards (100- or 150-μL aliquot) and was used to correct extract Q for calibration shift. A calibration may be stable for 3 weeks; however, NF outside the range of 95−107% indicated recalibration was needed.

Samples. About 48 dietary supplements containing ephedrine alkaloids and/or synephrine were purchased in 2001 through the Internet; a few products had been bought earlier at local health food stores. Capsules, caplets, tablets, powders and mixes, and liquids were represented. The contents of 15 capsules were emptied into a weighed (±0.01 g) vial, and, depending on size, a batch of either 15 or 20 caplets or tablets was weighed, pulverized with a mortar and pestle, and poured into a vial. Gram per unit was calculated. A canister of powder or mix product was vigorously shaken before portions were withdrawn from various locations and combined in a vial. These individual composites provided all of the sample test portions analyzed over time. Liquid test portions were withdrawn from the product bottle at the time of analysis. Experimentally, the 0.5-oz dispenser in the bottle of Liquid Health Diet delivered an average of 16.86 g/unit ($N = 4$, SD = 0.16). Vials and liquids were stored in a covered box in a drawer.

Analysis. One gram of sample test portion was weighed into a tared 125-mL Erlenmeyer flask (glass-stoppered). For high-potency products or if gummy ball(s) formed during extraction, the amount was reduced to 100 mg. For fortification trials, the desired fortification standards were pipetted onto the test portion and swirled to mix. A 100-mL amount of extraction solvent (decrease by the total volume of all fortification standards added) was added, the flask stoppered, and the mixture magnetically stirred rapidly for 0.5 h at room temperature. After 5 min had been allowed for solids to settle, ~5 mL was filtered through a 0.2-μm syringe filter (Titan, nylon 66, 13 mm; SRI, Eatontown, NJ), discharging the first milliliter to waste and collecting several milliliters in a glass vial. The precolumn loading sequence described earlier was followed. A total of 20−500 μL of extract was loaded such that the major alkaloids (−)-E and (+)-PE were quantitated near the upper end of the 100-fold calibration range, thereby improving the likelihood that the minor alkaloids fell within the range at the lower end. If the concentration of the minimum loading exceeded the calibration range, then 1:10 dilution with extraction solvent (5-mL volumetric flask) should be done before loading.

The level (mg/g) of ingredient I is determined from Q, loading volume L (μL), and test portion weight W (g) in a 100-mL extract according to

$$I = \left(\frac{Q}{L}\right) \times \left(\frac{100}{W}\right) \times \left(\frac{M}{\mathrm{NF}}\right)$$

where M is a factor that converted quantitation based on the hydrochloride salt to the equivalent free base [0.806 for (+)-NPE·HCl and 0.819 for (−)-E·HCl]. The averaged concentration (two signals) is reported only if the identity was confirmed by UV spectral match fit of 996−1000. All averaged and confirmed ephedrine alkaloid concentrations were summed ($\sum I$), and total ephedrine alkaloids or (±)-SYN contents in a serving were calculated by multiplying $\sum I$ or I by grams per unit times units per serving.

Comparative Analysis. Selected samples were analyzed by an independent LC-MS method (*8*). Briefly, ephedrine-d_5·HCl internal standard solution was added to the sample test portion, which was extracted (sonicated) with 4:1 (v/v) methanol/water, centrifuged, cleaned up on a propylsulfonic acid SPE column, and analyzed by using reversed-phase phenyl column LC-MS (full scan with in-source fragmentation by tube lens voltage) with confirmation of identity by three ion relative abundances.

RESULTS AND DISCUSSION

The ion exchange precolumn enriched and selectively gated cations to the analytical column. Extract solutes unable to form cations but held by secondary adsorption or solubility interactions with the packing material were flushed out during the ACN wash step of the loading sequence. The retained contents of the precolumn were reversed flushed onto the analytical column, avoiding degradation of chromatography by the precolumn's larger particle size. Unlike typical solid phase extraction cleanups, the on-line column was reusable, continuously regenerated by the mobile phase while coupled to the analytical column. The amino group of the ephedrine alkaloids and synephrine, being a strong organic base (large pK_a) and easily protonated in acidic media, existed predominately in the cationic form, readily available for ion exchange. However, the presence of additional cation-forming substances was expected in matrices as complex as dietary supplement finished products. Coextractive interference at the UV wavelength(s) used for quantitation should manifest itself through a resultant UV spectrum that could not be matched to the standard reference spectrum with high confidence (fit number ≥996). Without spectral confirmation of identity, quantitation was invalid, thereby averting a false positive. Furthermore, (−)-E, (+)-PE, and (±)-SYN, the analytes of greatest interest, were ingredients at nontrace parts per thousand, which reduced the risk of significant interference relative to that for a trace level (parts per million) measurement. Therefore, the choice to monitor at the ephedrine alkaloids' absorbance maxima of 205 and 210 nm was likely a good one, despite a preponderance of UV absorbers at low wavelengths. When criteria were met that (1) retention times of sample and standard peaks matched and (2) sample peak identity was confirmed by computer match of UV spectra, determination was deemed to be interference-free. In support of this conclusion, a large sample subset was analyzed by a highly selective LC-MS method.

Chromatography and Spectral Analysis. Figure 3 shows UV and fluorescence chromatograms of a calibration standard solution and three dietary supplement extracts analyzed by coupled-column SCX HPLC. Extract and standard component retention times may differ because analyses were spread over several days; fluorescence retention times were longer than UV times because the detector was downstream. Resolution of diastereomers was at or nearly baseline with only minor tailing of the latest eluting peak, (+)-N-MPE.

