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Quantitative and qualitative HPLC analysis of thermogenic weight loss products

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An HPLC qualitative and quantitative method of seven analytes (caffeine, ephedrine, forskolin, icariin, pseudoephedrine, synephrine, and yohimbine) in thermogenic weight loss preparations available on the market is described in this paper. After 45 min the seven analytes were separated and detected in the acetonitrile : water (80 : 20) extract. The method uses a Waters XTerra RP_{18} (5 μm particle size) column as the stationary phase, a gradient mobile phase of water (5.0 mM SDS) and acetonitrile, and a UV detection of 210 nm. The correlation coefficients for the calibration curves and the recovery rates ranged from 0.994 to 0.999 and from 97.45% to 101.05%, respectively. The qualitative and quantitative results are discussed.

1. Introduction

Obesity is on the rise in the US, and with that rise comes the craving for the elusive weight loss in a bottle. Companies are flooding the market with these thermogenic products, from the media present *Ephedra* containing products to the growing *Coleus forskohlii* products. Each of these products contains their own thermogenic blend of herbals, vitamins and minerals, which can make the ingredient label seem daunting to the consumer. Some of the plants used in thermogenic products and their active constituents are as follows: *E. sinica* (ephedrine and pseudoephedrine), *C. forskohlii* (forskolin), *Paullinia cupana* (caffeine), *Corynanthe yohimbe* (yohimbine), *Citrus aurantium* (synephrine), and *Epimedium* spp. (icariin). Many products may contain one or more of the above listed plants.

Ephedra sinica, known as *Ma Huang*, is one of the oldest medicinal herbs in Traditional Chinese Medicine (TCM). The most abundant biologically active analyte present in *E. sinica* is ephedrine (**1**). The pharmacological studies have shown **1** to be a sympathomimetic agonist at both the α- and β-adrenergic receptors which leads to an increased cardiac rate and contractility, to peripheral vasoconstriction, to bronochodilation, and to central nervous system (CNS) stimulation (Fouad-Tarazi et al. 1995; Walker et al. 1998). *Coleus forskohlii* Briq. (Lamiaceae) has been traditionally used to treat heart and lung disease, intestinal spasms, insomnia, and convulsions (Dubey 1981 et al.). Forskolin (**2**), a labdane diterpenoid, is considered the active secondary metabolite due to its ability to activate the enzyme adenylate cyclase (Seamon and Daly 1981). It has also been found to inhibit the platelet-activating factor (PAF) (Wong et al. 1993). *Paullinia cupana*, Guarana, contains caffeine (**3**) readily found in many of these products. Caffeine is widely known and used as a stimulant to increase one's energy (Sung et al. 1995). Increased blood pressure is associated with caffeine consumption (Sung et al. 1995). *Corynanthe yohimbe* contains within the bark the active constituent yohimbine (**4**). It has been commonly used for the treatment of impotence, orthostatic hypotension, and diabetic neuropathy (Wahrenberg et al. 1990; Verwaerde et al. 1997; Ernst and Pittler 1998). Yohimbine (**4**) increases the adrenergic α-1 activity and decreases the adrenergic α-2 activity (Millan et al. 2000). The effects of **1** are increased for a longer duration of time when used in combination with **4** (Jonderko and Kucio 1991). *Citrus aurantium*, bitter orange, contains the compound synephrine (**5**). Its structure is similar to ephedrine, and acts in much of the same pathways as **1** (Wu et al. 2003). Traditional uses of *C. aurantium* are also similar to those of *E. sinica*. *Epimedium* ssp., known as horny goat weed, contains the active secondary metabolite icariin (**6**) (Liang et al. 1997). Used in traditional medicine for kidney disorders, icariin shows androgenic, anti-estrogenic and antioxidant activity (Wu et al. 2003).

Analytical methods have been developed for the quantitation of these compounds in their respective plant extracts, but there is not one method reported which can detected all the compounds listed in a single procedure. Thus, a method has been developed using RP HPLC with PDA detection at 210 nm. The method was used to analyze 26 thermogenic products on the market claiming to contain one or more of the above listed genera for quantity and quality.

