ORIGINAL ARTICLE

COMPARATIVE ANALYSIS BY HPLC-UV AND CAPILLARY ELECTROPHORESIS OF DIETARY SUPPLEMENTS FOR WEIGHT LOSS

AMELIA TERO-VESCAN[1], CAMIL-EUGEN VARI[2]*, SILVIA IMRE[3], BIANCA-EUGENIA ŐSZ[2], CRISTINA FILIP[1], GABRIEL HANCU[4]

University of Medicine and Pharmacy, Faculty of Pharmacy, 38 Gheorghe Marinescu Street, Târgu Mureş, 540139, Romania
[1]Biochemistry and Chemistry of Environmental Factors Department
[2]Farmacology and Clinical Pharmacy Department
[3]Organic Chemistry Department
[4]Pharmaceutical Chemistry Department

**corresponding author: camil.vari@yahoo.fr*

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Abstract

The legislation for dietary supplements (DS) is extremely permissive although some of these supplements may contain pharmacologically active, alleged vegetal compounds such as caffeine (CF), yohimbine (Y) or ephedrine (EPH), and the lack of strict control raises the question of contamination with prohibited substances in the European Union. This paper proposes a comparative analysis of weight loss DS by two separation techniques, capillary electrophoresis and high performance liquid chromatography with UV detection. The results obtained by the two methods show a higher dose of CF than stated on the label, as these DS contain CF pure substance and CF-containing extracts (matrix), while for Y the determined content is lower than the amount specified on the label. The two methods give comparable results in the analysis of DS, any statistically significant differences can be related to the uniformity of the analysed samples.

Rezumat

Legislaţia în domeniul suplimentelor alimentare este extrem de permisivă, deşi unele dintre aceste suplimente pot conţine compuşi de origine vegetală, dar farmacologic activi cum ar fi cafeina, yohimbina sau efedrina, iar lipsa unui control strict ridică problema contaminării lor cu substanţe interzise în Uniunea Europeană. Lucrarea propune o analiză comparativă a unor suplimente pentru slăbit prin două metode de separare, metoda electroforezei capilare şi a cromatografiei de lichide de înaltă performanţă. Rezultatele obţinute prin cele două metode arată o depăşire a dozei de cafeină menţionată pe etichetă, suplimentele conţinând pe lângă cafeina substanţă pură şi extracte vegetale cu conţinut de cafeină (matrice), iar în cazul yohimbinei, aceasta se găseşte în cantităţi mai mici decât se menţionează pe etichetă. Rezultatele obţinute prin cele două metode sunt comparabile, eventualele diferenţe cu semnificaţie statistică apărute fiind puse pe seama neuniformităţii probelor analizate.

Keywords: weight-loss dietary supplements, HPLC, capillary electrophoresis, caffeine, yohimbine

Introduction

Weight loss dietary supplements (DS) often contain stimulants which increase basal metabolic rate and caloric needs of the body covered by lipolysis. DS are used by amateur or professional athletes and people who want to improve their physical appearance.

World Anti-Doping Agency (WADA) annual list includes several banned substances to athletes during competition and between competitions, often these legislative changes taking sport officials by surprise. Meanwhile, detection methods applied to test athletes are increasingly effective, selective and precise. All these increase the popularity of DS among athletes based on the assumption that being of vegetal origin are virtually free of side effects and the active substances contained are not subject to doping suspicions.

Many DS contain plant extracts derived from *Garcinia cambogia, Capsicum anuum, Camellia sinensis, Arctostaphylos uva-ursi, Zingiber officinale, Citrus paradisi*, whose standardization is not rigorously followed and no measurable clinical data regarding their effects are provided.

In case of some compounds, most often of vegetal origin with proved pharmacological action like caffeine (CF), ephedrine (EPH) or yohimbine (Y), labelling and recommended dosage could lead to confusion favouring overdose (from pure substance and similar compounds from matrix) and an indication regarding the daily recommended dose has not been established.

The abundance of DS containing vegetal extracts on the market raises the question of their efficacy and safety on long-term use, especially if the

exposure of the target population and the total cumulative dose are very difficult to estimate [4].