In **Figure 4**, the normalized UV spectra of chromatographic peaks detected at standard component retention times (**Figure**

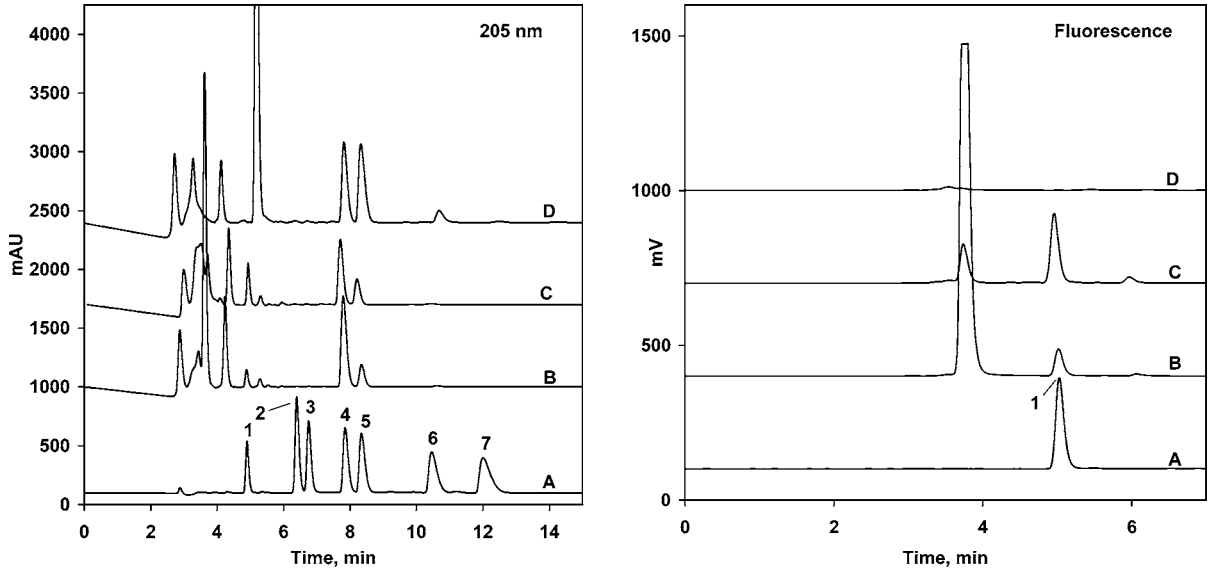


Figure 3. SCX UV and fluorescence chromatograms of (A) standard solution (~3 μg per component, except 1.6 μg of component **1**), (B) Xenadrine RFA-1, (C) Xetalean, and (D) Ultra Diet Pep. Analytes: **1**, (±)-SYN; **2**, (–)-NE; **3**, (+)-NPE·HCl; **4**, (–)-E·HCl; **5**, (+)-PE; **6**, (–)-N-ME; **7**, (+)-N-MPE.

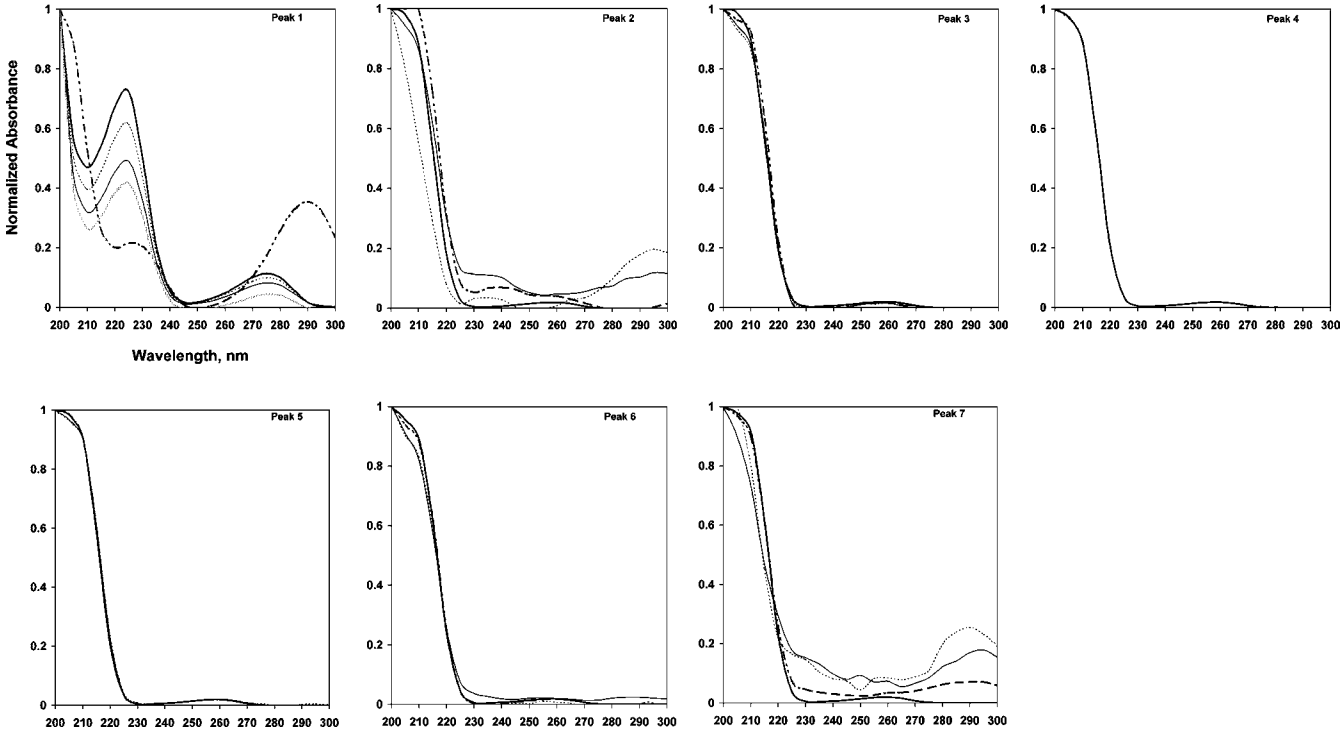


Figure 4. Normalized UV spectra of chromatographic peaks from **Figure 3** for identity confirmation by visual matching: (—) highest calibration standard [~3 μg, except 1.6 μg of (±)-SYN]; (•••) Xetalean; (—) Xenadrine RFA-1; (— - - —) Ultra Diet Pep; (shaded curve) 0.016 μg of (±)-SYN. See text for comparison to numerical value of computer match.