2. Investigations, results and discussion

2.1. Method development

Methods have already been developed for the quantification of the analytes in this study, but each analyte has its own method. In many cases, the thermogenic products on the market contain two or more of the analytes, such as one which contains ephedrine (**1**), forskolin (**2**), caffeine (**3**), and synephrine (**4**). Time is saved by producing one method for all six analytes instead of analyzing the product for each analyte.

The method development involved a set of various reverse phase columns (Phenomenex Synergi MAX, Luna C18(2), Lichrospher RP 18e, Prodigy ODS 3, Supelco Discovery C18, and Waters XTerra). The Waters XTerra RP_{18} 150×4.60 mm (5 μm particle size) was chosen. Initially, retention times of **1** and **5** were a problem since the compounds were not retained on the column long enough for quantification. A method reported earlier by Gurley et al. (1998) used sodium dodecyl sulfate (SDS) as a buffer to increase the retention time of the ephedrine alkaloids on reversed phase columns. This increased the retention times of **1** and **5** from approximately 1 min to approximately 33 and 27 min respectively. Due to the variety in physical properties of the six analytes, a gradient system using water buffered with SDS and trifluoroacetic acid (TFA) and acetonitrile was developed. TFA was added to the mobile phase to sharpen the resolution of the analytes. Initially the method was held at 95% water content in order to ensure an elution of **3** at approximately 5 min. Then the gradient gradually changed to 40% water content over 45 min to elute the remaining analytes. A chromatogram of the six analytes is shown in Fig. 1.

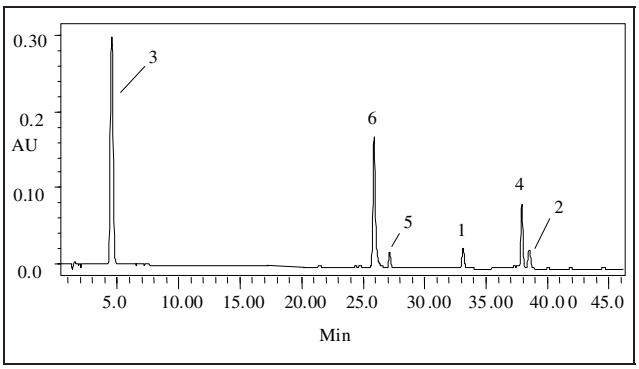

Fig. 1: Chromatograms of the analytes at 210 nm

The extraction method also had to be analyzed due to the differences in physical properties of the six analytes. Initially, acetonitrile was used for extraction purposes, but **1** and **3** had a low solubility in this solvent. Also, using water as the extracting solvent was a problem since many of the commercial products clumped into a solid mass upon adding the water. In a 80 : 20 mixture of acetonitrile : water, **1** and **3** were soluble and the samples did not clump.

The method was validated by a random triplicate injection of all samples. Intra- and inter-day extractions were also analyzed, followed by spiking experiments to confirm extraction efficiency. All RSDs were less than 5%. The presence of the analytes were confirmed by a comparison of retention times with the standard compounds as well as a comparison of the UV spectra of the analytes and those detected in the products. The UV spectra are shown in Fig. 2.

