

Adulteration of Dietary Supplements by the Illegal Addition of Synthetic Drugs: A Review

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Abstract: In the last few years, the consumption of dietary supplements, especially those having plants as ingredients, has been increasing due to the common idea that they are natural products posing no risks to human health. In the European Union and the United States, dietary supplements are legally considered as foods/special category of foods, thus are not being submitted to any safety assessment prior to their commercialization. Among the issues that can affect safety, adulteration by the illegal addition of pharmaceutical substances or their analogs is of major concern since unscrupulous producers can falsify these products to provide for quick effects and to increase sales. This review discusses the various classes of synthetic drugs most frequently described as being illegally added to dietary supplements marketed for weight loss, muscle building/sport performance and sexual performance enhancement. Information regarding regulation and consumption is also presented. Finally, several conventional and advanced analytical techniques used to detect and identify different adulterants in dietary supplements and therefore also in foods, with particular emphasis on plant food supplements, are critically described. This review demonstrates that dietary supplement adulteration is an emerging food safety problem and that an effective control by food regulatory authorities is needed to safeguard consumers.

Keywords: adulteration, analogs, dietary supplements, food safety, pharmaceutical drugs, plant food supplements

Introduction

In the last few years, the consumption of dietary supplements, in particular those labeled as being plant food supplements (PFS), has been increasing worldwide. Since ancient times, botanicals and botanical preparations have been used for health purposes, either for nutritional objectives to maintain well-being and prevent ailments or as medicines used to cure a disease or relieve its symptoms (Eussen and others 2011). The use of plants is mainly related to their composition in different compounds that are known to have physiological effects in humans, including major nutrients, vitamins, minerals, and other biologically active substances (Silano and others 2011). Besides being used as food and in traditional medicine, more recently, botanicals and preparations thereof have also found several other applications including cosmetics, homeopathic products, biocides, extraction of compounds for the pharmaceutical industry, and as ingredients in dietary supplements (Garcia-Alvarez and others 2014). In particular, during the last few years botanicals have become increasingly available on worldwide markets in the form of PFS, which are legally considered as foods, both in the European Union (EU) and the United States under Directive 2002/46/EC and the Dietary Supplement Health

and Education Act (DSHEA), respectively, thus not requiring any safety assessment prior to their commercialization. Because the formulation of PFS includes plants or plant extracts, they are often advertised as being “natural” with many consumers perceiving these products as being “healthier” and safer compared to conventional pharmaceutical preparations. However, besides the risks inherent to the consumption of botanicals, such as possible side effects and interaction of biologically active phytochemicals with prescription drugs, in the last few years different studies have been reporting cases of PFS adulteration with different synthetic drugs (pharmaceuticals or their analogs). This type of adulteration is a major food safety and public health concern considering both the massive growing consumption of PFS and the fact that consumers are not aware of the risks associated with the possibility of pharmaceutical drugs being illegally added. Therefore, several works have been performed in the last decade reporting the development and application of new and advanced techniques for the detection of adulterants in dietary supplements, with special focus on PFS. This review intends to provide an overview on recent information regarding this subject, comprised of data on the most frequently reported adulterants in PFS and the currently available analytical techniques used for the detection and identification of synthetic drugs illegally added to these products. General information about legislation and consumption of dietary supplements (including PFS) is also presented. Since PFS are frequently categorized according to their advertised purpose, this review will focus on the ones used for weight loss, muscle building/sport performance, and sexual performance enhancement purposes, as they

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represent the majority of consumed PFS, thus the most prone to be adulterated.

Regulation, Consumption, and Adulteration of Dietary Supplements

Regulation on dietary supplements (including PFS)

In the EU, food supplements (which includes PFS) are regulated by the Directive 2002/46/EC which defines these products as being “. . . foodstuffs the purpose of which is to supplement the normal diet and which are concentrated sources of nutrients or other substances with a nutritional or physiological effect, alone or in combination, marketed in dose form, namely forms such as capsules, pastilles, tablets, pills and other similar forms, sachets of powder, ampoules of liquids, drop dispensing bottles and other similar forms of liquids and powders designed to be taken in measured small quantities.” This Directive also specifies “nutrients” as being vitamins and minerals and establishes the maximum and minimum levels for the allowed compounds, yet it does not mention what can, or cannot, be accepted as being “other substances with nutritional or physiological effect.” Thus, several different substances are generally accepted as being included in this definition such as amino acids, enzymes, pre- and probiotics, essential fatty acids, and botanicals or botanical extracts, with their use frequently depending on national legislation (Silano and others 2011). The lack of specification and harmonization among the different EU countries regarding the usage of botanicals or botanical extracts in dietary supplements results in discrepancies among states with the inclusion of some botanicals in PFS being allowed in some countries and prohibited or non-regulated in others. Additionally, based on the current legislation in EU, botanicals and extracts thereof can be used either in PFS or as traditional herbal medicines (regulated by the Directive for traditional medicinal products (Directive 2004/24/EC)), with the distinction between both being somehow blurred, as the borderline between food and medicinal uses is often established based on national habits and interpretation of definitions in legal provisions (Silano and others 2011). Being considered as foods in the EU, PFS are subjected to the dispositions of the General Food Law (Regulation (EC) 178/2002), with the responsibilities for food safety issues mainly relying on food business operators. This also implies that PFS must comply with other food legislation such as hygiene regulations (Regulation (EC) 852/2004), maximum level of contaminants (Regulation (EC) 1881/2006), and novel foods regulation (Regulation (EC) 258/97) among others. Despite the wide range of regulations, food safety problems may arise in different member states, leading the EU to operate a rapid alert system for food and feed (RASFF). The RASFF provides the supervisory authorities with a quick and efficient mechanism for exchanging knowledge on the notifications issued by the different EU member states every time a food presents a serious risk to public health due to contamination, adulteration, or lack of framing in the above-mentioned laws. With this information, the member states are able to take immediate action, warning consumers, and causing products withdrawal from the market (Petroczi and others 2011). The RASFF database can be searched by food type and product category in which “Dietetic foods, food supplements, fortified foods” is included.

In the United States, both finished dietary supplements (including PFS) and their ingredients are regulated by the Food and Drug Administration (FDA). However, they are covered by a regulatory framework signed into law in 1994, the “Dietary Supplement Health and Education Act of 1994” (DSHEA), which recognizes

dietary supplements as a separate category of foods and establishes its own requirements for safety and labeling, thus limiting FDA’s ability to regulate supplements. The DSHEA states that a dietary supplement is a product (other than tobacco) intended to supplement a diet, as long as it bears or contains 1 or more of the following dietary ingredients: vitamins; minerals; herbs or other botanicals; amino acids; dietary substances used by man to supplement a diet by increasing the total dietary intake; concentrates, metabolites, constituents, extracts, or a combination of the ingredients referred to above, and is intended to be taken by mouth as a pill, capsule, tablet, or liquid (USA 1994). Furthermore, according to DSHEA, this type of product is not represented for use as a conventional food or as a sole item of a meal or the diet and should be labelled as a dietary supplement (USA 1994). Additionally, under this framework, a company is responsible for determining that the dietary supplements it manufactures or distributes are safe and that any claims made are substantiated by adequate evidence (Silano and others 2011). In the U.S., similar to what happens in the EU, dietary supplements do not require any approval from FDA before being introduced in the market. Manufacturers do not have to provide evidence for the safety and effectiveness of the products, but they are prohibited to market unsafe or ineffective products (Rapaka and Coates 2006). Nevertheless, if a dietary supplement includes in its formulation any ingredients marketed after 1994, which are considered as “new ingredients,” the manufacturer must first notify FDA and provide information regarding reasonable evidence that it is safe for human use (Rapaka and Coates 2006). According to Wheatley and Spink (2013), DSHEA significantly weakened FDA’s authority over dietary supplements and created opportunities for consumer deception, in particular in what concerns imported supplements. The recent review of Silano and others (2011) can be consulted for more detailed information regarding regulations applicable to PFS, including in other countries not mentioned in this paper, and the review by Wheatley and Spink (2013) for more information regarding dietary supplements in the United States.