3) are overlaid for comparison, to relate the quality of spectra superimposition (extract and standard component) with the fit number generated from the computer match. The simplicity of the UV spectra of the ephedrine alkaloids indicated that fit numbers of near perfect match (996−1000) were required for confidence in identity confirmation. In contrast, confirmation of synephrine was easier because of a more feature-rich spectrum (more bands for matching) and a required fluorescence peak, without which confirmation was impossible. For example, even though the UV spectrum of peak 1 for Xetalean and Xenadrine RFA-1 but not Ultra Diet Pep compared well to the (±)-SYN standard spectra, the fluorescence chromatograms in **Figure 3** significantly increased confidence that only the first two products contained synephrine. Note the concentration dependence of (±)-SYN standard spectra, which likely was influenced by proximity to and tailing of the solvent front. Therefore, the UV reference library included (±)-SYN spectra at the five calibration levels. Computer matching of peak 1 of Xetalean [quantitated at 0.91 μg of (±)-SYN] to the UV spectrum of the 1 μg standard gave a 996 fit number; peak 1 of Xenadrine RFA-1 [quantitated at 0.34 μg of (±)-SYN] was matched to the UV spectrum of the 0.5 μg standard also with a 996 fit.

Found in trace amounts, (−)-NE (peak 2), (+)-NPE (peak 3), and (+)-N-MPE (peak 7) were more susceptible to interference from coextractives (see **Figure 4**). The presence of (−)-

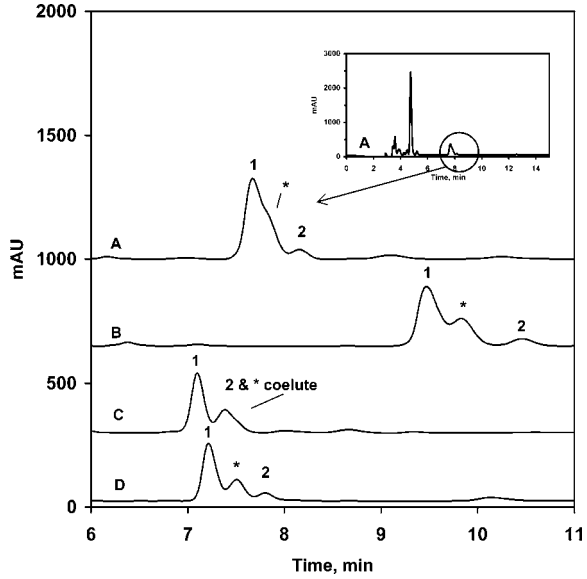


Figure 5. Chromatograms at 205 nm of Ripped Fuel chocolate-flavored protein drink mix illustrating interference by an unknown matrix component (peak ∗) in the (–)-E (peak 1) and (+)-PE (peak 2) elution region for two Zorbax 300-SCX columns (column 1, A and B; column 2, C and D). Elution by method-specified mobile phase (A and C) and alternative mobile phases (B and D) is discussed in text. Inset is full chromatogram.

NE was not confirmed in these samples mainly due to extraneous absorption bands above 225 nm. Computer-matched fits were 913 for Xetalean, 994 for Xenadrine RFA-1, and 995 for Ultra Diet Pep, suggesting that (−)-NE may be present in the latter two samples but that quantitation error was likely. (+)-Norpseudoephedrine was confirmed in Ultra Diet Pep with a 997 fit and in Xetalean and Xenadrine RFA-1 with a 999 fit. (+)-*N*-Methylpseudoephedrine was confirmed in Ultra Diet Pep (996 fit) only marginally because of poor overlap seen in the very weak bands beyond 225 nm but was not confirmed in Xetalean (919 fit) or Xenadrine RFA-1 (942 fit), for visually obvious reasons. Because the UV spectra of the ephedrine alkaloids, unlike synephrine, showed negligible concentration dependence, spectra from a single (middle) calibration point were sufficient for the reference library.

Spectra of extract peaks 4 and 5 overlaid standard (−)-E and (+)-PE spectra, respectively, almost perfectly, as reflected by computer-matched fit numbers between 998.8 and 999.9. Peak 6 was identified as (−)-N-ME in Xenadrine RFA-1 (998 fit) and Ultra Diet Pep (1000 perfect match) but was not confirmed in Xetalean (985 fit) because of subtle differences visible in the major and minor absorption bands. Note that spectra of the ephedrine alkaloid standards (**Figure 4**, peaks 2−7) were virtually indistinguishable without retention time data.

Figure 5 shows interference with UV detection of (−)-E or (+)-PE in the chromatographic analysis of Ripped Fuel chocolate-flavored protein drink mix (vanilla and fruit punch flavors behaved similarly). Besides herbal blends, this product included mixtures of vitamins, minerals, trace elements, milk and egg protein, and 20 amino acids. Expanded chromatograms A and C reveal slight differences in Zorbax 300-SCX column-to-column selectivity, as the unknown eluted near (−)-E on one column but near (+)-PE on the other. Spectral matching failed to confirm analyte identity. In chromatogram B, changing the mobile phase to 50:40:10 partially separated the unknown sufficiently for acceptable quantitation of (−)-E. In chromatogram D, a 75:15:10 mobile phase eluting the other column has nearly baseline resolved the unknown and (+)-PE. In both column instances, identity was confirmed because of these slight adjustments in mobile phase proportioning. For all other products examined, no change in the normal 50:35:15 mobile phase composition was required.