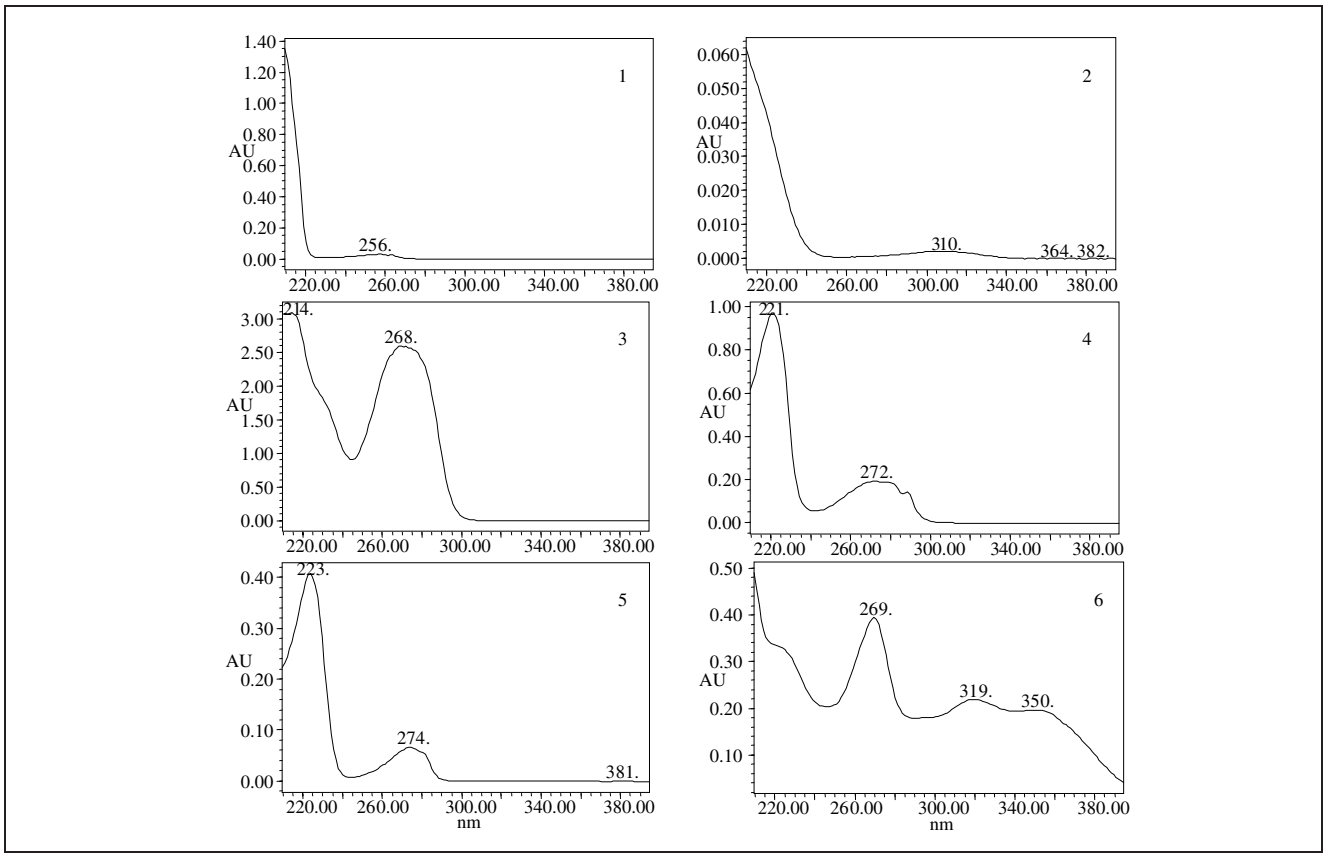

Fig. 2: UV spectra of the six analytes

Table 1: Concentration (mg) of analytes determined in thermogenic products by HPLC with PDA at 210 nm

Product	1	2	3	4	5	6
T1					9.6 (15.0)	
T2					9.9 (27.0)	
T3					0.3 (−)	
T4						8.2 (25.0)
T5						8.4 (25.0)
T6						9.5 (25.0)
T7				3.5 (8.0)		
T8				4.0 (8.0)		
T9		14.2 (15.0)				
T10	7.0 (20)	10.9 (8.3)	18.4 (200.0)		0.8 (5.0)	
T11				0.1 (0.2)		0.1 (−)
T12		ND*			8.6 (−)	
T13		21.7 (20.0)			4.4 (5.0)	
T14			0.6 (11.0)		1.5 (8.0)	
T15			1.0 (3.7)			
T16	4.0 (6.7)	4.5 (5.6)	16.8 (66.7)		0.5 (1.7)	
T17	6.3 (6.7)		17.7 (66.7)			
T18	8.3 (6.7)		18.2 (100.0)		1.7 (2.5)	
T19	4.8 (6.7)	1.4 (1.7)	15.4 (50.0)			
T20	13.0 (15.0)					
T21	5.4 (10.0)		17.9 (100.0)			
T22	9.2 (12.5)	11.7 (10.0)	15.2 (62.5)			
T23	10.0 (−)		16.9 (−)			
T24	8.3 (10.0)		19.7 (99.0)		15.8 (−)	
T25	7.4 (10.0)		12.6 (16.0)			