Consumption of dietary supplements (with particular emphasis on PFS)

Over the last decade, it is undeniable the existence of notorious growth in the consumption of dietary supplements. Among these, PFS consumption showed a strong increase, with its highest consumption records occurring in the U.S. and EU (Egan and others 2011). The increased acceptance of these types of product by different consumer groups has been associated with a variety of factors including (1) a rising mistrust in conventional medicine and pharmaceutical drugs together with a higher demand and interest for alternative therapies, (2) the perception that “natural” is “healthy” and that plant products are safe, (3) a rising tendency for self-medication aiming for increased control over one’s own health and decisions that may affect it (Ritchie 2007; Egan and others 2011; Vargas-Murga and others 2011).

Dietary supplements are used by the population in general for a variety of purposes, including for balancing the diet, to compensate for the lack of nutrients or exercise or unhealthy lifestyle, health maintenance, to prevent chronic diseases, improve appearance, improve wellness including mental conditions, for sexual performance enhancement and sports performance enhancement, among others (Egan and others 2011; Petroczi and others 2011). Different studies regarding the consumption of dietary supplements have been carried out, most of them focusing on specific population groups such as children, pregnant women, the

elderly, individuals with chronic diseases, cancer patients, and athletes (Petroczi and others 2011; Vargas-Murga and others 2011). However, most information on the prevalence of the use of dietary supplements in general, and the intake of PFS in particular, come from the U.S. with data being obtained as part of large surveys, such as the Natl. Health and Nutrition Examination Surveys (NHANES), the Health and Diet Surveys, and the Natl. Health Interview Surveys (NHIS) (Egan and others 2011; Garcia-Alvarez and others 2014). Based on data from NHANES (2003 to 2004 and 2005 to 2006), it was estimated that approximately 49% of the U.S. population used dietary supplements, with 14% reporting the use of PFS, while in NHIS 2007, almost 18% of the surveyed adults reported the use of PFS. In 2002, a prevalence study regarding the usage of nonvitamin nonmineral supplements among 1000 students from a U.S. university with average age of 26-y-old, showed that more than 26% used that kind of supplements with ginseng, echinacea, protein/amino acids, and ginkgo biloba being the most frequently reported (Perkin and others 2002). In the EU, as part of the FP7 project PlantLIBRA, a retrospective survey concerning the type and frequency of PFS consumption among 6 EU countries (Finland, Germany, Italy, Romania, Spain, and the United Kingdom) was recently reported (Garcia-Alvarez and others 2014). The study, which included a total of 2359 participants, showed that almost 19% used at least 1 PFS, with higher values being observed in Italy (22.7%) and lower ones in Finland (9.6%), and that PFS including ginkgo biloba, evening primrose, and artichoke, in the form of capsules or tablets, were the most frequently used.

Although the factors leading to PFS consumption may vary according to demographic and health factors, among others, the information available in the literature regarding the characteristics of PFS consumers seem to show that, in general, higher consumption is found for women, older adults, individuals generally having a higher education and socioeconomic level, being more likely to self-report their health status as being "good," being physically active while being less likely to smoke (Schaffer and others 2003; Radimer and others 2004; Nielsen and others 2005; Egan and others 2011; Garcia-Alvarez and others 2014). A high intake of dietary supplements (including PFS) is also reported to take place among athletes in order to improve their training performance (Petroczi and others 2008, 2011; Kiertscher and DiMarco 2013).

Adulteration in dietary supplements

With an increasing consumption of dietary supplements, safety in production and marketing, namely in what concerns the quality and levels of physiologically active ingredients in these products as well as labeling compliance, is a general concern for the different stakeholders worldwide including consumers, health professionals, and regulators (Sullivan and Crowley 2006). In particular, different safety issues have emerged regarding PFS, which are considered derivatives from plants and therefore are frequently labelled as "natural" products, thereby transmitting a false sense of security to the consumer since much toxicity is embedded in nature (Liang and others 2006; Di Lorenzo and others 2014). Among such issues, adulteration of PFS, including either the addition of illegal substances or the intentional swap or misidentification of plant material, is a major concern. Considering the economic value associated with the global trade of dietary supplements (in the United States, it is estimated that consumers spend over \$20 billion each year on these products), they are very prone to be adulterated for economic reasons and profit increases (Wheatley and Spink 2013). One of such adulterations encompasses the ille-

gal addition of synthetic drugs since unscrupulous producers can augment dietary supplements to provide for quicker effects. In fact, for some products as, for example, weight-loss dietary supplements, consumers tend to quit using those products if they don't realize any initial effects. On the contrary, if the supplement quickly succeeds in providing the desired results, more units are likely to be sold, thus increasing the seller's profit.

Several studies performed over the last decade have been showing intentional adulteration of dietary supplements by the addition of pharmaceutical drugs, especially in the case of PFS as they have a more complex matrix, thus making adulterant detection more difficult to accomplish. Pharmaceutical adulterants include appetite suppressors, stimulants, antidepressants, anxiolytics, diuretics, and laxatives in weight-loss PFS, phosphodiesterase type-5 enzyme (PDE-5) inhibitors in sexual performance enhancement, and anabolic steroids and prohormones in supplements used for muscle building/sports performance enhancement. An additional problem concerns the use of analogs of those substances, for which no pharmacological studies are available, and also the use of counterfeit drugs of doubtful quality.

As referred to in the previous sections, being legally considered as foods in several countries, dietary supplements (including PFS) do not require any kind of permission to be placed on the market, but the legal responsibility for their safety lies with the business operators. Consequently, in the EU several phytoformulations are being sold under the guise of PFS allowing them to circumvent the requirements and official registration procedure needed if they were considered as being traditional medicinal products. Additionally, nowadays PFS are widespread in the global market, being easily accessible to consumers in supermarkets, drugstores, natural health/food stores, herbal shops, and gyms possible to be purchased through television sales and online by using the Internet. In the last decade, the spread of dietary supplements coming from the black market has also suffered a significant increase (Geyer and others 2008; Petroczi and others 2011; Gilard and others 2015; Odoardi and others 2015).

In the U.S., under the Dietary Supplement and Nonprescription Drug Consumer Protection Act, signed into law in December 2006, the manufacturer, packer, or distributor of a dietary supplement, whose name appears on the label of a dietary supplement marketed in the U.S., must report to FDA, within 15 d, any serious adverse events (events that result in death, a life-threatening experience, hospitalization, a persistent or significant disability or incapacity, a congenital anomaly or birth defect, or requires, based on a reasonable medical judgment, a medical or surgical intervention to prevent any of the referred outcomes) that are reported to them by consumers or health care professionals (FDA 2015c). If the adverse events are not considered serious in accordance with the 2006 act, the firm can still complete and submit a voluntary adverse events report (AER) form at its discretion. FDA reviews all AERs in a postmarket surveillance effort to track safety issues that require intervention, however an AER by itself does not demonstrate a causal relationship between the dietary supplement and the reported health problem (GAO 2013). Consumers and health professionals are strongly encouraged to voluntarily report adverse effects to FDA since dietary supplements adulterated with active pharmaceutical drugs or their analogs can become apparent through AER surveillance, thus assisting FDA in identifying potential safety concerns.

