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Determination of caffeine and identification of undeclared substances in dietary supplements and caffeine dietary exposure assessment

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ABSTRACT

Caffeine is one of the most consumed stimulants in the world, and is a frequent ingredient of dietary supplements. The aims of this work were to validate a GC-MS method for the quantitation of caffeine and identification of other substances in supplements, mainly weight loss products, and to estimate the caffeine intake by consumers. Sample preparation included extraction with chloroform:water in ultrasonic bath, centrifugation and analysis of the organic layer for caffeine quantitation, and extraction with methanol for identification of other substances. A total of 213 samples of 52 supplement products not registered in Brazil and seized by the Brazilian Federal Police were analyzed. From the 109 samples that declared the amount of caffeine present, 26.6% contained more than 120% of the specified content. Considering the maximum recommended dose stated on the product labels, the consumption of 47.9% of the samples would lead to a daily intake of caffeine above the safe limit of 400 mg. Undeclared drugs, including sibutramine, phenolphthalein, amphepramone and femproporex were found in 28 samples. These results show that consumers of dietary supplements should be aware that these products might contain caffeine at levels that could represent potential health risks, in addition to undeclared pharmaceutical drugs.

1. Introduction

Caffeine, or 1,3,7-trimethylxanthine, is one of the most consumed and studied stimulants in the world. It is present in a wide variety of foods and beverages, as well as in about 60 plant species (Schwenk and Costley, 2002; Gurley et al., 2015). Caffeine has central nervous system stimulating properties, it is diuretic, decreases fatigue, enhances mental focus and athletic performance, and presents thermogenic effects (Rang et al., 1997; Greenway, 2001). There is also evidence suggesting that the consumption of caffeine seems to reduce caloric intake, which is why it may contribute to weight loss (Westerterp-Plantega et al., 2006). When consumed in moderate doses (around 200 mg/day), caffeine has an excellent safety profile (Gurley et al., 2015). However, in higher dosages (more than 2000 mg/day), it can cause severe hypertension, arrhythmias, seizures and even death. Individuals that are more sensitive may present adverse effects at lower dosages (Schwenk and Costley, 2002; Holmgren et al., 2004; Kerrigan and Lindsey, 2005; Liddle and Connor, 2013; Gurley et al., 2015).

Caffeine is a major component of dietary supplements, mainly in products for weight loss, energetics and athletic performance enhancers (Gurley et al., 2015). Caffeine was frequently associated with herbal extracts from the *Ephedra* family that contain ephedrine alkaloids, since this association was considered to be more efficient for weight loss than caffeine or ephedrine alone (Greenway, 2001; Gurley et al., 2015). In 2004, however, the US Food and Drug Administration (FDA) removed all products containing *Ephedra* extracts or ephedrine from the market, since they presented an unreasonable risk of illness or injury under the conditions of use recommended or suggested on the product label (USA, 2004; Gurley et al., 2015). Ephedra and ephedrine are also forbidden as a food ingredient in several European countries, Canada, Australia and New Zealand (EFSA, 2013).

After the banning of *Ephedra*, a new generation of "ephedra-free" supplements came to the market, containing several natural sources of caffeine and other herbal extracts with substances with pharmacologic action (such as synephrine and yohimbine). The amount of caffeine in these supplements usually exceeds that found in beverages and foods, but most products do not declare the

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caffeine content (Schwenk and Costley, 2002; Gurley et al., 2015).

In Brazil, caffeine can only be commercialized as a supplement under the "caffeine supplements for athletes" category; the products cannot contain any other substances and must declare the amount of caffeine present, which must be between 210 and 420 mg per serving (Brazil, 2010a). In the US, however, products do not need to state their caffeine content if it is included in a proprietary blend, sufficing to state the total amount of that blend (USA, 1994). There are few papers reporting the quantification of stimulants in supplements, focusing mainly on *Ephedra* and *Citrus aurantium* alkaloids, and the information is frequently limited to a low number of samples. Studies that did quantify caffeine in supplements found major variations between declared and detected contents; in products that did not declare the caffeine content, the compound was present at varying levels or even absent (Haller et al., 2004; Marchei et al., 2005; Seeram et al., 2006; Andrews et al., 2007; Evans and Siitonen, 2008; Viana et al., 2015).

Furthermore, the presence of undeclared drugs in dietary supplements is another point of concern. While both the United States and the European Union have effective systems for detecting and divulging these occurrences to the public, Brazil does not have such a system (Neves and Caldas, 2015). This does not mean that adulterated products are not present on the Brazilian market. Neves and Caldas (2015) evaluated data from forensic reports issued by the Brazilian Federal Police (BFP) from 2007 to 2013 and found 180 cases of supplement adulteration. De Carvalho et al. (2012) analyzed 106 weight loss supplements acquired on the internet from nine different Brazilian states, and found four of them to be adulterated with femproporex or sibutramine.

The aims of this work were to develop and validate a GC-MS method for the quantification of caffeine and identification of other substances present in dietary supplements, and to analyze samples seized by the BFP and sent for forensic analysis by the National Institute of Criminalistics.

2. Material and methods

2.1. Standards and reagents

Caffeine standard (98.5% purity, confirmed by Nuclear Magnetic Resonance) and dipentyl phthalate, used as an internal standard (IS; 97% purity), were from Acros Organics (Geel, Belgium). HPLC grade chloroform and methanol were purchased from Tedia (Fairfield, OH, USA) and water was produced by a Milli-Q Direct-Q system (Millipore, Bedford, MA, USA). Hexane used for capsule cleaning was purchased from J. T. Baker (Phillipsburg, NJ, USA). Working standards of 1,3-dimethylamylamine (DMAA), sibutramine and ephedrine (seized materials sent for forensic analysis by the BFP and chemically characterized prior to use) were used for retention time comparison during screening analysis.