* ND = Not detected
The quantities of analytes claimed by the label in the product are given in parentheses.

2.2. Product analysis

A number of commercial products (25) were analyzed using the developed method. The products varied in composition, either containing one of the desired analytes, or a mixture of analytes. Product T16 contained the highest number of analytes (4). Table 1 lists the quantification results and the chromatograms of a few selected products are shown in Fig. 3. The values in Table 1 are the concentrations (mg) of the analytes. The values in parentheses are the quantities listed on the labels of the commercial products.

Products T1, 2, and 3 were bitter orange products, and compound **5** was detected in all three samples, but in lower quantities than listed on the label. There was no concentration listing on the label of T3. Horny goat weed was the individual herb present in products T4, 5, and 6. The detected quantities of **6** were roughly the same in all three products, but significantly lower than the concentrations reported on the labels. Each capsule was stated to contain 25.0 mg of **6**, the product manufacturers likely purchased the raw starting material from the same distributor. Products T7 and 8 were Yohimbe bark products, and **4** was detected in both products at about half the quantity that should have been present according to the label claim. The only other single component product was T9, which contained *Coleus forskohlii*. Forskolin (**2**) was detected in one capsule (14.2 mg), which was a relatively close to the amount listed on the label (15.0 mg).

The remaining products (T10 to T25) are mixtures of plants containing various analytes of interest. Ephedrine (**1**) was detected in eleven of the products (T10, 16, 17, 18, 19, 20, 21, 22, 23, 24, and 25). Only about half the amount of **1** claimed on the labels was detected in T10 and T21. Six of the products contained *Coleus forskohlii* (T10, 12, 13, 16, 19, and 22). Forskolin (**2**) was detected in all but product T12. The detected concentrations of **2** were also approximately the same as given on the labels of the products. Twelve of the products (T10, 14, 15, 16, 17, 18, 19, 21, 22, 23, 24, and 25) tested positive for **3** in much lower concentrations than listed on the labels. Caffeine (**3**) was present from either a green tea extract, a guarana extract, or from the addition of pure caffeine (**3**). The concentration of **3** in the products was high and exceeded the upper quantification limit of the detector. If the extract was further diluted, the other analytes fell below the limit of quantification. The detected concentration of **3** in T25 was similar to that listed on the label. Besides products T7 and T8, product T11 contained **4** by detection, and the concentration was similar to that listed on the label. Seven of the products (T10, 12, 13, 14, 16, 18, and 24) contained detectable amounts of **5**, and only the amount detected in T13 was similar to that listed on the label. Icariin (**6**) was also detected in T11, but the label did not list a concentration for **6**. In all 25 products, the presence of analytes claimed by the product labels was confirmed, and none of the analytes were detected in any of the products in which the analyte was not listed.

2.3. Conclusions

This is the first single method which allows to confirm the presence of six analytes known to be in thermogenic weight loss herbals within 45 min. By a simple sonication extraction, the confirmation of an analytes presence in a product is complete within approximately 90 min, extraction to analysis. This is an advantage over using multiple methods in order to detect each individual analyte. The method may also aid in detecting adulteration of a product with synthetic ephedrine or yohimbine.