Despite the potentially serious health risks, very little is known about the prevalence or common adverse effects of dietary supplements adulterated by the illegal addition of pharmaceuticals. A

2007 retrospective study concerning patients who were referred to the Hospital Authority Toxicology Reference Laboratory of Hong Kong from 2004 to 2006, described a positive result for the presence of pharmaceutical drugs or their analogs (either on urine samples, on the consumed dietary supplement, or in both) for 28 individuals from a total of 42 patients suspected to have clinical problems related to the use of weight-loss products (Yuen and others 2007). The authors also described the identification of sibutramine, fenfluramine and a fenfluramine analog in 3 slimming products, and the hospitalization of 4 patients after taking those products (Yuen and others 2007). More recently, Cohen and others (2012) assessed the prevalence of use and associated side effects of Pai You Guo, a weight-loss dietary supplement manufactured in China that was found to be adulterated, by performing a cross-sectional study using an anonymous questionnaire distributed among Brazilian women living in a U.S. community. From the 565 valid surveys, 130 respondents confirmed using this supplement, corresponding to an overall prevalence of Pai You Guo use of 23% (Cohen and others 2012). The vast majority of women using this supplement (85%) reported experiencing at least 1 side effect during use, the most frequent being dry mouth (59%), anxiety (29%) and insomnia (26%) (Cohen and others 2012). Even though the authors did not analyze any sample of the PFS used by those women, the FDA has previously found sibutramine in Pai You Guo. As a consequence, in late 2009, the FDA took multiple steps including a safety alert to consumers and a recall of the product due to serious safety concerns. Despite this, in the study of Cohen and others (2012) 61% of users purchased the dietary supplement after the FDA recall and none of the respondents were aware of the FDA alert. Subsequently, to investigate if supplements still on sale after FDA recalls are free of adulterants, Cohen and others (2014) evaluated the presence of banned drugs in 27 dietary supplements purchased at least 6 mo after being recalled. The authors found that 18 of those recalled dietary supplements still available for purchase remained adulterated, with 12 products containing the same adulterant previously identified by the FDA and 6 products containing a different analog, or the same compound previously identified added with a new adulterant.

For the mentioned reasons, there has been a recent major interest in the development of analytical techniques aimed at an accurate, quick, and effective screening of illegal substances in dietary supplements, with special focus on PFS, in order to provide adequate tools for regulatory agencies to control the existence of fraud by detecting tainted PFS. In the following sections, information regarding the substances most frequently used to adulterate different PFS are presented from published studies focusing on the evaluation of PFS samples, as well as the techniques used to detect those substances.

PFS Often Adulterated Weight-loss supplements

Overweight and obesity are major risk factors for several chronic diseases and have been recognized by the World Health Organization as an increasing public health issue affecting millions of individuals, especially in Western developed countries. Additionally, for health reasons today's society strongly promotes having a normal weight and a slim figure. The loss of weight or the maintenance of an ideal weight are both commonly associated with diet and exercise, often requiring significant changes in eating behavior and lifestyle (Wing and Phelan 2005). In the search for alternatives to a quicker weight loss and to simultaneously avoid lifestyle changes, people are increasingly resorting to the so-called "quick-

fix" slimming agents (Tang and others 2011). As a result, several PFS are currently being sold with alleged weight-loss promise. Since these products have plants or plant extracts in their composition, they are often advertised as containing "purely natural ingredients," which is generally perceived by many consumers as having no risks and being safer products than pharmaceutical drugs. However, they can cause adverse reactions or interfere with conventional pharmaceutical therapies, even though both cases are considered as uncommon by sellers (Di Lorenzo and others 2014). More importantly, recent studies have shown that weight-loss PFS are frequently found to be adulterated by the illicit addition of synthetic drugs (Table 1). Since the sale of PFS advertised for weight loss has become a very lucrative business, manufacturers can be tempted to increase profits by doping PFS with drugs in order to achieve quicker effects and to advertise the effectiveness of their products (Chen and others 2009; Tang and others 2011; Deconinck and others 2012a). The drugs most generally associated with weight-loss PFS adulteration include anorexics (such as sibutramine, orlistat, diethylpropion (amfepramone), rimonabant, fenproporex, phentermine, and mazindol, and so on) but also stimulants (ephedrine, norephedrine, and synephrine), anxiolytics (mainly benzodiazepines such as diazepam), antidepressants (fluoxetine, sertraline), diuretics (such as furosemide and hydrochlorothiazide), and laxatives (including phenolphthalein). Several of these drugs are considered by regulatory agencies as being controlled substances or prescription drugs and others were banned/removed because of their adverse effects in humans (De Carvalho and others 2011). Of these substances/adulterants, the most frequently used are anorexics derived from amphetamines (De Carvalho and others 2011), with sibutramine being the most commonly detected in PFS. Sibutramine is considered an anorexic structurally related to amphetamines; it acts as a neurotransmitter reuptake inhibitor, reducing the reuptake of serotonin, norepinephrine, and noradrenalin, resulting in higher concentrations of these compounds at the synaptic clefts, thus leading to a reduction in appetite (Deconinck and others 2014). This compound was approved by FDA in 1997 and was legally prescribed and sold for the treatment of obesity until 2010 when Abbott Laboratories (Ill., U.S.A.) voluntarily withdrew sibutramine from the market due to the high risk of heart attacks and strokes, especially in patients with a history of cardiovascular disease (Csupor and others 2013). In the same year, the European Medicines Agency (EMA) issued a statement for the removal of sibutramine from the European market considering that the drug's benefits did not justify the potential risk of heart attacks (EMA 2010). Even though sibutramine has been banished from the EU and U.S. markets, both scientific studies and regulatory agency controls show that this drug continues to be fraudulently added to PFS. According to the FDA list of tainted dietary supplements, from a total of 416 public alerts launched between 2010 and 2015, 37% corresponded to adulterated weight-loss products, from which most cases (87%) involved the illegal addition of sibutramine (FDA 2015a). In the same period, the EU rapid communication system on food and feed (RASFF) notified that 64 weight-loss dietary supplements imported from different countries (mainly China and the United States, but also from Thailand, the Philippines, the Netherlands, the United Kingdom, Kosovo, and Australia) were contaminated with sibutramine (RASFF 2015). This drug was also reported as the most frequently used anorexic in a 2009 study (before it was withdrawn) after the evaluation of 105 PFS, from which 35 samples were shown to contain adulterant slimming agents (Chen and others 2009). Since the illegal addition of sibutramine is known to

Table 1—Summary of studies performed on the analysis of adulterants in weight-loss dietary supplements and used methodologies.