Assessment of nutritional behaviour in athletes and identification of the main DS used and legally purchased by them, online or from the black market, is one of the study premises, especially given that many alleged products from plants are contaminated with synthetic chemical compounds highly pharmacologically active. Also the presence on the market of products, which may illicitly contain different compounds in order to improve their effectiveness, is a public health issue.

A general strategy to perform analysis on multicomponent DS products starts with the development of efficient and selective screening method for a wide range of compounds.

A large variety of methods exist in the literature dealing with the detection and determination of different compounds of natural origin from DS, the large majority using high performance liquid chromatographic (HPLC or UPLC) techniques, but also capillary electrophoresis (CE) which has proved to be an alternative and also a complementary method for the analysis of these particular types of substances [9].

Even if there are relatively numerous methods of determination for CF [10], Y [6], EPH [15] and SB [18] by CE, no CE method for the simultaneous qualitative or quantitative determination of the four compounds mentioned above has been reported so far in the literature.

The purpose of this study was to analyse the quality of several marketed DS in order to evaluate the recommended dose by the manufacturer taking into account the actual content of active substance and the dose normally administered by the consumer. The identification of possible interference and/or associated risks between the restrictive diets or some particular food behaviour and the use of DS was also investigated. The analysis was based on two screening analytical methods, CE and HPLC, respectively, for the qualitative and the quantitative determination of compounds of vegetal origin, legally introduced in DS for athletes (CF, Y), and some possible contaminants, not declared by the manufacturer, which are prohibited by WADA, such as EPH of vegetal or synthetic origin and sibutramine (SB). The chemical structures of the four studied compounds are presented in Figure 1.

The purpose of this study was to develop screening methods of legal substances according to the anti-doping legislation, but whose labelling might lead to confusion (e.g. CF content associated with plant extracts containing CF, the total cumulative dose is not quantifiable) and of undeclared substances, but prohibited (SB, EPH). Two analytical screening methods were used in parallel to identify suspicious DS (amounts different from those stated or containing prohibited substances) for further analysis of these DS, the most sensitive and validated methods will further be used.

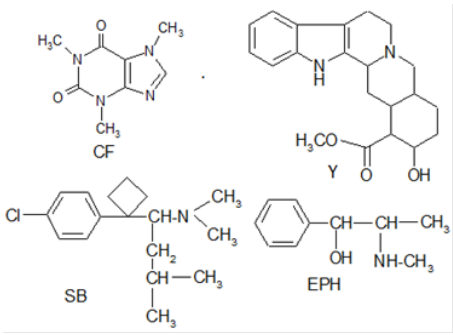

Figure 1.
Chemical structures of the studied compounds

As for most of the compounds present in the form of extracts there is a threshold dose recommended daily, overcoming it may be associated with health risks, especially in case of physical effort or in combination with restrictive diets that promote weight reduction consecutive to lipolysis (caloric restriction, limited carbohydrate intake – e.g. the ketogenic diet). In these situations, the excessive consumption of substances promoting lipolysis like CF, considered safe, may have adverse metabolic and cardiovascular consequences. Association of anorectic compounds (SB) or which increase the metabolic rate (EPH) undeclared can induce severe side effects, including sudden death by cardiac arrhythmias in individuals with full apparent health.

Materials and Methods

Standards and reagents

Standards were of minimum 98.0% purity and were acquired as follows: SB hydrochloride from Chemos GmbH, CF from Fluka, Y hydrochloride from Alfa Aesar, and EPH hydrochloride from Sigma-Aldrich.

All HPLC grade solvents and reagents - methanol, acetonitrile, phosphoric acid 85%, sodium tetraborate, disodium hydrogenophosphate, sodium dihydrogeno-phosphate, sodium hydroxide and ammonium hydroxide, were bought from Merck (Merck KGaA, Darmstadt, Germany). Purified water used was obtained from a purified water system (Millipore SA, Molsheim, France).

DS sample analysis

Six DS used by athletes, available on the Romanian market at the time of the study which can be obtained legally (not black market) were selected. DS containing only vitamins, minerals, amino acids and proteins were excluded.