3. Experimental

3.1. Chemicals and reference compounds

HPLC grade acetonitrile was obtained from Fisher Scientific (Fair Lawn, NJ, USA). Trifluoroacetic acid (TFA), sodium dodecyl sulfate (SDS), and caffeine were purchased from Sigma (St. Louis, MO, USA). Ephedrine, pseudoephedrine, forskolin, and yohimbine were purchased from Chroma-

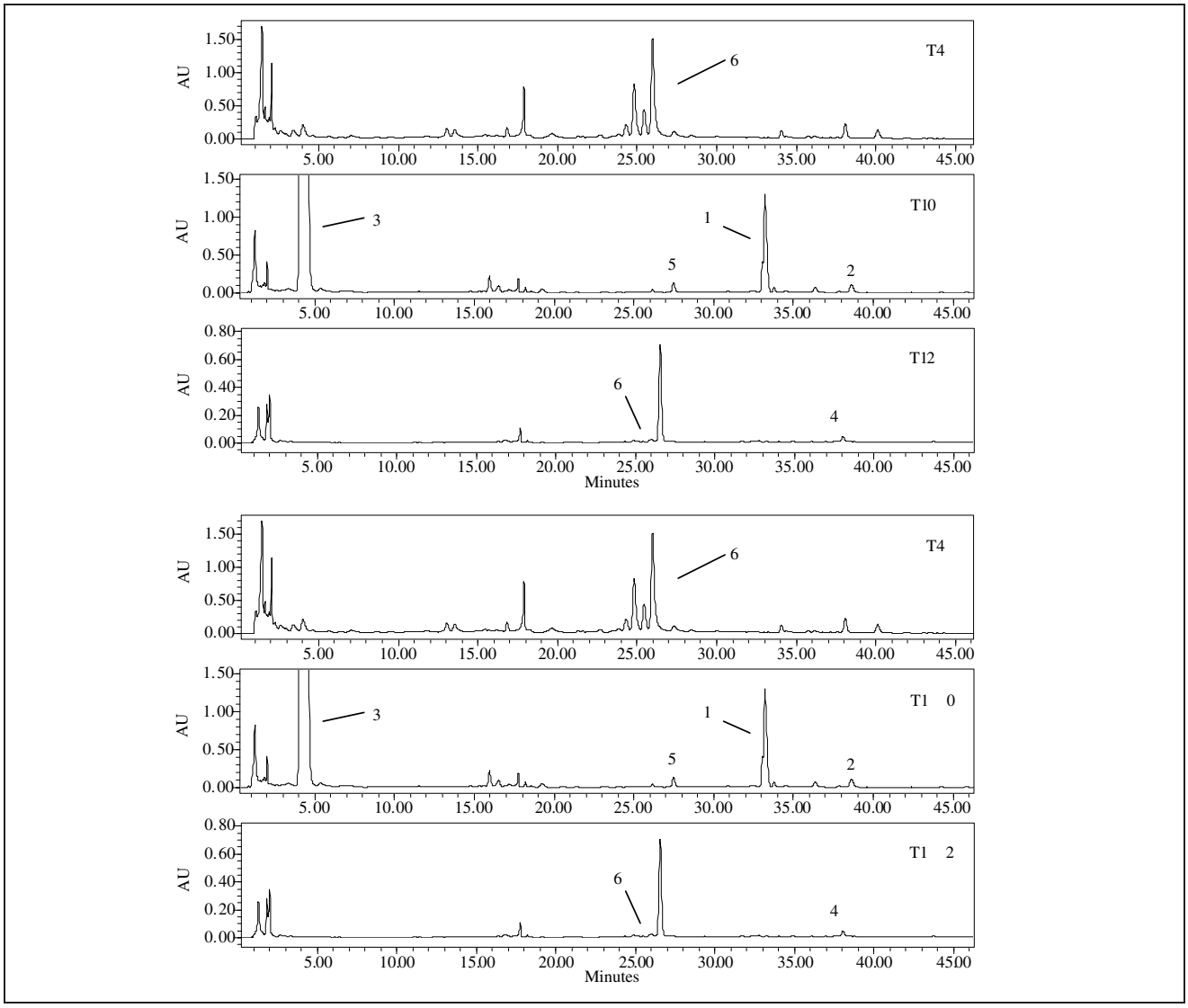

Fig. 3: Chromatograms of selected products at 210 nm

Dex (Santa Ana, CA, USA). Icariin was provided by Prof. M. Iinuma from Gifu Pharmaceutical University (Gifu, Japan). HPLC grade water was prepared by filtering nanopure water through a 45 μm membrane filter.