Adulterants of interest	Formulation type	Sample preparation	Method	Adulterated samples / total samples	Reference
Sibutramine	Capsules	Sample suspended in 1 mL methanol (for GC-MS) or 1 mL mobile phase (for HPLC), centrifuged and analyzed (after dilution 1:50 v/v, for GC analysis)	HPLC-DAD; GC-MS	1/1	Jung and others (2006)
Anorexics (sibutramine, <i>N</i> -di-desmethylsibutramine, <i>N</i> -mono-desmethylsibutramine, fenfluramine), antiobesity drug (orlistat), laxative (phenolphthalein)	Capsules, teabags	Sample extracted with 20 mL methanol for 10 min, centrifuged, supernatant diluted with methanol and solution filtered (0.45-mm nylon membrane)	LC-ESI-MS	11/22	Wang and others (2008)
Untargeted screening of adulterants	Capsules, tablets, powder	Sample dissolved in 5 mL of CD ₃ CN:D ₂ O (80:20 v/v), stirred 10 min, sonicated 10 min, centrifuged and the supernatant (550 mL) analyzed.	DOSY ¹ H-NMR; MS/MS	14/20 ^a	Vaysse and others (2010)
Anorexics (sibutramine, fenfluramine), stimulants (ephedrine, norpseudoephedrine), diuretic (clonamide) and others (natural laxatives rhein, emodin, chrysophanol)	Tea powder, capsules, tablets	Sample (0.1 g) extracted with 8 mL 70% methanol aqueous solution (v/v), sonicated for 30 min at room temperature and filled to volume (10 mL); 1 mL of extracted solution was centrifuged and 10 μL supernatant solution analyzed.	HPLC-ESI-MS/MS	12/12	Shi and others (2011)
34 Compounds including anorexics (amfepramone, phentermine, rimonabant, 2,4-dinitrophenol, fenfluramine, sibutramine), stimulants (amphetamine, caffeine, synephrine, ephedrine, pseudoephedrine), laxative (phenolphthalein), diuretics (althiazide, bumetanide, furosemide, spironolactone, triamterene) and antidepressant (fluoxetine)	Capsules, tablets, powders	Sample was dispersed in mobile phase (acetonitrile / phosphate 50 mM buffer solution, 5/95 v/v), stirred, sonicated, centrifuged, clear supernatant was filtered through a 0.45-mm pore size GHP membrane filter and suitably diluted before analysis.	UHPLC-DAD	20/20	Rebire and others (2012)
Anorexics (amfepramone, sibutramine, fenproporex) and antidepressants (fluoxetine, paroxetine, sertraline, bupropion)	Not referred	Sample dissolved in 25 mL of methanol, filtered using 1st cotton and then cellulose acetate membranes (0.45 mm) and 1 mL analyzed.	Capillary Electrophoresis	4/106	De Carvalho and others (2012)
<i>N</i> -desmethylsibutramine	Not referred	Pulverized samples (200 mg) dissolved in 2.0 mL of methanol, sonicated 30 min, methanolic extracts evaporated to dryness and dissolved in methanol	LC-PDA; LC/MS	1/27	Park and others (2012)
Anorexics (sibutramine, <i>N</i> -desmethylsibutramine, <i>N</i> -didesmethylsibutramine), laxative (phenolphthalein)	Capsules, tablets	Sample extracted with 20 mL acetonitrile, sonicated, centrifuged, supernatant diluted 2000- or 5000-fold with acetonitrile and filtered (0.2 μm PTFE filter)	FI-MS/MS (confirmation using LC-MS/MS)	11/17	Song and others (2014)

(Continued)

Table 1—Continued.

Adulterants of interest	Formulation type	Sample preparation	Method	Adulterated samples / total samples	Reference
29 Drugs including anorexics (sibutramine, desmethyisibutramine, didesmethylsibutramine, diethylpropion, fenfluramine, mazindol, phentermine), stimulants (caffeine, ephedrine, pseudoephedrine, phendimetrazine), antidepressants (bupropion, fluoxetine, paroxetine, sertraline), laxatives (bisacodyl, phenolphthalein, sennosides)	Capsules, powder, tablet, granule and liquid	Sample (1 g) diluted with 50 mL methanol, sonicated 30 min and filtered (0.22 µm Millipore membrane)	LC-MS/MS	62/188	Kim and others (2014)
Anorectic drug (lorcaserin)	Capsules	Sample (100 mg) dissolved in 1 mL CD ₃ CN:D ₂ O (80:20 v/v), vortexed 1 min, sonicated 5 min, centrifuged and supernatant analyzed	NMR; MS/MS	1/1	Hachem and others (2014)
Anorexics (sibutramine, desmethyisibutramine, didesmethylsibutramine, rimonabant) and laxative (phenolphthalein)	Capsules, tablets, powder sachets	Sample sonicated with methanol, diluted 100 times using methanol/0.1% formic acid (buffered at pH 4) and filtered (0.45 µm filter)	HPLC-DAD-MS/MS	24/50	Reeuwijk and others (2014)
Sibutramine	Powder and encapsulated liquids	Sample (200 mg) suspended in 10 mL methanol, vortexed 30 s, supernatant collected and directly deposited onto HPTLC plates	HPTLC-UV densitometry; TLC-MS interface	28/52	Mathon and others (2014)
96 compounds including anorexics (fenfluramine, phentermine, rimonabant, sibutramine, topiramate), stimulants (amphetamine, 1,3-dimethylphenethylamine, β-methylphenethylamine, evodiamine, norephedrine, methamphetamine, cathine, ephedrine), antiobesity drug (orlistat), antidepressants (fluoxetine, sertraline), anxiolytic (diazepam), diuretics (hydroflumethiazide, bumetanide, chlorthalidone), hydrochlorothiazide, indapamide, methyclothiazide, metolazone)	Tablets, capsules, softgels and liquids	Samples (1.00 g added with 10 mL deionized water containing 2% formic acid for tablets, capsules and previously defatted softgels and 10 mL for liquids) were added, with 10 mL acetonitrile (tablets, capsules, and softgels) or acetonitrile containing 2% formic acid (liquids), shaken 30 min using a Glas-Col digital pulse mixer, 4 g anhydrous MgSO ₄ and 1 g NaCl were added, tubes were vigorously shaken for 1 min, centrifuged, and the acetonitrile extract was filtered	UHPLC-Q-orbitrap MS	3/23	Vaclavik and others (2014a)
Anorexics (benfluorex, phentermine, phenmetrazine, phendimetrazine, fenfluramine, fencafamine, mephentermine, sibutramine), stimulants (ephedrine, caffeine)	Powder, oily capsules and tablets	Sample (100 mg) added with internal standard, extracted with 5 mL methanol, vortexed 20 s, centrifuged, methanolic phase evaporated to dryness and reconstituted in 2 mL sodium hydroxide solution 1 M. Solution extracted with 5 mL pentane/ethyl ether (9:1) under agitation for 1 h, centrifuged, organic phase evaporated to dryness and reconstituted with 100 µL methanol/water/formic acid (6:4:0.03)	LC/HRMS	3/36	Strano-Rossi and others (2015)

^a Including food supplements and herbal medicines.

be recurrent in dietary supplements, this drug is generally included in the set of substances screened as adulterants in weight-loss PFS (Table 1).

Other amphetamine derived anorexics known to induce appetite loss but also having several side effects, such as fenproporex and amfepramone, have also been described as adulterants in slimming phytopharmaceutical products (De Carvalho and others 2011). As of 2010, several RASFF notifications were also made for products containing stimulants such as the alkaloids ephedrine (3 notifications) and synephrine (26 notifications). Among other compounds used to adulterate is phenolphthalein, a drug used as laxative and banned due to carcinogenicity concerns: it is frequently listed in public notifications from the FDA and RASFF. Since 2010 and considering a total of 155 weight-loss products listed by FDA as being adulterated, 9 products were shown to contain phenolphthalein and 48 products to contain this compound in combination with other drugs, such as sibutramine. In the same period, a total of 19 incidents were reported in RASFF for the detection of phenolphthalein in dietetic foods, food supplements, and fortified foods. Recently, the FDA also reported the presence of the antidepressant fluoxetine in dietary supplements. Anxiolytics, such as the 2 benzodiazepines diazepam and flurazepam, are also described as being associated with weight-loss PFS since they help to reduce anxiety, which is common in obese patients, while simultaneously covering the stimulating effects caused by added anorexics (De Carvalho and others 2011).

Body-building and athletic performance enhancement supplements

Dietary supplements are used by a large percentage of general consumers. Nevertheless, evidence suggests that an even larger usage rate occurs among athletes, although consumption prevalence varies with the type of sport, gender, and level of competition (Maughan and others 2011). Most athletes are very scrupulous with their body image and often choose to maintain certain restrictive diets, avoiding some foods in order to achieve a desired physical constitution, which is considered by some athletes as an important factor for their performance improvement. Such diets are often very strict and sometimes unbalanced, therefore leading to a nutritional failure of essential vitamins and minerals that can endanger both performance and health. To fulfill their nutritional needs, athletes may use a dietary supplement in order to diminish micronutrient deficits. However, regardless of their nutritional status, some athletes believe that taking supplements improves body appearance and physical performance, thus resorting to dietary supplements even if they are not needed (Kiertscher and DiMarco 2013).

Besides competitive sports, where the use of dietary supplements is considered to be widespread, these products are also commonly used by recreational gym users and amateur athletes (Goston and Correia 2010). Among dietary supplements, vitamin and mineral supplements are possibly the most consumed ones as they are generally perceived as being safe/harmless, though many PFS are also used. In particular, the so-called "fat-burning" and weight-loss products are considered to be PFS that are extremely popular among athletes (Maughan and others 2011).