The active substances were extracted by mixing with the mobile phase at its initial composition in an ultrasound bath for 20 minutes. The resulting

mixture was centrifuged for 15 minutes at 3500 rpm. The clear supernatant was filtered and used for analysis.

CE method

The measurements were performed on an Agilent 6100 CE system (Agilent Technologies) equipped with a DAD detector. The separation was carried out on uncoated fused-capillaries of 48 cm (effective length 40 cm) x 50 µm I.D. The electro-pherograms were recorded and processed by Chemstation 7.01 (Agilent Technologies) software. The pH of the buffer solutions was determined with a Terminal 740 pH–meter (Inolab, Germany).

Stock solutions containing 1 mg/mL of each compound were prepared in methanol and later were diluted conveniently for the analysis. The samples were introduced in the system at the anodic end of the capillary by hydrodynamic injection. All the samples and buffers were filtered through a 0.45 µm syringe filter and degassed by ultrasounds for 5 minutes, before use.

The capillaries were conditioned before use with 0.1 M sodium hydroxide for 30 minutes and with the background electrolyte (BGE) used in the analysis for 30 minutes. The capillary was rinsed for 1 minute with 0.1 M sodium hydroxide and buffer solutions before each electrophoretic, separation.

In order to find the suitable conditions for the separation a series of preliminary experiments were conducted at different pH values and buffer compositions. In the preliminary analysis there were used 25 mM phosphoric acid (pH 2.1), 25 mM sodium dihydrogenophosphate (pH 4.8), 25 mM disodium hydrogenophosphate - sodium didydrogeno-phosphate (1:1) (pH 7) and 25 mM sodium tetraborate (pH 9.3) as BGEs and we adjusted the pH of the buffer by adding 0.1 M sodium hydroxide solution. We applied "standard" electrophoretic conditions for capillary zone electrophoresis (CZE) analysis: temperature 20ºC, applied voltage + 25 kV, injection pressure/time 50 mbar/3 sec, sample concentration 25 µg/mL.

The UV spectra of the four studied substances in methanol were previously recorded and the absorption maximum at 273 nm for CF, at 264 nm for EPH, at 226 and 283 nm for Y and 223 nm for SB, were determined; consequently, 210 nm as control wavelength and a specific wavelength for each analyte as detection wavelength were selected for detection.

HPLC method

Measurements were performed on a LaChrom Merck Hitachi chromatographic system consisting of: L-7100 quaternary pump with in-line degasser L-7612, L-7200 automatic injector with thermostat L-7350, and L-7455 DAD detector.

The separation was carried out on a Kromasil 100-RP8 chromatographic column, 150 mm x 4.6 mm, 5 µm with Kromasil RP 8 precolumn, at 25ºC.

The analysis was performed by using as mobile phase a mixture of sodium dihydrogen phosphate 50 mM pH = 5 and acetonitrile at a flow rate of 0.9 mL/min in a linear gradient as follows: 80 - 35% mobile phase A in 15 minutes, followed by column wash and reequilibration period. Detection was set between 201 and 400 nm and specific wavelengths for each analyte were selected.

Stock solutions of 1 mg/mL in methanol were prepared from SB, CF, Y, and EPH. Working solutions were prepared by diluting the stock solution with methanol to obtain the calibration concentration interval between 20 - 100 µg/mL.

Other used equipments were: AB54S balance (Mettler-Toledo), pH-meter MP225 (Mettler-Toledo), centrifuge 2 - 15 (Sigma), mixer 10 (Falc Instruments), water purification device Direct Q (Millipore), ultrasonic bath Transsonic T700H (Elma).

Results and Discussion

CE method

As far as we know no CE method for the simultaneous determination of CF, Y, SB and EPH was reported. The development of the method started from individual separation data available on the scientific literature database. The CE methods published in literature for the separation of CF, use capillary zone electrophoresis (CZE) or micellar electrokinetic chromatography (MEKC) techniques and alkaline pH of the BGE [12, 17]. CF can be determined successfully using an alkaline borate BGE as it was already reported in the literature, but at BGE'pH above 5, EPH, SB and Y migrate very close or even with the electroosmotic flow (EOF) and their separation can't be resolved.