Table 1. Calibration Statistics for the Ephedrine Alkaloids in the 0.030–3.0 μg Range and for (±)-Synephrine in the 0.016–1.6 μg Range

analyte	signal, nm	av[a] slope[b]	av[a] intercept[c]	av[a] *r*	% RSD[a] slope	% RSD[a] intercept
(–)-NE	205	2.71E+06	2.12E+04	0.99998	4.0	32.9
	210	2.46E+06	2.99E+04	0.99993	3.9	25.5
(+)-NPE·HCl	205	2.16E+06	4.75E+02	1.00000	3.9	1951.3
	210	1.97E+06	6.16E+03	1.00000	3.7	156.9
(–)-E·HCl	205	2.29E+06	2.50E+04	0.99998	3.5	63.8
	210	2.10E+06	2.90E+04	0.99997	3.3	51.3
(+)-PE	205	2.73E+06	9.88E+03	1.00000	9.1	125.2
	210	2.51E+06	1.56E+04	0.99999	8.8	82.5
(–)-N-ME	205	2.64E+06	1.34E+04	0.99999	5.6	31.3
	210	2.47E+06	1.59E+04	0.99998	5.7	22.7
(+)-N-MPE	205	2.79E+06	1.07E+04	1.00000	4.4	43.9
	210	2.61E+06	1.23E+04	0.99999	4.4	22.6
(±)-SYN	225	2.87E+06	2.18E+04	0.99998	2.4	36.9
fluorescence		1.97E+06	1.26E+04	0.99993	4.6	51.3

[a] $N = 15$ calibrations over 1.5 years, except $N = 10$ for (±)-SYN. [b] Peak area counts per microgram. [c] Peak area counts.

Calibration, Extraction, and Enrichment. Statistics for external standard calibration presented in **Table 1** indicate excellent linear correlation and good repeatability of slope for all components over several generations of standard solution preparations. Variation in the average correlation coefficient occurred in the fifth decimal place. Although the intercepts were quite variable, the much larger slope values were not: typically, the RSD was <6%. Normalizing quantitation of analyte in extracts to daily quantitation of the respective component in two calibration standards relative to its true amount reduced error due to calibration shift. Delaying the calibration for several days after the preparation of standard solutions to maximize cation availability of synthetic (+)-PE significantly reduced systematic error in the quantitation of natural (+)-PE in product extracts.

A gummy ball formed during the extraction of 1 g of Fat Burner Energizer or Ephedra 850 + Tyrozine + LPC product but not during extraction of a 100-mg test portion. The interior of the ball mass was incompletely extracted, as determinative values were comparatively smaller for the larger test portion size. Similar extraction difficulties were not experienced with any other product.

Precolumn cation enrichment was directly proportional to mass loaded, until ion exchange capacity or linear calibration (or both) was (were) grossly exceeded. An extract of a high-potency product was used to study precolumn loadings. The normal loading for analysis of this product required 10-fold dilution such that a 40-μL loading placed (−)-E response near the upper bounds of calibration, whereas (+)-PE and (−)-N-ME responses were within range at the lower end. Not until the loading was 5 times normal did (−)-E quantitation, although 3 times above the calibration range, deviate from linearity. At 10 times normal loading (used undiluted extract and 40-μL aliquot), (+)-PE quantitation, still within its calibration range, increased by the same factor, indicating sufficient ion exchange capacity. At 25 times normal loading, (−)-N-ME quantitation, slightly beyond the calibration range, had increased 24.7 times, almost in direct proportion. Therefore, dilute components can be measured by simply loading a larger volume of undiluted

Table 2. Recovery of (±)-Synephrine and the Ephedrine Alkaloids Added to Dietary Supplements after Correction for Control Levels

	fortification, mg/g			recovery, %						
sample	(±)-SYN	(−)-E	others	(±)-SYN	(−)-E	(−)-NE	(−)-N-ME	(+)-N-MPE	(+)-PE	(+)-PE next day[a]
Red X	5.2			96.7						
Sizzle	5.2			97.5						
Xenadrine RFA-1	5.2			96.4						
Liquid Health Diet	2.6		1.0	94.0		96.1	104.7	89.1	84.7	99.2
Liquid Health Diet	2.6		1.0	93.2		95.1	104.2	89.1	86.7	99.2
Hydroxycut	2.6		1.0	95.7		96.1	104.7	88.6	71.3	103.7
Hydroxycut	2.6		1.0	96.3		96.1	103.7	88.6	73.3	98.7
Red X			1.0			88.1	96.2	102.3	80.8	105.7
Liquid Health Diet		0.82	1.0		100.6	102.4	101.0	101.9	58.6	100.1
Liquid Health Diet		0.82	1.0		102.7	106.4	105.2	104.1	73.6	104.4
Ultimate Orange		0.82	1.0		102.2	105.3	96.0	100.2	69.3	103.5
Optibolic Ephadrene		4.1	1.0		100.2	95.3	106.7	101.0	57.5	NA[b]
Hydroxycut		4.1	1.0		98.5	103.4	102.0	102.2	51.7	103.6
Hydroxycut		4.1	1.0		98.2	102.4	101.0	100.7	60.4	103.6
Hydroxycut		8.2	1.0		111.9	80.8	105.1	102.4	46.1	141.2[c]
Hydroxycut		8.2	1.0		96.3	84.3	92.2	102.3	48.5	97.7
Ultimate Orange		8.2	1.0		100.4	104.3	94.4	98.8	88.9	112.8
Red Rage		8.2	1.0		100.3	92.4	99.0	103.4	41.2	118.6
Solaray Ephedra		8.2	1.0		97.0	102.8	91.9	95.0	43.0	89.4
Solaray Ephedra		16.4	1.0		95.9	102.8	89.4	91.6	48.9	96.2
N				7	12	17	17	17	17	15
av				95.7	100.3	97.3	99.8	97.7	63.8	102.4
SD				1.5	4.2	7.5	5.5	5.9	16.0	6.9

[a] Raw extract reanalyzed the day after preparation; stored at ambient temperature in the dark. [b] Not Analyzed. [c] Outlier at 0.5% risk of false rejection by Dixon test ($n = 16$, $t_{22} = 0.652 > 0.624$ critical value) and Grubbs test ($n = 16$, $T = 3.095 > 2.845$ critical value).