Recent studies have shown that a wide range of substances can be present in dietary supplements advertised for body building and athletic performance enhancement, such as anabolic agents, stimulants, and anorexics, these often found in PFS (Maughan and others 2011). The 1st cases concerning the presence of anabolic androgenic steroids (AAS, also known as prohormones)

in dietary supplements were reported in 1999 (Geyer and others 2008). In subsequent years, different studies were performed, including the analysis of a large number of dietary supplements acquired in the U.S. and several European countries, clearly demonstrating that some products contained hormones or prohormones that were not declared on the label (Maughan 2005). However, most of these substances were found at very low levels and in varying concentrations for the same product, thus suggesting a cross-contamination instead of fraudulent admixtures. Nevertheless, it was demonstrated that the consumption of such supplements taken in the recommended doses could result in positive antidoping controls tests. This entails a major risk for high-level competition athletes since the strict liability principle applied by the World Anti-Doping Agency (WADA) does not distinguish between deliberate and inadvertent doping due to food supplements consumption, with all kinds of responsibility lying with the athlete taking the supplement (WADA 2015). In other cases, high amounts of WADA-prohibited substances, such as methandienone, have also been detected, sometimes in amounts considerably higher than the normal therapeutic doses, and these could jeopardize an athlete's health (Maughan and others 2011).

A wide range of stimulants, steroids, and other agents currently included on WADA's prohibited list have been identified as adulterants in dietary supplements. According to WADA, these substances are described as being performance enhancing drugs (PEDs), which are defined as being any pharmacological substance listed in the World Anti-Doping Code, or that has not been approved by a governmental regulatory health authority for human therapeutic use. Adulteration of dietary supplements with PEDs has been reported by several authors (Pipe and Ayotte 2002; Geyer and others 2004; Maughan 2005; van der Merwe and Grobbelaar 2005), and this can lead to a positive antidoping test. Besides substances with no current approval for human therapeutic use by any governmental regulatory health authority (generally considered as "designer" drugs), WADA's prohibited list includes an enormous diversity of chemicals, namely anabolic agents (AAS, clenbuterol, selective androgen receptor modulators, tibolone, zeranol, zilpaterol), peptide hormones, growth factors, and related substances (growth hormone, erythropoietin, chorionic gonadotropin), β -2 agonists, hormones, and metabolic modulators (aromatase inhibitors and selective estrogen receptor modulators), diuretics, and certain masking agents (such as acetazolamide, carmerone, indapamid, and plasma expanders). Moreover, many substances are included that are prohibited in-competition, namely stimulants (amfepramone, meferox, pseudoephedrine, sibutramine), narcotics (such as buprenorphine, dextromoramide, methadone, morphine, oxycodone), cannabinoids, and glucocorticosteroids (WADA 2014). Among the mentioned substances, the use of new/modified or "designer" steroids (such as prostanazol, methasterone, andostatrienedione, among others) is of higher concern because so little is known about their pharmacology and possible side effects (Geyer and others 2008).

When weighting the risks and advantages of using dietary supplements, in particular those purchased from "shady" sources such as Internet Web sites selling all kinds of "natural" products, athletes should consider the possibility that some of the above-mentioned substances can be found in dietary supplements, and in PFS in particular, most often to potentiate the "performance enhancing" effect advertised in the product. Those substances can cause secondary effects or drug interactions with pharmaceutical medicines or with the botanicals' active substance and, in the case of competition athletes, usage may lead to their detection in antidoping tests.

Table 2—Summary of studies performed on the analysis of steroid adulterants in dietary supplements and methodologies.

Adulterants of interest	Formulation type	Sample preparation	Method	Adulterated samples/total samples	Reference
25 Anabolic steroids	Solid nutritional supplements: protein concentrate and creatine monohydrate	Sample extracted with ethyl acetate; crude extract is purified using dispersive solid-phase extraction (SPE) with primary secondary amine (PSA) as sorbent	GC × GC-TOFMS	–	Stepan and others (2008)
49 Anabolic compounds	Capsules, tablets and liquids (some labelled as containing prohormones)	Sample extracted twice with methanol, centrifuged, evaporated to dryness, redissolved methanol and 100-fold dilution in methanol/water (65/35, v/v)	LC-MS/MS	11/19	Van Poucke and others (2007)
11 Anabolic androgenic steroids	Capsules, tablets, powders, fluids	Samples extracted with methanol, centrifuged, evaporated to dryness, sodium hydroxide solution 0.1 M and n-pentane added to the residue, shake and centrifuge; transfer n-pentane layer, add methanol/water solution (95:5, v/v); shake and centrifuge; discard n-pentane layer; add methanolic solution of 1-(<i>N</i> , <i>N</i> -diisopropylamine)-alkanes, evaporate to dryness and residue its derivatized	GC-MS	94/634	Geyer and others (2008)
Unknown androgens and androgen derivatives	Not referred (4 herbal mixtures and 4 sport supplements)	Sample sonicated with methanol and sodium acetate buffer (0.25 M; pH 4.8), centrifuged and acetic acid (4.0 M) added to supernatant (reaching pH 4.8); SPE purification (1st on C18 cartridge followed by 2nd SPE on Isolute NH ₂ cartridge), eluate evaporated to dryness	bioassay-guided fractionation combined with UHPLC/TOFMS	5/8 (compounds were tentatively identified)	Peters and others (2010)
88 Steroids	Tablets, capsules or powder	Sample extracted with ethyl acetate/acetic acid (99:9.0.1, v/v), centrifuged, upper layer eluted through NH ₂ SPE, eluate evaporated to dryness, residue dissolved in mobile phase and centrifuged (Ultrafree®-MC centrifugal, 0.22 μm, Millipore)	UPLC-MS	6/8	Becue and others (2011)
Androgenic and estrogenic (designer) steroids	Not referred	Sample extracted (ultrasonic bath and head-overhead mixing) with methanol and sodium acetate (12.5 mM, pH 4.8), centrifuged, supernatant diluted 5 times with HBS-EP buffer	bioaffinity LC-MS screening coupled to chip-UPLC(NanoTile)-Q-ToF-MS	8/22	Aqai and others (2013)
35 Anabolic steroids and clenbuterol	Powders, capsules, energy bars and tablets	Sample dissolved in methanol, evaporated to dryness, diluted in sodium hydroxide solution, extracted with pentane/ethyl ether 9:1	LC-HRMS in APCI mode	16/30 (some samples with multiple compounds)	Odoardi and others (2015)

Different studies recently available in the literature have shown the presence of anabolic steroids and designer drugs in dietary supplements, such as androstenedione, 5-androsten-3 β -ol-17-one (dehydroepiandrosterone; DHEA), methandienone, testosterone esters, androst-4-ene-3 β -17 β -diol, boldenone, among others (Geyer and others 2008; Becue and others 2011; Aqai and others 2013; Odoardi and others 2015). Table 2 shows a compilation of studies reporting the presence of illegal substances in dietary supplements. Additionally, regulatory agencies have also reported the presence of adulterants in such products advertised for body building/athletic performance enhancement. In the last 5 y, from a total of 416 public notifications issued by FDA, 18 concerned the presence of steroids or aromatase inhibitors in muscle building products (FDA 2015a). Although corresponding only to 4.3% of the total notifications, it should be noticed that there is no information regarding the total number of samples analyzed by type of supplement (weight loss, sexual enhancement, or muscle building).