As it is well known, the electrophoretic mobilities and ionization behaviour of analytes are the key factors driving separations in CZE. In CZE, the selectivity of the method is fundamentally based on charge-to-volume ratios, as separation occurs due to the differences between the own electrophoretic mobilities of the analytes. Knowledge of these basic physicochemical properties of the analytes gives valuable information about their nature and makes it easier to choose the appropriate experimental conditions for their separation. All four studied compounds have basic properties (amines) and will ionize in an acidic BGE.

Selectivity in CZE can be controlled by BGE composition and concentration, BGE pH, organic modifiers, applied voltage, system temperature and injection parameters. All these parameters were varied and their influence upon separation was

established in order to increase resolution and decrease migration times.

In order to develop a screening method for the simultaneous determination of the four studied compound by CZE we choose a slight acidic phosphate BGE, in which all the analytes are ionised and exhibit different electrophoretic mobilities.

Using a 50 mM phosphate buffer solution, at a pH of 4.8, applying a voltage of +25 kV at a temperature of 20°C and an injection pressure/time of 50 mbar/1 second, we achieved the simultaneous separation of the studied substances in approximately 6 minutes, the order of separation being: EPH, SB, Y, CF. The electropherogram of a mixture of standards using the optimized conditions is shown in Figure 2.

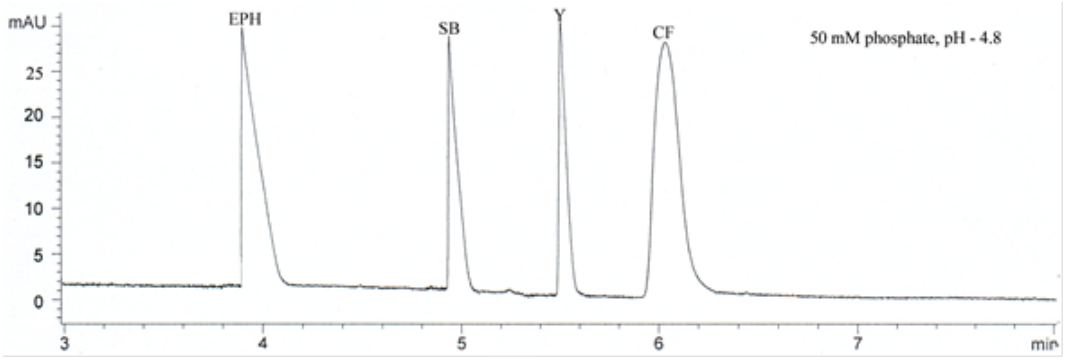

Figure 2.
Electropherogram of a standard methanolic solution with the concentration of 50 µg/mL EPH (MT = 3.95 min), SB (MT = 4.90 min), Y (MT = 5.50 min), CF (MT = 6.05 min) at 210 nm

The linear regression equations Area = f (concentration) for each substance were calculated according to six concentrations in a specific range (2.5 - 100 µg/mL) and three replicates per concentration. The linear regression coefficients were always above 0.99.

The optimized CZE procedure was applied to the analysis of the studied substances found in DS. Six samples of each DS were analysed, and three injections were performed to obtain the average values of the drug concentration. The peaks obtained from the samples were very similar with those obtained from standards and there were no noticeable interferences from the matrix (Figure 3).