Table 3. Average Level (Milligrams per Gram) and Precision (% RSD) for Analyses of Selected Dietary Supplements

		(−)-E		(+)-PE		(−)-N-ME		(+)-NPE		(±)-SYN	
supplement	*N*	av	% RSD	av	% RSD	av	% RSD	av	% RSD	av	% RSD
Liquid Health Diet	3	0.99	1.5	0.13	11.5	0.049	6.3				
Hydroxycut	4	5.09	11.1	0.55	13.7	0.18	11.4				
Ultra Diet Pep	4	5.20	6.9	5.94	7.1	1.19	3.6	0.078	20.7		
MetaboSurge	3	7.83	5.8	1.19	11.0	0.19	29.4			1.68	10.8
Xetalean (interday)	4	8.34	5.0	3.53	5.1					3.68	6.2
Xetalean (intraday)	4	8.24	1.1	3.41	1.0					3.68	2.6
Solaray Ephedra	5	8.53	1.9	4.02	5.8	1.29	1.7	0.55	6.3		
Pro-Ripped Ephedra	4	9.20	4.9	4.77	4.3					3.95	6.1
Xenadrine RFA-1	5	11.7	4.4	3.06	3.9	0.22	25.3			1.38	5.7
Thermadrol X	3	15.8	3.3	3.27	3.5	0.50	11.0	0.18	23.4		
Dymetadrine	3	25.7	7.1	3.62	9.8	1.10	3.5				
Sizzle	4	29.9	1.1	8.62	3.7	1.14	7.6				
EPH 1000	4	38.8	1.6	10.4	4.0						
Red X	5	43.0	4.3								
EPH 833	4	63.2	2.8	2.55	0.1	4.03	4.6				
av interday precision		4.2		6.0		10.4		16.8		6.3	

extract (>500-μL not recommended), thus avoiding reanalysis using a larger sample test portion.

Recovery. Listed in **Table 2** are analyte fortifications of and recoveries from diverse types of dietary supplement products. Recoveries averaged 96−102% for seven analytes. Ephedrine recovery was independent of fortification level from 0.8 to 16 mg/g. Full recovery of added synthetic (+)-PE was achieved the day after extraction (compare last two columns), probably due to the aging effect observed for calibration standard solutions prepared from the same material and described earlier. To test this hypothesis as well as rule out the possibility that measured concentration of natural (+)-PE had increased with extract aging, the extract of the control test portion was also reanalyzed the day after extraction by the LC-UV determinative step. There was no significant difference between the mean concentrations of natural (+)-PE for a set of 37 products when measured in freshly prepared extracts and again a day later (two-sided paired *t* test, 95% confidence, 35 df, 2.032 critical *t* vs 1.005 calculated). Therefore, the (+)-PE aging phenomenon was associated with synthetic material. A more detailed study was beyond the scope of this paper.

Precision. Statistics for replicate analyses of >12 products are arranged in **Table 3** by ascending mean (−)-E level. Test portions were taken from the original sample composite and analyzed over 5−7 months. Variables such as analyte stability in aging sample matrices and composite homogeneity (e.g., flakes suspected as tablet binders were nonuniformly distributed in some composites) were not controlled. Hydroxycut stood out as the sample with quantifiable ingredients consistently determined imprecisely (11−14% RSD). Nonetheless, interday precision typically averaged 4−6% RSD for the determination of major ingredients (−)-E, (+)-PE, and (±)-SYN. Trace ingredients (−)-N-ME and (+)-NPE were determined with poorer precision because the method was optimized for higher concentrations. Intraday precision of 1−3% RSD was demon-

Table 4. Ephedrine Alkaloids and (±)-Synephrine Found in 48 Dietary Supplements with Comparison to Declared (dcl) Amount per Serving (Unless Indicated Otherwise, Findings Were Confirmed at 996–1000 Computer Fit of Suspect Analyte and Standard UV Spectra)