Sexual performance enhancement supplements

Erectile dysfunction (ED) is a disease that affects 150 million men worldwide (Schramek and others 2014) being characterized by the inability to create or maintain penile erection during sexual activity. The treatments currently known for this problem encompasses the administration of PDE-5 inhibitors drugs. These compounds act by inhibiting the mentioned enzyme, which is responsible for the degradation of cyclic guanosine monophosphate (cGMP) to guanosine monophosphate (GMP), thus causing a rise of cGMP levels resulting in smooth muscle relaxation of helicine arteries followed by an increase of blood, thus enhancing normal erectile function (Codevilla and others 2013). Presently, the PDE-5 inhibitors legally commercialized worldwide are sildenafil citrate (Viagra[®]), tadalafil (Cialis[®]), vardenafil hydrochloride (Levitra[®]), udenafil (Zydena[®]), mirodenafil (Mvix[®]), lodenafil carbonate (Helleva[®]), and avanafil (Stendra[®] in the United States or Spedra[®] in EU) (Patel and others 2014). However, in the United States and EU only sildenafil, tadalafil, vardenafil, and avanafil are approved by the competent authorities (FDA and EMA, respectively) for the treatment of ED (EMA 2015; FDA 2015b). The side effects of prescription PDE-5 inhibitors and possible interactions with other drugs are well documented. These drugs can cause headaches, flushing, dyspepsia, nasal congestion, and visual disorders, and because they were shown to potentiate the hypotensive effects of nitrates and α -blockers, the concomitant use of PDE-5 inhibitors with such drugs should be avoided (Gur and others 2013). Moreover, since sildenafil, tadalafil, and vardenafil are mainly metabolized by the cytochrome P450 3A4 pathway, drugs that inhibit this pathway may decrease metabolism of PDE-5 drugs and increase their plasma concentrations (Schwartz and others 2010).

During the last few years, the demand for PDE-5 inhibitors has been increasing worldwide, not only for treating patients with ED but also because they are sometimes being used by young men without ED to enhance sexual performance for recreational purposes, occasionally associated with the intake of alcohol or other drugs (Korkes and others 2008; Bechara and others 2010). Even though these substances are considered as controlled prescription drugs, for several reasons including the stigma associated with sexual dysfunction and/or personal lack of confidence to openly speak with the doctor, lack of information, drug costs, and availability from easily accessible, cheaper, and discrete sources such as Internet Web sites, the supply of these products without medical

prescription through unofficial methods/parallel markets has been increasing during the last decade (Campbell and others 2013; Fejos and others 2014; Patel and others 2014). It is estimated that in Europe alone more than 6 million illegal products containing PDE-5 inhibitors are being purchased outside the official health system (Schnetzer and others 2010; Venhuis and de Kaste 2012) and that in the United States more than half a million of such uncontrolled tablets are being sold every month (Dorsey and Hellstrom 2007). Since these counterfeit products are not under any quality control program, there is an inherent risk of buying a poor-quality product, for example, having impurities, lacking sample homogeneity, or incorrect dosage. Besides the problematic use of counterfeit pharmaceutical products, several recent studies have shown the illegal presence of PDE-5 inhibitors and/or its analogs in PFS (Table 3). These types of supplements are increasingly popular since they are advertised as "natural products" for sexual performance enhancement leading to a false sense of security in consumers (Liang and others 2006; Singh and others 2009; Campbell and others 2013; Fejos and others 2014). Since the existence of demand leads to a rise in supply, a growing number of products have recently been advertised on the Internet and on television, usually marketed as a "natural" resolution for sexual problems (Liang and others 2006; Singh and others 2009; Petroczi and others 2011; Strano-Rossi and others 2015). However, to boost the performance of such products, unscrupulous producers can dope PFS with PDE-5 inhibitors (Table 3). Since 2010, FDA issued 229 public notifications for sexual enhancement dietary supplements (corresponding to more than 55% of total public notifications regarding dietary supplements for that period) due to the positive detection of approved PDE-5 inhibitors or its analogs (mainly sildenafil, but also tadalafil and the analogs sulfoildenafil, aminotadalafil, hydroxythiohomosildenafil, sulfosildenafil, dimethylsildenafil, sulfohydroxyhomosildenafil, dimethylacetildenafil, noracetildenafil, desmethyl carbodenafil, desmethylcarbodenafil, sulfosildenafil, and propoxyphenyl sildenafil). In the same period, 81 notifications were issued by RASFF regarding the presence of PDE-5 inhibitors or their analogs in dietetic foods, food supplements, and fortified foods.

Adulterated sexual enhancement PFS impose a very high risk to consumers' health as they are buying and consuming products that can cause adverse health effects, in particular in the case of individuals with cardiovascular diseases medicated with nitrates or α -blockers (Champagne and Emmel 2011; Venhuis and de Kaste 2012). As those individuals are advised not to take PDE-5 inhibitors, they can be tempted to try other alternatives advertised as being natural products and inadvertently put their lives at risk due to drug interactions caused by the adulterated PFS. Moreover, according to Venhuis and de Kaste (2012) PFS are most frequently adulterated with analogs rather than with approved PDE-5 drugs. These analogs are generally synthesized based on the PDE-5 approved drugs, with minor changes on their structures, with some analogs also corresponding to substances described in patents from pharmaceutical companies (Venhuis and de Kaste 2012). These unapproved analogs raise additional safety concerns as their pharmacokinetics and safety profile are mostly unknown. On the other hand, from the point of view of manufacturers performing PFS adulteration, the use of new and exotic analogs reduces the chances of being caught as these substances are generally more difficult to be detected in routine inspections using standard protocols (Patel and others 2014). Therefore, to protect consumers from fraudulent products, adequate methods for dietary supplements monitoring, including screening methods, strategies for the identification of

Table 3—Summary of studies performed on the analysis of adulterants in sexual performance enhancement dietary supplements and used methodologies.

Adulterants	Formulation type	Sample preparation	Method	Adulterated samples/total samples	Reference
3 PDE-5 inhibitors	Tablets, capsule, liquids	The sample was extracted in 5 to 10 mL of acetonitrile:water (50:50 v/v) with sonication for 15 min and the extract filtered (0.2 µm nylon syringe filter).	LC-ESI-MS; LC-UV	19/40	Gratz and others (2004)
Sildenafil	Capsules	Sample was ultrasonicated with MeOH for 15 min and the clear supernatant (1 mL) was transferred to a 25-mL flask and diluted to volume with MeOH.	HPTLC	3/3	Aboutashed and others (2007)
35 Compounds including 2 PDE-5 inhibitors and methyltestosterone	Capsule, tablets, pill, granules, oral solution	Sample was extracted with methanol (10 mg/mL; for oral solutions 1 mL was extracted with 4 mL methanol), sonicated 15 min, centrifuged, and supernatants diluted with mobile phase before analysis.	QTRAP LC-MS/MS	11/29 ^a	Chen and others (2009)
2 PDE-5 inhibitors, 5 sildenafil analogs and 1 vardenafil analog	Not referred	Sample (0.1 g) was extracted with 10 mL of ammonium formate (10 mM): acetonitrile (50:50 v/v), vortexed, sonicated, further diluted 100 to 1000 times and the resulting solution was filtered (0.45 µm Teflon filter).	LC-MS/MS	some products/> 100 ^b	Ng and others (2010)
3 PDE-5 inhibitors	Capsule, tablets, herbal oils	Samples were extracted with 5 mL methanol by sonication for 40 min; extract was centrifuged and supernatant directly injected to HPLC and LC-MS.	LC-MS/TOF; HPLC-DAD	1/85 ^c	Savaliya and others (2010)
3 PDE-5 inhibitors and yohimbine	Pill, soft capsules, hard capsules, oral drinks	Sample was extracted with 80 mL methanol, sonicated 30 min, diluted to 100 mL, 1.0 mL of this solution was diluted to 10 mL with 0.1 acetic acid solution:methanol (1:1, v/v) and solution was filtered (0.45 µm membrane).	HPLC-MS/MS	21/26	Zhang and others (2010)
3 PDE-5 inhibitors	Capsules, tablets	Sample was extracted with 20 mL acetonitrile:water (1:1 v/v), sonicated 30 min, centrifuged and filtered (0.2 µm membranes).	FI-MS/MS	1/13	Song and others (2012)
2 Tadalafil analogs	Capsules	Samples (0.1 g) were extracted with 5 mL 50:50 (v/v) CH ₃ CN:DIH ₂ O and shaking; extracts were filtered (0.2 µm PTFE syringe filters) and further diluted with extraction solvent.	LC-ESI-MS ⁿ	2/2	Toomey and others (2012)
18 Compounds including PDE-5 inhibitors, analogs and yohimbine	Capsules, tablets	Sample (0.2 g) was added with 5 mL methanol, vortexed 60 s, sonicated 15 min, cooled to room temperature and evaporated methanol added to initial total weight.	UFLC-ESI-MS/MS	9/16	Ren and others (2012)

(Continued)

Table 3—Continued.