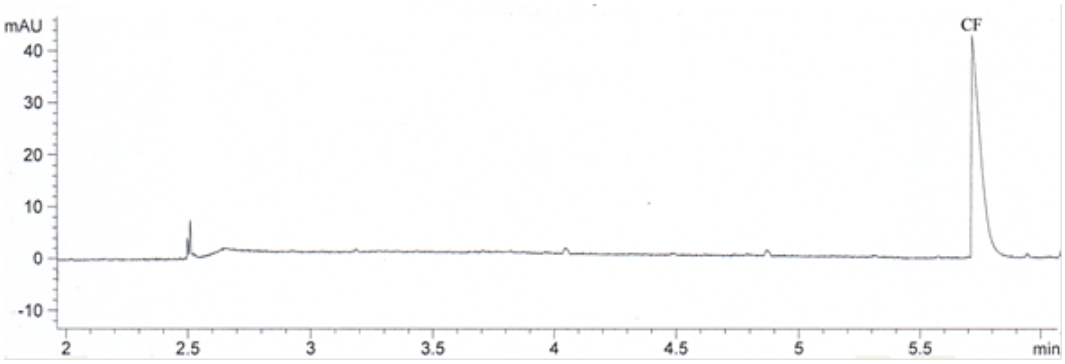

Figure 3.
Electropherogram of DS containing CF at 270 nm

HPLC method

A reference HPLC screening method was developed in order to confirm the CE data and to detect possible contaminants presented in DS in lower concentrations, not detectable by CE. The proposed chromatographic method allows the separation of the four compounds with good resolution and the gradient elution was applied in such manner that similar structural contaminants could be easily separated. The method proved good screening performances for a classical HPLC method. By applying the same principles on an UPLC equipment, a shorter run time could be achieve, as in Rebiere *et al*. method published relatively recently [16]. A chromatogram of a mixture of standards at reference wavelength of 229 nm is shown in Figure 4.

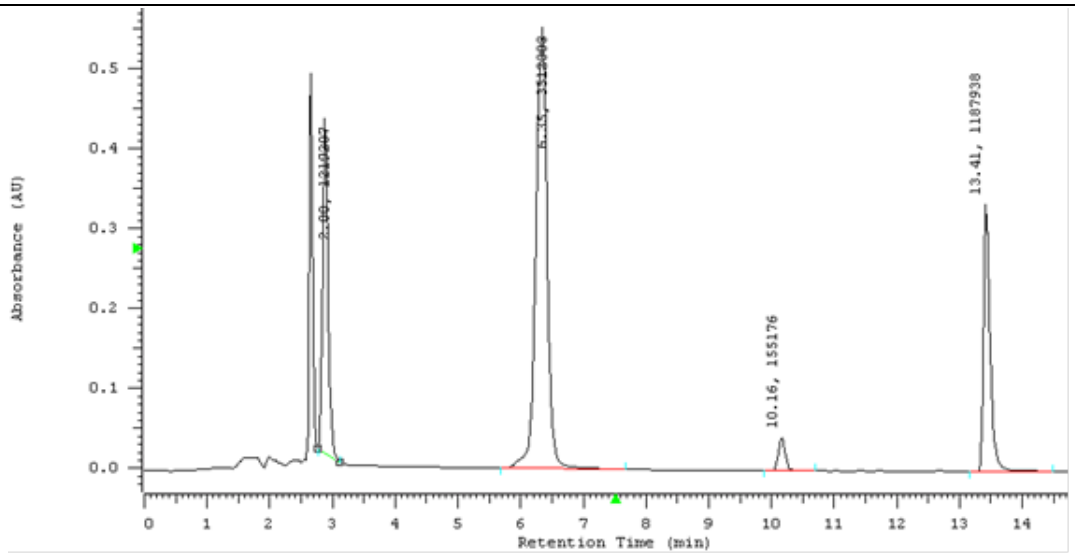

Figure 4.

Chromatogram of a standard methanolic solution with the concentration of 50 µg/mL EPH (RT = 2.88 min), Y (RT = 6.35 min), CF (RT = 10.16 min) and SB (RT = 13.41 min) at 229 nm

The calibration curve was constructed as Area = f (concentration) for five levels of concentration. The average calibration curve and the coefficient of determination are shown in Table I. The developed HPLC screening method was applied to the analysis of the studied substances found in dietary supplements (Figure 5).

