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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