Adulterants	Formulation type	Sample preparation	Method	Adulterated samples/total samples	Reference
2 Sildenafil analogs	Capsules	Sample (10 mg) was dissolved in 1.5 mL of CD ₃ CN, CD ₃ OD or CD ₃ CN:D ₂ O (80:20 v/v), vortexed 10 min, sonicated 10 min, centrifuged and 500 µL of supernatant analyzed (NMR analysis). Sample (58 mg) was stirred 40 min in 10 mL methanol, sonicated 5 min, centrifuged, 1 mL of extract was evaporated to dryness and residue dissolved in 1 mL methanol-d ₄ (quantitative and MS analyses). Compounds were also isolated and purified.	NMR; LC-MS; MS/MS; IR	1/1	Vaysse and others (2012)
PDE-5 inhibitors and analogs 3 PDE-5 inhibitors and 10 analogs	Capsules, tablets, pills Bulk powder, capsules	Not referred Sample was dissolved with methanol—water-formic acid (52:46:0.1 v/v/v), sonicated 10 min and appropriately diluted. Sample was added with 50 mL of water/ACN/formic acid (50:50:0.1, v/v), shaken 30 min, sonicated 5 min, centrifuged and supernatant diluted 5-fold with water/ACN/formic acid (50:50:0.1, v/v).	HPLC-DAD-ESI-MS HPLC-CAD	74/91 13/24	Campbell and others (2013) Poplawska and others (2013)
1 Vardenafil analog (Hydroxythiovaridenafil)	Capsules	Sample was added with 50 mL of water/ACN/formic acid (50:50:0.1, v/v), shaken 30 min, sonicated 5 min, centrifuged and supernatant diluted 5-fold with water/ACN/formic acid (50:50:0.1, v/v).	LC-UV-MS/MS; NMR; IR	1/1	Jankovics and others (2013)
3 PDE-5 inhibitors, 24 sildenafil analogs, 5 tadalafil analogs and 5 vardenafil analogs	Pills, soft capsules, hard capsules, bulk powders	Sample (0.5 g) was extracted with 25 mL MeOH/water (70:30, v/v), sonicated 30 min, centrifuged, solution was filtered (0.2 µm PVDF filter) and diluted to appropriate concentration for analysis.	LC-ESI-MS/MS	45/52	Lee and others (2013a)
6 PDE-5 inhibitors, 26 sildenafil analogs, 7 tadalafil analogs, 5 vardenafil analogs; 4 other drugs for ED	Capsules, tablets, powder, film, liquid	Sample (0.5 g) was dissolved in 25 mL 70% MeOH, sonicated 30 min, filtered (0.20 µm PVDF membrane syringe filter) and appropriately diluted with MeOH.	LC-MS/MS	77/164	Lee and others (2013b)
Sildenafil and analogs	Capsules, tablets, liquids	Sample (half a dose unit) was sonicated with methanol, diluted 100 times with MeOH/0.1% formic acid (pH = 4) and filtered.	LC-DAD-MS/MS	23/71	Reeuwijk and others (2013)
Diethylaminopretadalafil	Capsules	For LC-UV analysis, sample was sonicated with 25 mL methanol and filtered (0.45 µm nylon syringe filter) (the compound was also isolated and purified).	LC-UV; NMR; HRMS	1/1	Zhang and others (2014)
4 Sildenafil analogs	Capsules	Sample was mixed with 10 mL methylenechloride and 2 mL 2 M sodium hydroxide solution; organic layer was collected and the aqueous layer was reextracted with 5 mL methylenechloride, combined organic phase was dried under a stream of nitrogen, residue was reconstituted in 4 mL tetrahydrofuran, solution was diluted with acetonitrile and filtered (0.45 µm Spartan filter).	LC-DAD; LC-MS; NMR	1/1	Schramek and others (2014)

(Continued)

Table 3–Continued.

Adulterants	Formulation type	Sample preparation	Method	Adulterated samples/total samples	Reference
82 Compounds including 71 erectile dysfunction active substances (PDE-5 inhibitors and analogs)	Tablets, liquid-gel capsules, oral liquids, herbal samples	Sample was added with 10 mL MeOH:H ₂ O:ACN (70:20:10 v/v) containing 0.1% to 1% formic acid, vortexed 2 min, sonicated 10 min and vortexed 3 min. Samples were centrifuged (if needed) and supernatant was filtered (0.45 μm PTFE syringe filter).	LC-MS/MS	~35 ^d	Lebel and others (2014)
3 PDE-5 inhibitors and 11 analogs	Tablets, capsules	Sample (dosage unit) was agitated with MeOH:ACN (50:50 v/v) 30 min, sonicated 5 min, centrifuged and an aliquot of the clear supernatant was diluted 2.5-fold with MeOH:ACN (50:50 v/v)	HPLC-UV	5/-	Fejos and others (2014)
3 PDE-5 inhibitors and 2 analogs	Tablets, capsules, powders, soft capsules	Samples (5 to 7 mg) were dissolved in 10 mL methanol, sonicated for 15 min, 20 μL of this solution was diluted up to 700 μL with methanol and filtered (0.2 μm PTFE filter) into autosampler vials.	UPLC-TOF-MS; GC-MS	3/11	Damiano and others (2014)
3 PDE-5 inhibitors	Powders, oily capsules, tablets	Sample (100 mg) added with internal standard was extracted with 5 mL methanol, vortexed 20 s, centrifuged, methanolic phase evaporated to dryness and reconstituted in 2 mL sodium hydroxide solution (1 M). Solution extracted with 5 mL pentane/ethyl ether (9:1), shaken 1 h, centrifuged, organic phase evaporated to dryness and reconstituted with 100 μL methanol/water/formic acid (6:4:0.03).	LC-HRMS	4/36	Strano-Rossi and others (2015)
21 Compounds including 3 PDE-5 inhibitors and 5 analogs, yohimbine, phenolamine and mesylate	Capsules, tablets, powders, granules, drinkable liquids, chewing-gums, strips, gel	Samples (100 mg or 1 mL liquid preparations lyophilisate) were mixed with 1 mL CD ₃ CN:D ₂ O (80:20, v/v), stirred for 10 min, sonicated for 10 min, suspension was centrifuged and the supernatant analyzed. TSP was added before NMR analysis. For quantitative analysis sample (15 to 20 mg) was dissolved in 5 mL of methanol, stirred, sonicated, 1 mL was evaporated to dryness and residue dissolved in 1 mL MeOH-d ₄ . And added with 0.05 mL of TSP solution (10 mM).	¹ H NMR	104/150 (some samples with multiple compounds)	Gilard and others (2015)

^a Given values comprise only samples related with ED adulterants; in this study 35 adulterated samples were detected among 105 PFS samples.

^b More than 100 samples of health supplements and Chinese herbal drugs samples were analyzed with analogs being detected in some products (number not reported).

^c Herbal formulations.

^d The number of positive samples was not reported; samples included herbal medicines, dietary supplements, and legitimate or counterfeited trademark products.