A mixture of cellulose (Merck - Darmstadt, Germany), lactose (Sigma-Aldrich - St. Louis, MO, USA), starch (J. T. Baker Phillipsburg, NJ, USA) and mannitol (bulk material sent for forensic analysis by the BFP and chemically characterized prior to use) was used as blank matrix for tablets/capsules (pharmaceuticals); a supplement containing *Tribulus terrestris* extract (GC-MS analysis showed it contained no caffeine) was used as blank matrix for herbal extract tablets/capsules, and glycerin (Cinética – Jandira, SP, Brazil) as a blank matrix for capsules with liquid content.

2.2. Standard solution preparation

All standard solutions and sample extracts were prepared using a solution of the internal standard (IS) dipentyl phthalate in chloroform at 50 μg/mL (henceforth called "IS solution"). A stock solution of 250 μg/mL of caffeine was prepared by weighing 12.5 mg of the caffeine standard and solubilizing it in 50 mL of the IS solution. Every time a new IS solution was prepared, a new caffeine stock solution was also prepared. Caffeine stock solutions and IS solutions were kept at room temperature and consumed within one week, a period during which their stability was assessed and considered satisfactory (less than 2% degradation of caffeine; data not shown). Calibration points at 25, 50, 100 and 175 μg/mL were prepared by diluting the stock solution with IS solution; the highest calibration point was the undiluted stock solution itself.

2.3. Samples

Usually, dietary supplements are seized and sent for forensic analysis by the BFP whenever there is a suspicion that they may be counterfeited, adulterated, smuggled into the country, contain any proscribed or controlled substances or cannot, for any reason, be commercialized in Brazil. This study focused on supplements claiming to aid in weight loss, but also included other kinds of supplements that did not declare caffeine on their labels (such as pro-hormones), but in which caffeine was detected during forensic analysis. The 213 samples analyzed in this study were seized from 2010 to 2016, and included tablets, capsules with powder content ("solid capsules") and capsules with liquid content ("liquid capsules"). All seized samples were stored at room temperature before analysis. The expiry date, when declared, varied from 2007 to 2020, and the samples were analyzed in September 2016; 83.6% of the samples were analyzed after their expiry date.

2.4. Sample preparation

The mean weights of tablet/capsule samples were determined by averaging the weight of five tablets or the content of five capsules. Three tablets or the contents of three liquid or solid capsules were ground and/or homogenized, and an amount equivalent to 1/10 of the mean weight was transferred to a 15 mL falcon tube; 1 mL of milli-Q water and 5 mL of the IS solution were added. Tubes were shaken manually, vortexed for 10 s, sonicated for 10 min, and centrifuged for 5 min at 3000 rpm; a 50 μL aliquot of the organic layer was added to a vial containing 950 μL of IS solution, to a final volume of 1 mL. If the concentration fell below the lowest calibration point, the organic layer was analyzed at a 500:500 dilution or undiluted; if the result was higher than the highest calibration point, a 25:975 dilution of the organic layer was made.

For the qualitative analysis of other substances, an amount equivalent to 1/20 of the mean weight of tablets and capsules was transferred to a 15 mL falcon tube and 3 mL of methanol was added. Tubes were shaken manually, vortexed for 10 s, sonicated for 10 min and centrifuged for 5 min at 3000 rpm. The solution was directly transferred to a vial and analyzed.

2.5. Equipment

GC-MS analyses were performed on a GC System 7890A, coupled with a 5975C Mass Spectrometer (operating at 70 eV) and an automated sample injector system CTC PAL G 6509-B (all Agilent Technologies, Santa Clara, California, USA). A HP5-MS (Agilent Technologies) capillary column was used (25 m × 0.20 mm i.d. x 0.33 μm film thickness). The injection port temperature was 280 °C, injection volume was 0.5 μL and split injection mode (50:1) was used. The oven temperature was programmed at 70 °C for two minutes, increased to 250 °C at 40 °C/minutes, held at 250 °C for 2 min, raised to 315 °C at 40 °C/minute and held at 315 °C for 3.875 min, with a total run time of 14 min.

Temperatures of the MS ion source and GC/MS interface were

230 and 280 °C, respectively. MS detector was used in Full Scan mode, with two different sets of parameters. At the beginning and the end of the run, a mass range of 20–400 m/z was monitored, operating with 1.95 sweeps per second (sampling rate of 3), to detect substances other than caffeine, including anorectics. During a 2-min interval in the middle of the chromatogram (from 6.3 to 8.3 min, the region where caffeine and the IS elute), the detector was set to monitor from 40 to 200 m/z, operating with 4.32 sweeps per second (sampling rate of 3), a range that comprises all expected fragments of caffeine (main fragments at m/z 194, 109, 67 and 55) and IS (main fragments at m/z 149, 150 and 43) mass spectra. This alteration led to a lower baseline in this region of the chromatogram. During the qualitative analysis, the detector operated at m/z 20 to 400 range during the entire run.

Quantification was performed considering the ratio between the caffeine peak area and the IS peak area. Peak areas were calculated from the Total Ion Chromatogram (TIC). Qualitative identification of other substances was conducted by comparing the mass spectrum with the National Institute of Standards and Technology (NIST) electronic library. Additionally, retention time information was used for identification of sibutramine, ephedrine and DMAA, for which working standards were available.