3.2. Dietary preparations

Products were purchased from Nutri-Mart (Diamond Bar, CA, USA), the Vitamin Shoppe (North Bergen, NJ, USA), and local herbal stores (Oxford, MS, USA). All samples are deposited at the National Center for Natural Products Research, University, MS.

3.3. Equipment

Sample analysis by HPLC was done on a Waters Alliance 2695 Separations Module with a Waters 996 PDA detector (Waters, Millford, MA, USA). The analysis software was Millennium[32] by Waters. The column was a Waters XTerra RP_{18} 150 × 4.60 mm (5 μm particle size) and the guard column was a Security Guard C18 cartridge system (Phenomenex, Torrance, CA, USA). Extraction took place in a FS20H Ultrasonic Cleaner (VWR Scientific Products, West Chester, PA, USA).

3.4. Sample preparation

Six of the commercial products tested were tablets, while the remaining products were encapsulated powder. Seventeen of the products were a mixture of plants, vitamins and minerals, while eight of the products contained one plant according to their labels.

3.4.1. Encapsulated powder sample preparation

The powder (0.4–1.0 g) was dumped into a 15 mL screw capped polypropylene centrifuge tube (Falcon tubes from VWR Scientific Products) and extracted three times with 3.0 mL of acetonitrile : water (80 : 20) by sonication for 10 min. The emulsion was centrifuged (5.0 min at 3000 rpm) and the supernatants were combined to a 10 mL volumetric flask by pipette and diluted to the final volume with acetonitrile and mixed thoroughly. All samples were filtered through a 0.45 μ PTFE syringe filter prior to injection.

3.4.2. Tablet sample preparation

One tablet was crushed to a fine powder in a mortar and pestle. After recording the weight, the sample was transferred to a 15 mL screw capped polypropylene centrifuge tube (Falcon tubes from VWR Scientific Products). The extraction procedure was then followed as above in the encapsulated powder sample preparation (*3.4.1.*).

3.5. Calibration

Approximately 5.0 mg of each standard compound was placed in a 10 mL volumetric flask and diluted in acetonitrile : water (80 : 20) (stock solution).

Table 2: Calibration data for compounds 1–7

Compd.	Regression equation	Correlation coefficient	Limit of detection (μg/mL)
1	$y = 2.65 \times 10^4 x$	0.999	0.01
2	$y = 2.65 \times 10^4 x$	0.998	0.01
3	$y = 1.52 \times 10^3 x$	0.999	0.01
4	$y = 6.51 \times 10^4 x$	0.994	0.01
5	$y = 3.92 \times 10^4 x$	0.999	0.01
6	$y = 1.77 \times 10^4 x$	0.999	0.01
7	$y = 2.52 \times 10^4 x$	0.999	0.01

Further calibration levels were prepared by diluting the stock solution with acetonitrile : water (80 : 20). Within the range of concentrations injected (650.0–5.0 μg/mL) the detector response was linear. The combined calibration data (regression equation, correlation coefficient, limit of detection) is listed in Table 1. The recovery rates were as follows: **1**, 99.5%; **2**, 99.1%; **3**, 100.3%; **4**, 101.1%; **5**, 99.5%; **6**, 98.8%; **7**, 99.8%.

3.6. Analytical conditions

The following conditions were optimal: solvent A aqueous 5 mM SDS (five drops of TFA to 1 L), solvent B was acetonitrile. Start the mobile phase at 90A/10B and hold for 5 min, then change to 40A/60B over 45 min. Total run time was 50 min. To wash the column, change the mobile phase to 5A/95B from 40A/60B over 5 min. Hold at 5A/95B for 5 min. After the wash time has elapsed, return the mobile phase to the starting amounts and allow it to re-equilibrate for 10 min prior to the next injection.

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