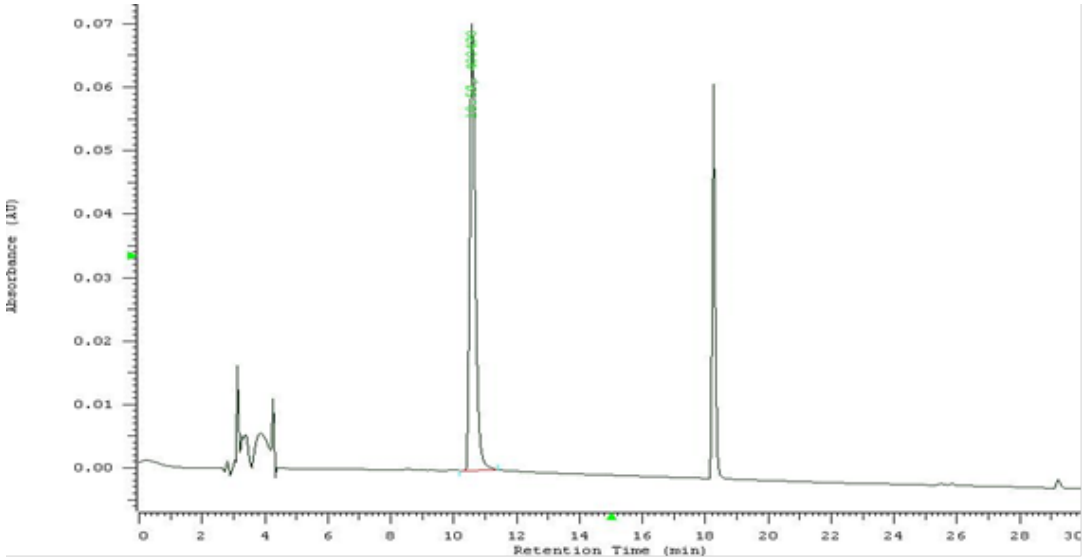

Figure 5.

Chromatogram of DS containing CF (RT = 10.51 min) at 254 nm

Both methods proved good linearity on the specific concentration range. Table I summarized as example, the linearity data of identified substances in DS.

Table I

Linearity regression data for CF and Y substances (n = 3, concentration range for CE method 2.5 - 100 µg/mL, concentration range for HPLC method 20 - 100 µg/mL)

	CE method		HPLC method	
Analyte	**Regression equation**	**R^2**	**Regression equation**	**R^2**
Y	y = 1.67 (± 1.06) x +3.17 (± 2.15)	0.9996	y = 1.18 (± 0.99)·10^4 x +4.6 (± 3.7)·10^5	0.9981
CF	y = 1.64 (± 0.98) x +7.22 (± 5.25)	0.9960	y = 1.24 (± 0.89) ·10^4 x +2.14 (± 1.52) ·10^5	0.9968

DS data analysis

The results of the DS analysis by the proposed methods are summarized in Tables II and III. DS legally obtained did not contain undeclared substances from the analysed group (EPH, SB).

Regarding the content of CF, in the case of all six DS, the determined amount was greater than the stated amount, but labelling may lead to confusion (CF + extracts containing CF obtained from *Guarana, Camellia sinensis, Yerba Mate*) [13]. However this way of labelling can create confusion and may predispose to overdosing. The International Society of Sports Nutrition declares that CF is effective in enhancing athletic performance in small or moderate doses 3 - 6 mg/kg bw, while high doses do not bring additional benefits (> 9 mg/kg bw) [5].

Table II

Determination of CF from DS

DS	Declared CF quantity (mg)	Found CF quantity (mg) ± SD (n = 3)				Statistical significance
		CE	**Diff %**	HPLC	**Diff %**	
DS1	135.00	265.10 ± 4.30	+96.37	223.89 ± 3.60	+65.84	$p < 0.05$ S
DS2	100.00	154.42 ± 5.60	+54.42	165.67 ± 2.03	+65.67	$p > 0.05$ NS
DS3	80.00	114.24 ± 3.98	+42.80	102.71 ± 0.38	+28.38	$p < 0.05$ S
DS4	160.00	205.62 ± 10.84	+28.51	211.98 ± 8.80	+32.48	$p > 0.05$ NS
DS5	112.50	169.06 ± 4.72	+50.27	174.61 ± 2.78	+55.20	$p > 0.05$ NS
DS6	200.00	302.72 ± 7.16	+51.36	282.77 ± 4.57	+41.38	$p > 0.05$ NS