2.6. Method validation

Method validation was performed following ANVISA guidelines for medicines (Brazil, 2003) and MAPA guidelines for drugs in veterinary products (Brazil, 2011).

Linearity of the calibration curve was assessed by preparing and analyzing six replicates of each calibration level (25, 50, 100, 175 and 250 μg/mL) in IS solution. Lower concentrations were tested but resulted in small and ill-shaped peaks. Data were evaluated for linear or quadratic relationships, using untransformed data, square root transformation and logarithm transformation. The quality of the regressions obtained was evaluated considering the correlation coefficient, Cochran tests and F tests for variances (to detect heteroscedasticity); analysis of variance (ANOVA) to evaluate lack-of-fit; sum of relative errors; graphic evaluation of the randomness of the residuals; and residual standard deviation (measures their dispersion throughout the regression curve and evaluates their absolute value).

Selectivity of the method was evaluated by analyzing blanks of the three matrices (pharmaceutical, herbal and glycerin) and the IS solution alone, investigating the presence of any interfering peaks in the caffeine or IS retention times. Extracts of the blank matrices were prepared according to the proposed sample preparation procedure (Section 2.4). Matrix effects were evaluated by comparing the results of samples fortified at 0, 25, 100 and 250 μg/mL caffeine prepared in IS solution and *in matrix* (three replicates at three levels each; each solution was injected three times), quantified with a calibration curve prepared in IS solution. The results were compared using a *t*-test (Brazil, 2011).

Precision and recovery studies were conducted together. Twelve aliquots of each blank matrix were weighed in 15 mL falcon tubes (approximately 70 mg each) and 1 mL of caffeine chloroform solution (at 2.5, 5, 10 and 25 mg/mL) used to fortify the aliquots at four different levels (25, 50, 100 and 250 μg/mL), three aliquots per level. The fortified blank samples were homogenized, left to dry for 48 h, extracted following the proposed method and caffeine quantified against a freshly prepared IS calibration curve (each solution was injected two times). This entire procedure was repeated after one week to evaluate the intermediate precision.

Recovery was the mean value (in % of the fortified level) and repeatability was the Relative Standard Deviation (RSD) obtained for the three replicates (two injections for each replicate) analyzed on the same day; intermediate precision was defined as the RSD of the six replicates prepared and analyzed on two different days. Limit of quantification (LOQ) was defined as the smallest concentration with acceptable repeatability, intermediate precision and recovery; threshold values adopted were 10% for repeatability, 15% for intermediate precision (Brazil, 2011) and 80–120% for recovery. Limit of detection (LOD) was established by analyzing successive dilutions of calibration standards and determined as the most diluted peak to yield a signal-to-noise ratio higher than 10 on the TIC.

3. Results

3.1. Method validation

Data obtained in the linearity study showed that the calibration curves were homoscedastic (experimental Cochran = 0.484; tabulated Cochran value = 0.5065; experimental F = 4.334; tabulated F value = 5.05). The residual plot from the linear regression had a characteristic curved shape indicating that a quadratic model was adequate; residuals from the quadratic regression were random and the quadratic term of the regression was significant according to its confidence interval. Since the quadratic regression still presented some lack-of-fit (Experimental F = 11.63; tabulated F value = 3.385), and the sum of the residual errors was still considered large (145.27; N = 30), data transformation was tested. Square root transformation did not yield good results, but logarithm transformation yielded significant improvements in the quadratic regression (Experimental F for lack-of-fit = 5.82; sum of residual errors 24.8; N = 30). Correlation coefficient (0.9973) and residual standard deviation (0.02508) were satisfactory, so this regression was chosen (log10 transformed, quadratic).

The method showed to be selective, as no peaks were detected on the blank matrix chromatograms. No matrix effects were observed, since t-tests showed there was no significant difference between results in the three matrixes compared with results obtained in IS solution.

Table 1 shows the results obtained for recovery, repeatability and intermediate precision. Adequate results were obtained at 25 μg/mL for all matrixes, so the LOQ was calculated as 25 mg caffeine/capsule or caffeine/tablet for the 50:950 sample dilution, and 1.25 mg caffeine/capsule or caffeine/tablet for the undiluted analysis. LOD of the equipment was determined as 10 μg/mL, which corresponded to 0.5 mg caffeine/capsule or caffeine/tablet (considering undiluted analysis).

3.2. Quantification of caffeine in dietary supplements

The proposed method was used to determine the caffeine content of 213 supplement samples (11 tablets, 97 solid capsules and 105 liquid capsules) of 52 different products sent to forensic analysis by the BFP. The product names and sample information are shown in the Supplemental Material. Most of the samples (201) were weight loss products (one did not declare the presence of caffeine), 9 were body building supplements (none declared the presence of caffeine) and 2 declared to have diuretic action and the presence of caffeine. One product had no identification. All samples declared to be manufactured in/for the United States, except the product without identification and the weight loss supplement that did not declare caffeine on the label, and stated to be of Brazilian origin.

Samples were prepared as described and analyzed in batches of up to 36 samples each. A fortified control sample was prepared by the addition of 100 mg of caffeine standard to the content of one of the blank herbal matrix capsules; the mixture was thoroughly

Table 1
Validation parameters for the quantification of caffeine in different kinds of dietary supplement matrixes by GC-MS.