		findings, mg/serving							ephedrine alkaloids			synephrine	
supplement	serving size	(−)-NE	(+)-NPE	(−)-E	(+)-PE	(−)-NME	(+)-NMPE	(±)-SYN	total	dcl	%	dcl	%
3Andro Xtreme[a]	2 capsules	–[b]	–[b]	25.9	2.95	0.89	–[b]		29.8	32	93.0		
AdipoKinetix	1 capsule	19.2	–[b]	–[b]	–[b]	–[b]	–[b]		19.2	20	95.4		
Amphetra-Lean	2 caplets	0.22	0.33	14.9	6.90	0.33	–[b]		22.7	24	94.6		
BetaLean HP	3 capsules	–[b]	–[c]	19.1	1.46	0.40	–[b]		21.0	20	104.9		
Clenbutrx[a]	4 cm^3 liquid	0.08 (NC)	0.04 (NC)	25.9	5.58	1.21	–[b]	12.7	32.7	–	–	–	–
Complete Diet Boost (lot 1)	2 capsules	C[d]	–[b]	15.6	2.04	0.57	–[b]	5.48	18.2	20	91.1	5.2	105.4
Complete Diet Boost (lot 2)	2 capsules	–[b]	0.29	12.4	4.39	0.44	–[b]	5.40	17.5	20	87.5	5.2	103.9
Dexatrim Natural	1 caplet	C[d]	0.087	8.21	2.95	0.58	–[b]		11.8	12	98.6		
Dyma-Burn Xtreme[a]	2 capsules	–[b]	–[b]	17.6	1.94	0.59	–[b]	5.47	20.1	21	95.8	5.2	105.3
Dymetadrine[a]	1 capsule	C[d]	–[b]	20.6	2.90	0.88	–[b]		24.3	20	121.7		
Energy Fuel (Ma Huang free)	2 caplets	–[b]	–[b]	–[b]	–[b]	–[b]	–[b]	13.4	0.0	0	–	19.5	68.7
EPH 1000[a]	1 capsule	–[b]	–[b]	35.3	9.49	1.08 (NC)	–[b]		44.8	73.8	60.7		
EPH 833[a]	1 capsule	–[c]	–[b]	50.0	6.78	2.20	–[b]		58.9	60.0	98.2		
Ephedra 850 + Tyrozine + LPC[a]	1 capsule	C[d]	–[b]	42.3	8.62	3.58	–[b]		54.5	64.0	85.2		
Fat Burner Energizer[a]	1 capsule	0.19 (NC)	0.25	4.07	12.6	0.78	0.088		17.8	25	71.1		
Hydroxycut[a]	4 capsules	–[b]	–[b]	18.8	2.05	0.63	–[b]		21.5	20	107.4		
Liquid Health Diet[a]	0.5 oz liquid	–[c]	–[b]	16.6	2.25	0.82	–[b]		19.7	12	164.3		
Metabolife 356	1 caplet	0.079	0.090	9.84	1.53	0.19	–[b]		11.7	12	97.7		
Metab-O-Lite	2 caplets	–[b]	0.26	18.4	4.26	0.65	–[b]		23.6	24	98.4		
MetaboSurge[a]	1 tablet	–[b]	0.23	10.2	1.55	0.25	–[b]	2.04	12.2	12	101.8	3.0	68.1
Optibolic Ephadrene[a]	2 tablets	–[b]	–[c]	10.8	7.51	0.41	–[b]		18.8	19.8	94.7		
Performance Orange Workout drink mix[a]	spoon (28.35 g)	–[b]	–[b]	9.64	4.54	–[c]	–[b]		14.2	24.9	56.9		
Phenyl XL	1 tablet	18.8	–[b]	–[b]	–[b]	–[b]	–[b]		18.8	20.1	93.1		
Pro-Ripped Ephedra (lot 1, purchase 1)[a]	2 capsules	–[b]	–[c]	11.8	6.10	0.24 (NC)	–[b]	4.92	17.9	19	94.1	4.0	123.0
Pro-Ripped Ephedra (lot 1, purchase 2)	2 capsules	0.1 (NC)	–[c]	12.2	6.28	0.1 (NC)	–[b]	5.07	18.4	19	97.1	4.0	126.8
Pure Ephedrene[a]	1 tablet	–[b]	–[b]	22.7	6.91	0.92	0.41 (NC)		30.5	25.0	122.2		
Red Rage Workout drink mix[a]	scoop (47 g)	C[d]	–[b]	13.0	1.65	0.48	–[b]	4.49	15.2	16.8	90.6	4.0	112.2
Red X[a]	1 tablet	–[b]	–[b]	18.1	–[b]	–[b]	–[b]	0.00	18.1	25	72.3	25	0.0
Ripped Fuel	2 capsules	NC	–[c]	16.2	4.90	–[b]	–[b]		21.1	20	105.4		
Ripped Fuel protein drink mix (chocolate)	scoop (67.1 g)	0.01 (NC)	–[b]	18.3	2.80	0.01 (NC)	0.02 (NC)		21.1	20	105.4		
Ripped Fuel protein drink mix (fruit punch)[a]	scoop (65.0 g)	–[b]	–[b]	15.4	5.30	0.005 (NC)	0.03 (NC)		20.7	20	103.7		
Ripped Fuel protein drink mix (vanilla)	scoop (65.5 g)	0.01 (NC)	–[b]	17.0	3.31	0.005 (NC)	0.02 (NC)		20.3	20	101.7		
Sizzle[a]	1 caplet	–[b]	0.21	23.1	6.64	0.88	–[c]	0.00	30.8	24.0	128.3	20	0.0
Solaray Ephedra (lot 1)	1 capsule	0.038	0.19	3.06	1.40	0.46	C[d]		5.1	5.6[e]	92.0		
Solaray Ephedra (lot 2)	1 capsule	0.091	0.22	3.23	1.41	0.35	–[b]		5.3	5.6[e]	94.9		
Stacker 2[a]	1 capsule	–[b]	–[b]	14.5	1.66	0.50	–[b]		16.6	25	66.6		
Stoked[a]	scoop (1 g)	–[b]	–[b]	0.56	14.8	0.17 (NC)	–[b]		15.3	20	76.6		
Thermadrene	1 capsule	C[d]	0.11	14.4	2.45	–[c]	–[b]		17.0	20	85.0		
Thermadrol X	1 capsule	0.20 (NC)	0.12	10.5	2.16	0.33	–[b]		13.1	12	108.8		
Thermic Combustion	2 capsules	–[b]	–[c]	10.8	3.93	–[c]	–[b]	16.9	14.7	16	91.8	16.0	105.7
Therma Pro (lot 1)	1 capsule	0.31	C[d]	15.8	4.42	0.20	–[b]		20.7	20	103.5		
Therma Pro (lot 2)	1 capsule	0.33	C[d]	16.2	4.58	0.21	–[b]		21.3	20	106.6		
Thermo Diet	2 caplets	–[b]	–[b]	15.4	1.81	0.40	–[b]		17.6	16	110.2		
Ultimate Energizer[a]	1 capsule	C[d]	0.31	16.4	4.33	0.27	–[b]	4.16	21.3	22	96.7	5.0	83.3
Ultimate Orange Workout drink mix	scoop (28.4 g)	–[b]	–[b]	19.7	0.57	–[b]	0.02 (NC)		20.3	20	101.5		
Ultra Diet Pep[a]	1 tablet	0.10 (NC)	0.10	6.29	7.20	1.41	0.11		15.1	18	84.0		
Xenadrine RFA-1[a]	2 capsules	–[b]	0.17	17.6	4.59	0.33	–[b]	2.02	22.7	20	113.3	5.0	40.5
Xetalean[a]	2 capsules	–[b]	–[c]	15.2	6.43	0.20 (NC)	–[b]	6.75	21.6	20	108.0	5.0	135.0

[a] Also analyzed by LC-MS. [b] Response, if any, was below the lowest calibration limit of 0.02 μg, and identity was not confirmed (NC) by UV spectral matching. [c] Inconsistent confirmation of identity among replicates with a quantifiable response. [d] Response was below the lowest calibration limit of 0.02 μg, but identity was confirmed (C) by UV spectral matching. [e] Midpoint of label range 3.75–7.5.

strated with replicate determination of the abovementioned major ingredients in Xetalean.