Table III

Determination of Y from DS

DS	Declared Y quantity (mg)	Found Y quantity (mg) ± SD (n = 3)				Statistical significance
		CE	**Diff %**	HPLC	**Diff %**	
DS6	3.00	1.18 ± 0.04	- 60.66	1.54 ± 0.04	- 48.66	$p < 0.05$ S

At high doses of DS, the maximum recommended dose by the 10th Edition of Romanian Pharmacopoeia and the 8th Edition of European Pharmacopoeia (500 mg) is exceeded. CF effectiveness as an ergogenic substance is explained by cardiac stimulation [11], bronchodilation and lipolysis with cAMP-dependent mobilization of energy deposits due to inhibition of phosphodiesterase plus central stimulation by purinergic receptor blocking mechanism [14]. Since 2014 CF is a monitored substance by WADA in order to highlight the *clichés* of misuse in sport [19].

Regarding Y in the only studied product in which the presence of Y was declared, the recovered amount was lower than that declared on the label. This product was withdrawn from the market later in 2014. Y is an alkaloid acting as antagonist on α1-adrenergic receptor, α_2-adrenergic receptors, 5-HT_{1B}, 5-HT_{1D}, 5-HT_{2A}, 5-HT_{2B}, D_2 [3]. Although it has a good therapeutic index (human $LD_0 = 0.643$ mg/kg bw) the effectiveness as a lipolytic substance is low, but Y enjoys a favourable reputation as an aphrodisiac preferred by athletes for this reason. Literature describes cases of acute Y over dosage [8].

The analysis of the 6 legal DS did not reveal the presence of EPH and SB. Although EPH use is forbidden in sports, DS containing Ma Huang or nose drops containing EPH (0.5% EPH nose drops, vial of 10 mL) are frequently used orally, equivalent to an oral dose of 50 mg. EPH presents alpha, beta-sympathomimetic effects causing tachycardia, bronchodilation, lipolysis, central stimulation and acting synergistically with CF [2]. EPH alone or in combination with CF is dangerous in case of physical effort predisposing to arrhythmias and with increased cardiovascular risk, literature data describe many life threatening cases [7].

Due to restrictive legislation on EPH, "fat burning" DS contain substances with similar effects, but of less intensity: pseudoephedrine (considered doping only if urinary concentration overcomes 150 ng/mL) - found in over the counter (OTC) medication for the treatment of colds and flu. Another ephedrine-like substance is synephrine, not included on the Prohibited List, but monitored since 2014 according to WADA (in order to highlight the *clichés* of misuse in sport) [19].

In the studied products SB was not identified (central anorectic drug withdrawn by The European Medicines Agency on August 2010 due to cardiovascular risks). In Romania 6 counterfeit allegedly vegetal products have been identified originating from China (such as the “Weight-loss capsule” or "Super Slim") containing SB and phenolphthalein [1].

Both analytical methods are affordable techniques in an analytical laboratory of any DS manufacturer and can be used for the assay of the four analytes in DS, taking in consideration both the advantages and disadvantages of each. While the HPLC method offers the advantages of lower limit of detection (LOD) and better reproducibility, the advantages of CE method are related to the low consumption of samples and reagents, fast analysis time and method development and high degree of resolution.

The conclusions drawn about the content of the analysed DS samples were confirmed by both methods. The differences between the levels of CF and Y determined by the two methods are statistically insignificant, with a few exceptions, which prove a low content uniformity as the analysed extracts by the two methods were obtained independently from the same product.

Conclusions

The two proposed screening methods, based on classical high performance liquid chromatography technique and capillary zone electrophoresis with UV detection, respectively, are designed to be simple and reliable and can be used, after full validation, in control laboratories for routine analysis of dietary supplements. As far as we are aware, the present paper proposes the first capillary electrophoresis method for the simultaneous determination of caffeine, yohimbine, sibutramine and ephedrine. The methods revealed the same issues regarding the analysed dietary supplements.

Acknowledgement

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