Matrix	Conc. (μg/mL)	Recovery (%) N = 3	Repeatability (RSD_r, %) N = 3	Intermediate precision (RSD_p, %) N = 6
Pharmaceutics	25	87.5	10.8	10.8
(tablets/solid capsules)[a]	50	94.6	3.3	3.8
	100	98.6	2.7	3.3
	250	93.5	3.2	2.3
Herbal	25	84.6	5.5	7.9
(tablets/solid capsules)[b]	50	94.3	1.8	3.4
	100	100.6	3.6	3.6
	250	92.4	3.5	2.9
Glycerin	25	84.4	4.7	8.2
(liquid capsules)	50	95.1	6.1	6.3
	100	101.1	4.4	5.6
	250	93.9	3.9	3.2

[a] Mixture of cellulose, lactose, starch and mannitol.
[b] *Tribulus terrestris* extract.

homogenized and an aliquot of one-tenth of the final mass of the mixture was analyzed within each batch as a quality control sample (QC). The results obtained for the QC samples (N = 6) were always within the acceptable range for recovery (80–120%) and intermediate precision (RSD < 10%). Fig. 1A shows the TIC of a caffeine standard in IS solution and Fig. 1B the TIC of a Lipo 6 Black® sample containing caffeine. Individual quantitative results for all samples are presented in the Supplemental Material. Caffeine levels detected ranged from 65.5 to 276.8 mg per tablet (Table S1), 0.5–389.4 mg per solid capsule (Table S2) and from 12.8 to 255.7 mg per liquid capsule (Table S3), with seven solid capsules not containing caffeine (<LOD), although they declared its presence. For samples that declared the amount of caffeine present (10 tablets, 68 solid capsules and 31 liquid capsules, Supplemental Material), the ratio of caffeine detected/declared was calculated and the samples were distributed in five ratio ranges (Fig. 2A); values between 80 and 120% were considered to be according to the label specification. Samples that declared the presence of caffeine but did not specify the amount present (1 tablet, 29 solid capsules and 74 liquid capsules) were distributed in 5 concentration ranges according to the amount of caffeine detected (Fig. 2B).

Most tablet samples (10 of 11) declared the amount of caffeine present, and from those, 50% contained less than 80% of the declared amount (lowest value 49.6%) (Fig. 2A). However, only two of these tablets were analyzed before their expiry date (one contained 72.2% and the other 111.6% of the declared caffeine amount), so caffeine degradation cannot be ruled out for the other samples (expiry dates from 2012 to 2015; analyses were performed on September 2016). The highest relative amount of caffeine found in a tablet was 114.9% of the declared value, within the acceptable variation (Table S1).

Most of the solid capsule samples (70.1%) declared the amount of caffeine present, from which 20.6% contained less than 80% of the stated amount (reaching < LOQ, regardless of the expiry date) and 19.1% contained more than 120% of the stated amount (up to 382.2%; Table S2). About 30% of the liquid capsules declared the caffeine amount, from which 19.3% contained less than 80% of what was stated (lowest value 47%, all expired) and 51.6% contained more than 120% of the stated amount (up to 197%; Table S3).

Eleven samples did not declare the presence of caffeine, all solid capsules (Table S2). Nine were bodybuilding supplements, in which the amount of caffeine present ranged from 0.5 mg/capsule to 294.8 mg/capsule, with an average of 115.6 mg/capsule. The presence of caffeine at 0.5 and 0.7 mg/capsule in two samples might be due to cross-contamination during the manufacturing process; the third lowest caffeine concentration detected in these samples was 18.9 mg/capsule, which is already a relevant amount that may denote intentional adulteration. It should be noted that the eight samples containing significant amounts of caffeine (from 18.9 to 294.8 mg/capsule) did not contain the pro-hormones declared on their labels. Finally, the product without any identification contained 49.3 mg caffeine/capsule.

The most frequent products analyzed in this study were Lipo 6 Black® (2 different formulations), Oxyelite Pro®, Lipo 6x® and Dyma-Burn Xtreme®. The number of samples and lots of these products, the mean caffeine contents and their variability (RSD) are shown in Table 2. The largest variability between the samples was found for the "old formulation" of Lipo 6 Black® (51.9%), which did not declare the caffeine content, followed by Lipo 6x® (27.1%).

The intra-lot variability (as %RSD) was evaluated for products for which at least three samples of the same lot were available for analysis (two lots of the "new" Lipo 6 Black®, one lot of the "old" Lipo 6 Black®, one lot of Oxyelite Pro® and one of Lipo 6x®). RSD was lower than 10% for the two new Lipo 6 Black® (n = 4 and 3) and the Oxyelite Pro® (n = 4) lots. The Lipo 6x® lot (4 samples) had a RSD of 29.2% and the old Lipo 6 Black® lot (3 samples) had the highest RSD (54.9%).

3.3. Caffeine intake from the consumption of dietary supplements

The Brazilian legislation states that caffeine supplements for athletes should provide between 210 and 420 mg caffeine per serving, added only as anhydrous caffeine (at least 98.5% purity; Brazil, 2010a). Six liquid capsule samples analyzed in this study (all Hydroxycut Hardcore®) provided more than 420 mg/serving (max. of 590.7 mg).

All except two samples analyzed were from the USA, a country that does not require the amount of caffeine present to be stated on the product, which may also contain other sources of caffeine, such as botanical extracts (USA, 1994). Most products analyzed in this study recommended the consumption of more than one tablet/capsule per day (up to 9). Using the maximum recommended dose on the product label and the caffeine concentration determined in this study, the daily caffeine intake from the consumption of each sample was estimated and is shown in Fig. 3.