Limit of Quantitation (LOQ). Method LOQ was assigned to the lowest calibration point, nominally 0.03 μg. The limit of determination was calculated to be 0.006 mg/g, based upon the LOQ loaded onto the precolumn from a 500-μL aliquot of 100-mL extract of a 1-g test portion.

Comparative Determination by LC-MS. A set of 25 samples (identified in **Table 4**) was analyzed concurrently by an independent method (*8*) using a d_5-labeled (−)-E·HCl internal standard, a phenyl LC column in reversed-phase mode, and a mass spectrometer in full-scan mode with in-source fragmentation by tube lens voltage for quantitation and confirmation (three ions). A single analytical determination of (−)-E, (+)-PE, and (±)-SYN by the UV method was plotted against the corresponding single MS finding in **Figure 6**. Accordingly, identity was confirmed with no discrepancy. Linear regression showed that (−)-E determination was more closely correlated (R^2 =

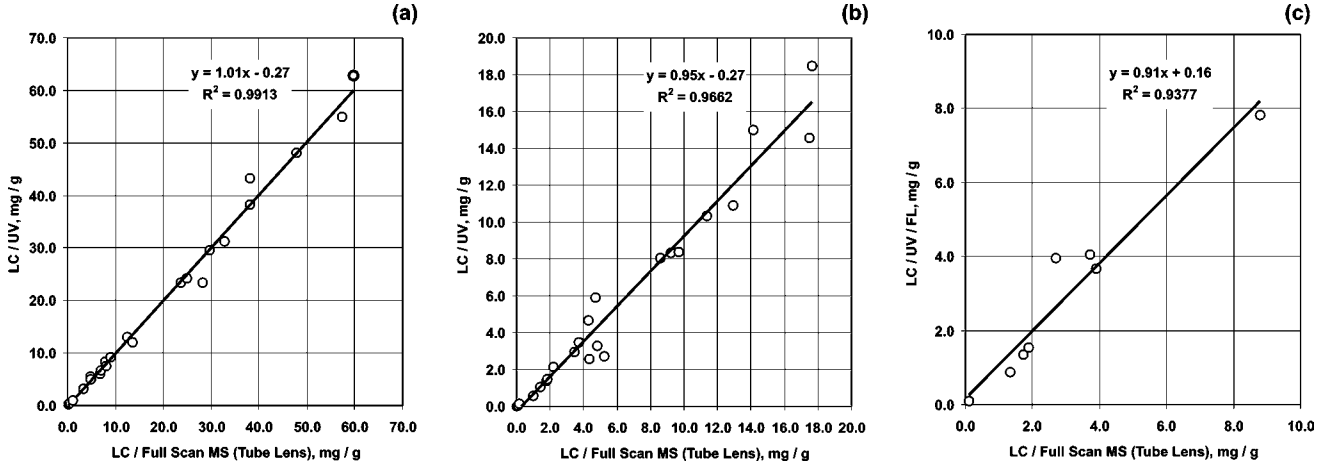


Figure 6. LC determination of (a) (–)-E, (b) (+)-PE, and (c) (±)-SYN in 25 dietary supplements by this method compared to MS method. Linear regression and correlation statistics are placed on each graph.

0.991) and had the smallest proportional bias (1%), followed by (+)-PE and finally by (±)-SYN. The synephrine comparison may have been compromised because fewer products contained it. The sample set mean for (−)-E, (+)-PE, and (±)-SYN determinations was calculated for each method, and the difference between the means was tested for significance at 95% confidence by a two-sided paired *t* test. There was no significant difference between sample means for UV and MS determination of (−)-E (df = 24, critical $t = 2.066$, calculated $t = -0.301$) or of (±)-SYN (df = 7, critical $t = 2.365$, calculated $t = -0.415$). However, determination of (+)-PE did show a statistical difference between the sample set means (df = 23, critical $t = 2.071$, calculated $t = -2.715$). When the batch of extracts was reanalyzed with the mass spectrometer configured in MS/MS mode (*10*), the difference between sample set means for (+)-PE determination by UV and MS/MS was no longer statistically significant (calculated $t = -0.226$) but was statistically significant for MS compared to MS/MS (calculated $t = 3.292$). Because no (+)-PE aging effect was observed with either mass spectrometric analysis (the same phenyl column was used; the primary separation mechanism was solute solubility compared to ion exchange in the UV method), the statistical comparison may indicate that MS/MS measurement (*11*) may be more reliable than that by the published MS configuration. In addition, sample set means for (−)-E and (±)-SYN were not significantly different for UV versus MS/MS, as was the case for UV versus MS already discussed. Too few occurrences of (−)-NE, (+)-NPE, and (+)-N-MPE prevented statistical comparison between methods. Tailing elution of (−)-N-ME gave rise to a weak MS quantitation signal in too many extract analyses such that comparison with UV was abandoned.