The safe daily intake of caffeine for adults is estimated to be 400 mg (EFSA, 2015), which would be equivalent to 6.7 cups of Brazilian expresso coffee (average of 59.8 mg caffeine/60 mL; Camargo and Toledo, 1998). Caffeine intake above the safe daily dose might lead to adverse effects such as tachycardia, insomnia, nervousness, headaches, abdominal pain, nausea, vomiting, diarrhea and diuresis (Nawrot et al., 2003; Higdon and Frei, 2006). Specific populations such as pregnant women, elderly people or hypertensive individuals may present adverse events at lower

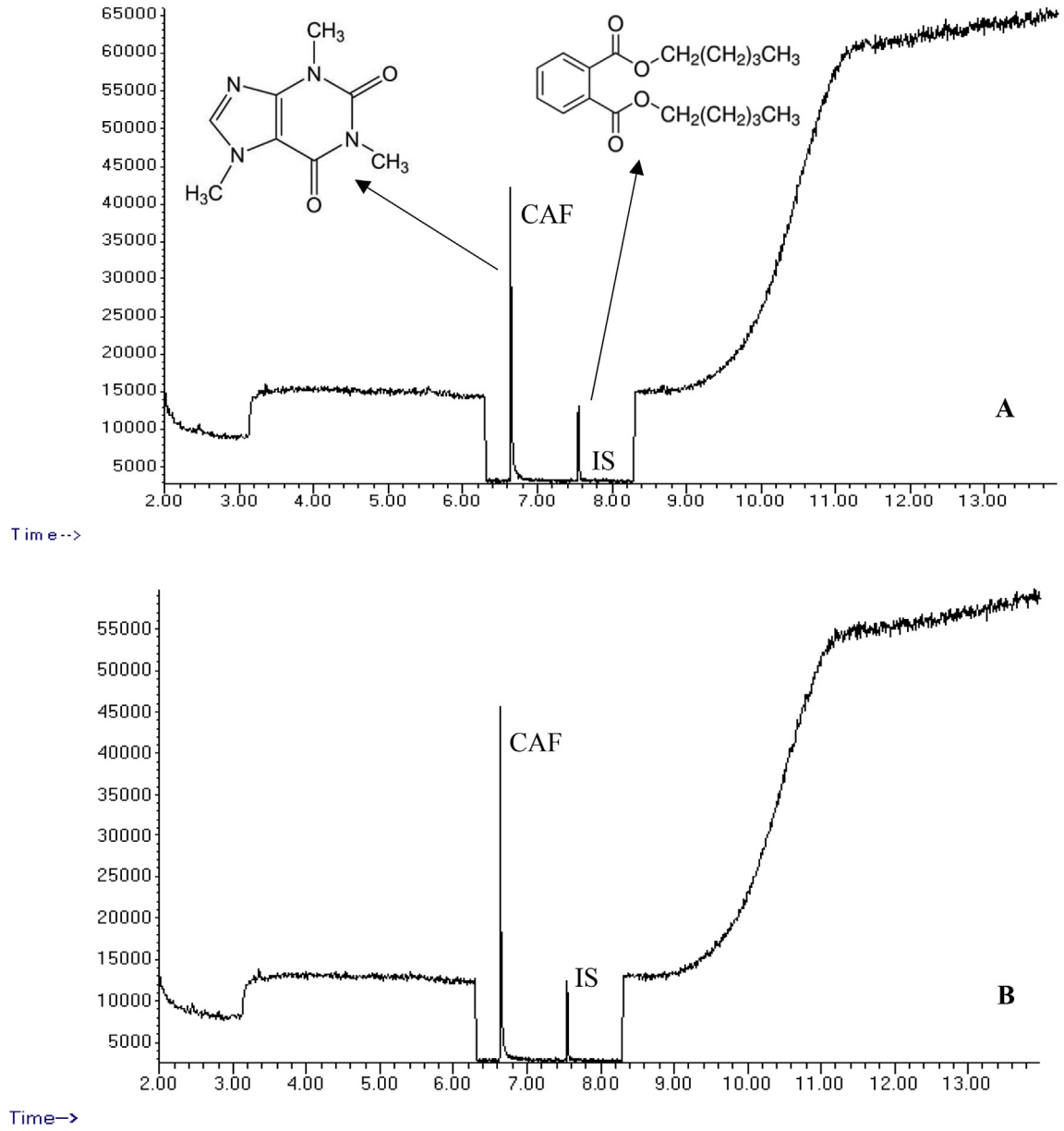

Fig. 1. (A) Total ion chromatogram of caffeine (CAF) standard at 100 μg/mL and dipentyl phthalate (IS) at 50 μg/mL; (B) Total ion chromatogram of a sample of Lipo 6 Black® containing 40.6 mg of caffeine/capsule.

doses, and pregnant women should not ingest more than 200 mg/day (EFSA, 2015).

The daily intake from the consumption of the supplements analyzed exceeded 400 mg caffeine for 101 samples: 36.3% of the tablets, 44.2% of the solid capsules and 52.4% of liquid capsules (Fig. 3). Daily intake varied from 220.2 to 553.6 mg/day for tablets (mean of 377.8 mg/day), from 0.7 to 1101.3 mg caffeine/day for solid capsules (mean of 449.4 mg/day) and from 76.8 to 1181.4 mg/day for liquid capsules (mean of 417.7 mg/day).

3.4. Screening for undeclared active ingredients in dietary supplements

After having their caffeine content determined, samples were reanalyzed after extraction with methanol, in a search for other compounds declared on the product labels and for undeclared substances. About 13% of all samples analyzed (28 out of 213) contained undeclared ingredients. The results of individual samples are shown in Supplemental material. Quantitative analysis was not performed for these compounds.

Several samples contained phenylethylamines (PEA), synephrine/methylsynephrine and yohimbine, all listed on the product labels (Tables S1, S2 and S3). 1,3-dimethylamylamine (DMAA), a substance that was present in many weight loss supplements before being banned by the FDA (and also in Brazil) in 2012, was found in 64 samples, in two of them undeclared (one tablet, one solid capsule). One sample declared the presence of DMAA, but did not contain this substance, and contained 15.4% of the stated caffeine amount (Oxyelite Pro® sample, Table S2). It is beyond the scope of this study to discuss the safety and efficacy of these substances for weight loss; none could be commercialized in Brazil as a dietary supplement (Brazil, 2010a).

Three out of the 11 tablets analyzed (21.4%) contained undeclared substances, with two cases of clobenzorex and one of

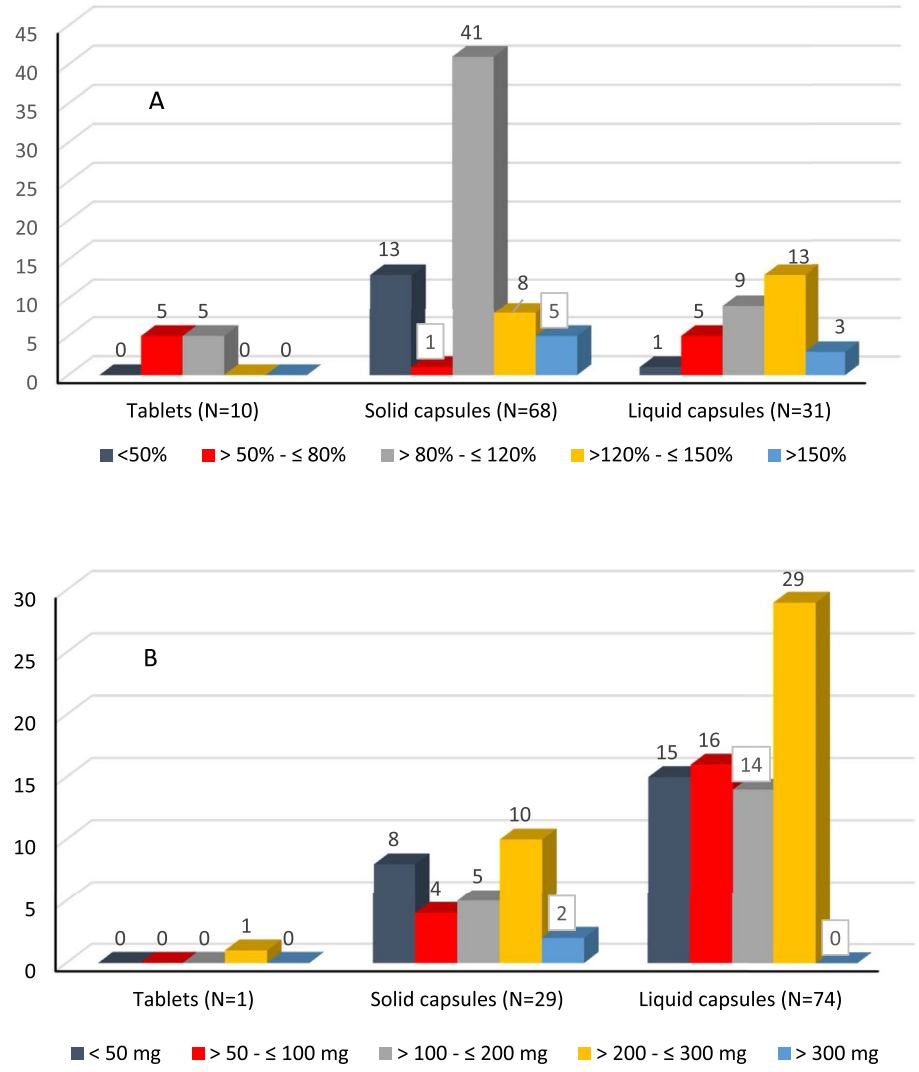

Fig. 2. Caffeine in 213 dietary supplement samples seized by the Brazilian Federal Police, distributed according to the (A) % detected in relation to the declared amount and (B) amount present for samples that did not declared the amount or the presence of caffeine.

Table 2
Caffeine content and its variability among the samples of the most frequent products analyzed.

Product	Number of samples (number of lots)	Mean caffeine ± sd, mg/capsule	Variability RSD, %[a]
Lipo 6 Black® (new formulation)[b]	40 (29)	203.1 ± 37.7	18.6
Lipo 6 Black® (old formulation)[b]	23 (13)	44.2 ± 22.9	51.9
Oxyelite Pro®[c]	17 (12)	113.6 ± 7.9	7.0
Lipo 6x®[c]	10 (7)	79.5 ± 21.6	27.1
Dyma-Burn Xtreme®[d]	10 (8)	182.1 ± 12.9	7.1

[a] Relative Standard Deviation.
[b] Content of caffeine not declared.
[c] Declared 100 mg caffeine/capsule.
[d] Declared 165 mg caffeine/capsule.

phenpromethamine (Table S1). Both substances are members of the phenylethylamine family and are banned by the World Anti-Doping Agency. All six cases of undeclared substances that were detected in the liquid capsule samples (5.7% of the 105 analysed) also referred to phenpromethamine (Table S3). In total, 14 solid capsule samples were adulterated (14.4% of the 97 analyzed), containing different undeclared compounds, including sibutramine, phenolphthalein, dipyrone, fluoxetine, aminopyrine, DMAA, phenprometamine, ketamine, clobenzorex, amphepramone and femproporex (Table S2). No quantitative analysis was performed for these substances, but they were mostly detected as major peaks in the chromatograms, as is illustrated in Fig. 4.