Survey. The results of a limited survey comprising 48 finished products are presented in **Table 4**, arranged in alphabetical order by product name and averaged from replicate analyses. Amount of active ingredient per serving was usually available from the label or was found from other sources such as manufacturer's literature on the Internet or a telephone call to customer service, except insufficient information was provided for Clenbutrx. Ingredients declared as herbal Ephedra or Ma Huang referred to total ephedrine alkaloid content. Solaray was the only product labeled with ephedrine alkaloid content in a range, the midpoint of which was used for comparison to analytical findings. AdipoKinetix and Phenyl XL claimed norephedrine−HCl as the sole ephedrine alkaloid, at 25 mg per serving; (−)-NE determination, after conversion to the hydrochloride salt, was 93−95% of the label. During chromatography of fresh Phenyl XL extract, the (−)-NE peak tailed so much that peak area integration was unreliable. This problem was overcome simply by either repeating the chromatographic analysis a few hours later or performing the method using a 100-mg test portion size. Both ways gave excellent peak shapes and equivalent content determination. The fact that this problem was not observed with AdipoKinetix extract suggests that some matrices may temporarily affect the cation exchange interaction between analyte and phase. From our experience with finished products, matrix interference with cation exchange of the ephedrine alkaloids, although rare, was easily overcome with simple adjustments to the procedure. As a minor component, (−)-NE was confirmed in four products at insignificant levels. Energy Fuel labeled as Ma Huang free was indeed found not to have any ephedrine alkaloids, but the (±)-SYN finding was two-thirds of the label claim.

A few products were spot-checked for manufacturing consistency in ephedrine alkaloid and synephrine content. Two different lots of Solaray and Thermo Pro were consistent in found levels of individual as well as total ephedrine alkaloids. Although two lots of Complete Diet Boost agreed in total ephedrine alkaloids per serving, levels of (−)-E and (+)-PE varied such that (−)-E was either 86 or 71% of the total; however, synephrine content was consistent. Another purchase of Pro-Ripped Ephedra had the same lot number and experimentally the same ephedrine alkaloid and synephrine findings, suggesting a homogeneously blended lot bottle-to-bottle. Time did not allow a more thorough study of the variation of the (−)-E level relative to total ephedrine alkaloid content over many lots of product, within the context of its natural variability in Ephedra herbs used in the formulations.

The products we analyzed clustered into three groups according to the amount of total ephedrine alkaloids in a serving size: group I delivered ≤15 mg/serving ($N = 10$), group II delivered 17−24 mg/serving ($N = 26$), and group III delivered ≥30 mg/serving ($N = 7$). If the two supplements containing only (−)-NE were omitted, then in 41 products the composition of total ephedrine alkaloids averaged 75.3% ephedrine (SD = 17.9) and 21.8% pseudoephedrine (SD = 17.4). As a rule of thumb, three-fourths and one-fifth of total ephedrine alkaloids delivered in a serving were (−)-E and (+)-PE, respectively. Exceptions included Red X and Ultimate Orange Workout drink mix, in which the total ephedrine alkaloids was virtually pure ephedrine; Stoked, Fat Burner Energizer, and Ultra Diet Pep had, respectively, total ephedrine alkaloids of 96, 71, and 47% pseudoephedrine. Persons consuming the least potent supple-

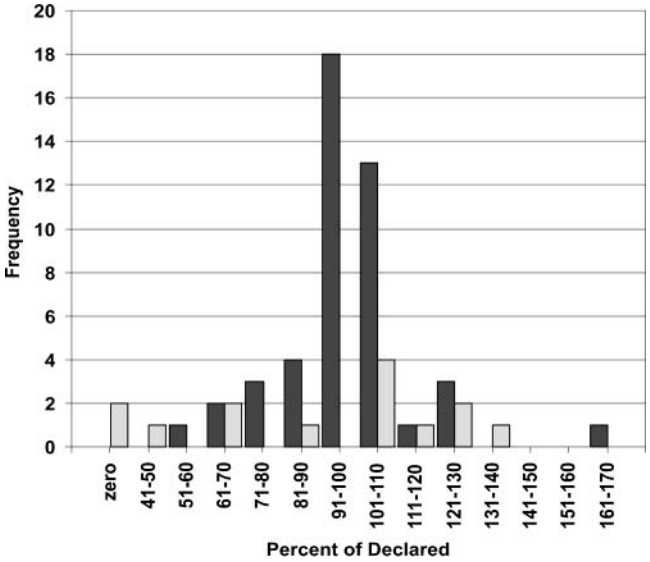


Figure 7. Analytical findings relative to product declaration: (black bars) total ephedrine alkaloids; (gray bars) (±)-synephrine.

ment in this survey were getting ~3 mg of (−)-E per serving as opposed to people receiving up to 50 mg of (−)-E per serving from the high-potency supplements.

Laboratory findings relative to product declaration (includes labeling and other sources of information) are presented as a histogram in **Figure 7**. In 46 products, 78% had total ephedrine alkaloids within an arbitrary ±20% range about the declaration. Gurley et al. (*12*) found 70% of 20 samples with declared value to be within this same range, suggesting that accuracy in labeling ephedrine alkaloids has improved incrementally. Nonetheless, 13% of our and their samples had <80% of the declared amount, and ~9% of ours exceeded 120% versus 15% of theirs. We found that synephrine content labeling was usually unreliable. No synephrine was found in Red X and Sizzle (including by LC-MS) despite, respectively, 20 and 25 mg/serving label claims. Synephrine added to test portions was recovered completely (see **Table 2**), demonstrating that had synephrine been present initially in these products, then it would have been recovered and measured. In 14 products with synephrine as a labeled ingredient, we found just 43% within ±20% of the declaration, 36% fell below 80%, and 21% exceeded 120%.

In summary, dietary supplement products containing ephedrine alkaloids and/or (±)-synephrine packaged in capsules, tablets, caplets, mixes, powders, and liquids were analyzed in 1 h using on-line cleanup and simple liquid chromatographic detectors calibrated with external standards. Results for (−)-ephedrine, (+)-pseudoephedrine, and (±)-synephrine were comparable to independent determination by isotope dilution mass spectrometric measurements on extracts cleaned up off-line. The method ruggedly handled diverse sample forms and concentrations of sought-for substances, including products fortified with numerous additional ingredients. Measurement reliability was achieved by rigorous criteria of retention time matching and near-perfect UV spectral match to a reference standard, for confirmation of peak identity and purity. In a survey of finished products, discrepancies with content claims outside ±20% were found in one-fifth of the products for total ephedrine alkaloids and in more than half of the products for synephrine, including two samples in which no synephrine was detected.

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