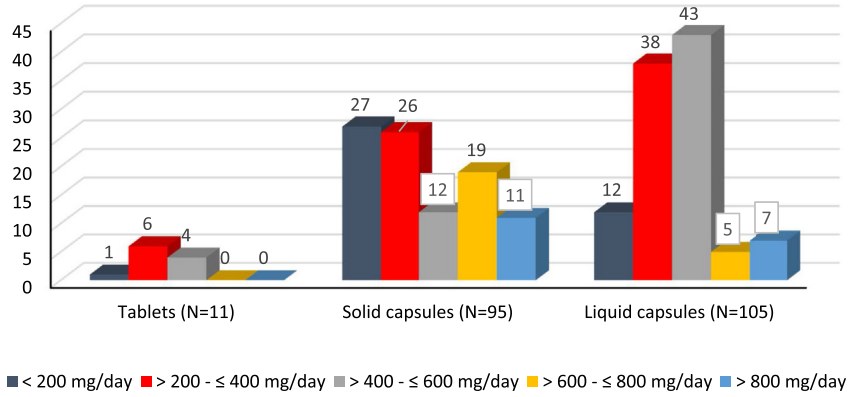

Fig. 3. Intake of caffeine from the consumption of dietary supplements (at the maximum recommended dose stated in the label).

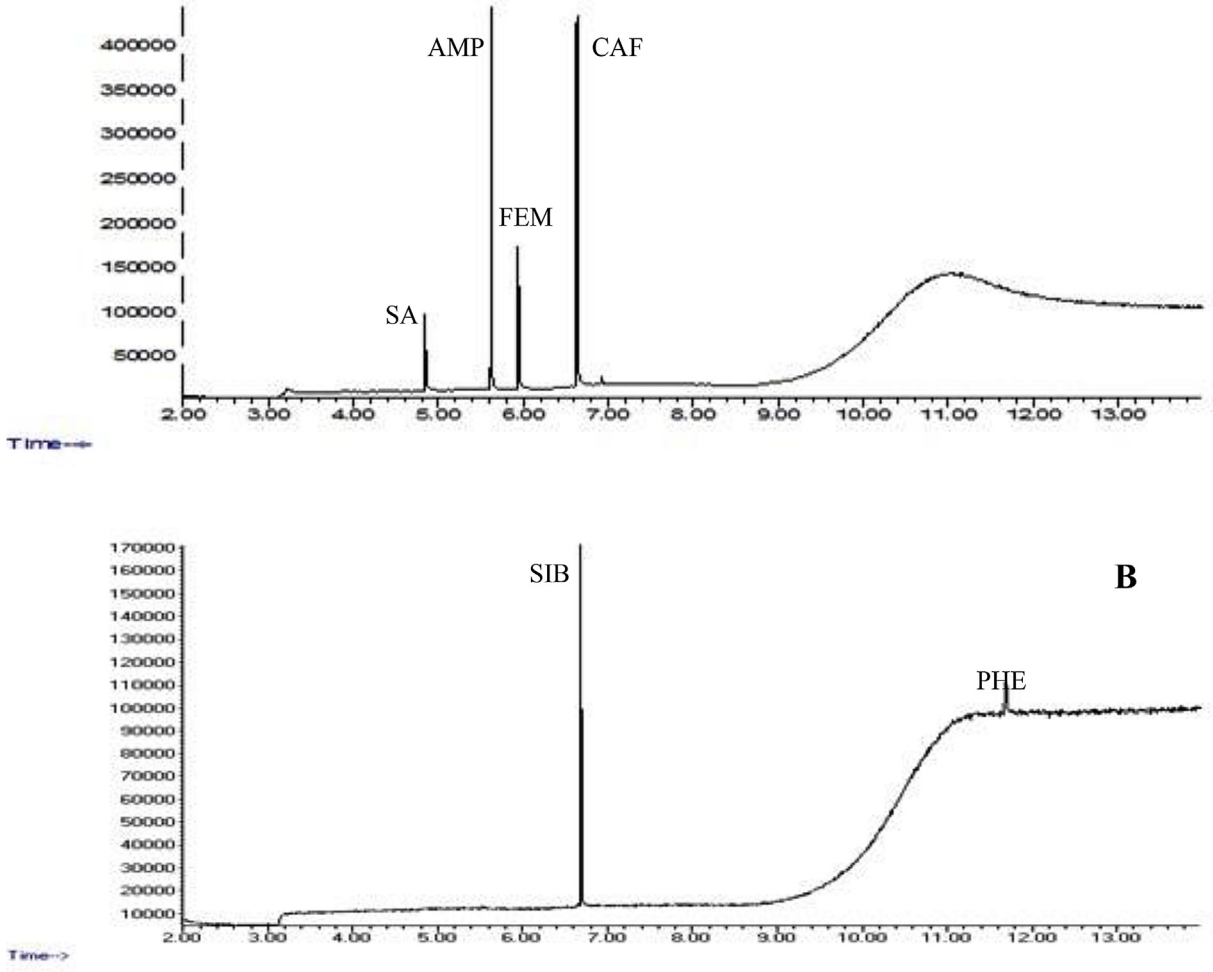

Fig. 4. (A) Total Ion Chromatogram (TIC) of a ECA Fuel® sample containing caffeine (CAF), salycilic acid (SA), amphepramone (AMP) and fempoporex (FEM); (B) TIC of a Stack Xtreme® sample containing sibutramine (SIB) and phenolphthalein (PHE).

4. Discussion

The results of this study confirm some studies conducted elsewhere, which indicated a wide range of caffeine levels present in supplements. Andrews et al. (2007) found concentrations of caffeine ranging from 0.07 to 307 mg/tablet in 53 supplements purchased in the USA, with concentrations up to 173% of what was declared. On the other hand, Haller et al. (2004) quantified caffeine and several *Ephedra* alkaloids in 35 samples of supplements from the US market, finding that 86% of samples contained less than 90% of the declared caffeine content, with one sample containing levels below 80% of the declared dose.

A study conducted with weight loss supplements acquired from Brazilian websites found that approximately 90% of the 46 analyzed

samples contained more caffeine than the maximum permitted level according to Brazilian legislation (420 mg/day; Viana et al., 2015). Most of these samples declared to be of Brazilian origin, and their consumption following label instructions could lead to a daily caffeine intake of up to 1476.7 mg/day (Viana et al., 2015), even higher than what was found in the present study (1181.4 mg/day). These results illustrate that both irregular foreign supplements smuggled into Brazil and national supplements manufactured in disagreement with sanitary legislation may contain caffeine at higher levels than what is described on the product labels. Although the number of samples within each lot available to this study was smaller than 10, as required by the Brazilian Pharmacopoeia for intra-lot homogeneity test (Brazil, 2010b), one lot of Lipo 6x® and one Lipo 6 Black® had a high variability of caffeine content between the samples (n = 3–4), indicating a poor manufacturing quality, a problem already identified for dietary supplements (Haller et al., 2004).

It is important to emphasize that supplement consumers should be aware that caffeine may cause adverse health effects at certain doses and that total caffeine intake also includes what is ingested through the diet. Sousa and Costa (2015) estimated the mean usual coffee consumption in Brazil as 163 mL/day, which according to the authors would be equivalent to 238 mg of caffeine. Previously, Camargo et al. (1999) had estimated that, on average, Brazilians ingest 171 mg caffeine/day from several dietary sources, including coffee, tea, chocolate and soft drinks. Using a comprehensive beverage survey in the USA, Mitchell et al. (2014) estimated a mean daily intake of 165 mg of caffeine for individuals aged 2 years and older, and of 200–225 mg for those over 35 years. Considering a mean caffeine daily intake of 200 mg from dietary sources, in addition to the intake from the consumption of supplements, the total caffeine intake would exceed the safe dose (400 mg/day) for 81% of the supplements analyzed in this work, and may represent a health concern.

Some of the other substances detected in the supplements can lead to serious side effects, which is why they are forbidden in several countries. Sibutramine can cause severe cardiovascular events, convulsions, mood disorders, anxiety and other effects (Negreiros et al., 2011), and is not allowed in several countries such as the United States and the European Union. DMAA was proscribed in Brazil in 2012, having the same legal status as cocaine (Brazil, 2012), and its consumption has been linked to cerebral hemorrhage and strokes (Gee et al., 2012). In the United States, the FDA considers supplements with DMAA to be illegal and is “doing everything within its authority to remove these products from the market” (USA, 2013). There are currently no medicines registered in Brazil, the United States or the European Union containing amphepramone or femproporex, anorectics that may cause a wide range of adverse effects, such as psychotic episodes, depression, anxiety, constipation or tachycardia (Negreiros et al., 2011). Finally, phenolphthalein was removed from the Brazilian market in 2002 due to increased risks of cancer (Brazil, 2002); it was also removed from the US market in 1999 (USA, 1999) and is classified as possibly carcinogenic to humans by the International Agency for Research on Cancer (IARC, 2000). The involuntary consumption of anorectics represents a health risk for inadvertent consumers since they may lead to serious adverse events. The commercialization of foreign supplements in which caffeine is associated with other substances is not allowed in Brazil; nonetheless, they are easily acquired on the clandestine market. Since they are advertised as foods, they tend to be perceived as safe and devoid of adverse effects.

One limitation of this study concerns the origin of the samples, since nearly all products declared to be manufactured in/for the United States, and the data reflect the profile of the products originating from this market. Another limitation is the fact that most samples were evaluated after their declared expiry date, which might impair the evaluation as to whether the detected amount is compatible with what was declared on the label. Caffeine is considered to be stable for 4 years at room temperature (Sigma-Aldrich, USA), and many samples whose expiry date was 2012, 2011 or even 2007 had detected/declared ratios of 100% or higher. It is not possible to confirm, however, if this is due to caffeine stability on the sample or if, originally, it contained more than the detected amount. Finally, since the amount of material available for the present study was limited due to legal reasons, it was only possible to use three tablets/capsules of each sample per analysis, which may have impaired the accuracy of the analytical results per sample.

5. Conclusion

This is one of the largest available studies concerning the quantitation of caffeine in dietary supplements. Samples were analyzed by a validated GC-MS method after a simple extraction procedure. Additionally, another extraction procedure was performed to identify the presence of other substances and undeclared pharmaceutical drugs. High amounts of caffeine were found in some samples, which might lead to excessive intake by consumers and represent a health risk. Additionally, undeclared drugs were detected in 28 samples, including anorectics and laxatives. Findings from this study should be used to raise awareness in government agencies and consumers of the risks implied in the consumption of dietary supplements.

Conflict of interest

The authors declare that there are no conflicts of interest.

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Appendix A. Supplementary data

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Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.fct.2017.03.063.

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