CD₃CN:D₂O, deuterated acetonitrile/deuterated water; TSP, sodium 2,2,3,3-tetrafluoro-3-(trimethylsilyl)propanoate; MeOH, methanol; ACN, acetonitrile; PTFE, polytetrafluoroethylene; PVDF, polyvinylidene difluoride.

new and unapproved compounds, and quantification of adulterants, are considered to be essential tools (Singh and others 2009; Fejos and others 2014; Johansson and others 2014; Patel and others 2014; Strano-Rossi and others 2015).

Determination of Adulterating Substances

The selection of sample preparation methodologies, as well as the analytical technique chosen for detection, identification and, eventually, quantification of the substances of interest, depends on several factors including the number of targeted compounds and different chemical families they belong to, required sensitivity, formulation type (tablets, oily capsules, liquids, and so on) and the complexity of the matrix (for example, some samples include a large number of different botanicals in their composition). In the following sections, an overview of different methodologies used for detecting synthetic adulterants in dietary supplements, focusing on various PFS, namely weight loss, muscle building/sport performance, and sexual enhancement performance is presented.

Extraction methods/sample preparation

As can be observed in Table 1 to 3, which show several studies that include the analysis of PFS samples aiming for the detection of illegally added synthetic drugs, most sample preparation methods generally comprise the extraction of analytes using an organic solvent, such as methanol or acetonitrile or their aqueous mixtures, with the obtained solution/suspension being agitated (shaking, vortex-mixed) or sonicated, centrifuged, further diluted, and filtered. Liquid samples are often simply diluted with the solvent, filtered, and directly injected for analysis. Despite the simplicity and fastness of such sample preparation methodologies, considering the matrix complexity several different phytochemicals can be co-extracted, which can influence the determination of the analyte(s) of interest. Therefore, besides simple extraction methodologies, clean-up procedures, and/or preconcentration steps maybe required depending on the detection technique used for analyzing the extract. In particular, when mass spectrometry (MS) is used co-extracted matrix components can potentially interfere with the detection of target analytes and cause severe matrix effects during electrospray ionization (ESI) either inducing suppression or enhancement of the signal, thus impairing the accuracy of the analysis (Vaclavik and others 2014a, 2014b). Matrix effects are generally minimized by using hyphenated techniques, namely, through the previous separation of compounds by chromatography. Additionally, the use of optimized sample extraction and clean-up protocols has been suggested by different authors, including the use of liquid-liquid extraction (Geyer and others 2008; Schramek and others 2014; Strano-Rossi and others 2015), solid-phase extraction (SPE) (Stepan and others 2008; Peters and others 2010; Becue and others 2011), and QuEChERS (quick, easy, cheap, effective, rugged, and safe) procedure (Vaclavik and others 2014a). When analyzing 88 steroid compounds in PFS using ultra-performance liquid chromatography-MS (UPLC-MS), Becue and others (2011) concluded that a clean-up procedure using a SPE NH₂-column was necessary for improved sensitivity and selectivity, since it allowed for a substantial reduction of background noise by retention of polar matrix effects. Stepan and others (2008) employed dispersive SPE using primary secondary amine (PSA) as a sorbent to effectively remove polar components such as sugars, 4-hydroxy-2-methoxycinnamaldehyde and partially remove fatty acids and vanillin. More recently, Vaclavik and others (2014a) used a QuEChERS procedure to extract target analytes from PFS samples. The QuEChERS procedure is based

on sample extraction using a mixture of acetonitrile and water and subsequent separation of phases by the addition of salts, with the polar matrix components being retained in the aqueous phase, while the analytes of interest are transferred to the organic phase. The employed procedure allowed an effective extraction of most analytes from the evaluated matrices with recoveries in the range of 80% to 120%, with the exception of highly polar analytes as they have more affinity for the aqueous phase. For the clean-up and purification of oily matrices from softgels, defatting with hexane and the use of a dispersive SPE purification step with Bondesil C₁₈ (octadecylsilane) sorbent showed to be effective, since it enabled the removal of some nonpolar co-extractants and slightly decreased the matrix effects while not decreasing recoveries of the analytes (Vaclavik and others 2014a).

Additionally to extraction, depending on the used technique, it can be necessary to include a derivatization reaction step during sample preparation. In the case of gas chromatography-MS (GC-MS) detection methods, still frequently used for the detection of steroids and prohormones, considering the poor volatility of most adulterants, derivatization is most often needed, which slightly increases total sample preparation time (Geyer and others 2008; Vaclavik and others 2014b).

Techniques used for the detection and identification of adulterants

Chromatographic methods. *Thin-layer chromatography (TLC).* TLC has long been used in the characterization of plant drugs with pharmacologically active components in botanical formulations and standardized extracts. TLC can also be used for the preliminary identification of herbal products adulterated with pharmacological drugs (Cai and others 2010; Csupor and others 2013). TLC is considered to be a simple, easy, rapid, and inexpensive technique for the preliminary screening of compounds, however the availability of standards for those substances is mandatory and, above all, it has a very low sensitivity (Ariburnu and others 2012). Recently, Lv and others (2015) proposed a method which combined TLC and surface-enhanced Raman scattering (SERS) to directly identify stimulant alkaloids (ephedrine, pseudoephedrine, methylephedrine, and norephedrine) as trace adulterants in PFS. The method used SERS technology which relies on the specific vibrational spectroscopy with ultra-high sensitivity at molecular level based on 8 common Raman peaks for the compounds under evaluation. This allowed for a simple, rapid, and accurate methodology, with results from real samples analysis being confirmed by UPLC quadrupole time of flight MS (UPLC-QTOF/MS).

The use of high-performance TLC (HPTLC) also allows improving sensitivity compared to TLC, thus it has already been reported as a screening method for herbal products adulterated with pharmacological drugs (anorectic and PDE-5 inhibitor drugs) (Abourashed and others 2007; Sanzini and others 2011; Ariburnu and others 2012). Recently, Mathon and others (2014) proposed an HPTLC-ultraviolet (UV) densitometric method for the quantification of sibutramine in PFS, with its unequivocal identification being confirmed by MS using a TLC-MS interface. The method was applied to the analysis of 52 weight-loss supplements obtained via the Internet showing that half of those were adulterated with sibutramine, with some products containing amounts 3 times higher than the dosage prescribed as an appetite suppressant drug prior to its withdrawal (Mathon and others 2014). The authors reported the nonexistence of significant statistical differences among the quantitative results obtained using the validated

HPTLC method with those obtained by HPLC-UV and HPLC-MS/MS.

Irrespectively of the PFS type (weight loss, muscle building, or sexual performance), since in both methods (TLC and HPTLC), reference standards are required for a positive identification, they are not considered appropriate for screening adulterations with new pharmacological drugs (designer drugs).

High-performance liquid chromatography (HPLC). HPLC is a well-established and widespread technique for the routine analysis of different compounds all over the world (De Carvalho and others 2011). In the case of complex matrices, such as PFS, the chromatographic step prior to analyte detection/identification enables the separation of several phytochemicals from the bulk sample matrix that could interfere or suppress the signals of the compounds of interest (Vaclavik and others 2014a, 2014b). Liquid chromatography (LC), namely HPLC and more recently ultra-high-performance LC (UHPLC), is the most commonly reported separation technique applied for the analysis of pharmaceutical adulterants in dietary supplements, in particular those used for weight loss and sexual performance enhancement (Table 1 and 3). The use of HPLC coupled to ultraviolet detection, especially HPLC with a diode array detector (DAD), has been recognized for the preliminary identification and screening of adulterants in weight-loss products (De Cock and others 2001; De Carvalho and others 2011; Deconinck and others 2012a; Rebiere and others 2012; Csupor and others 2013) and sexual PEDs (Singh and others 2009; Ortiz and others 2010; Savaliya and others 2010; Sacré and others 2011; Venhuis and others 2011a, 2011b; Wollein and others 2011; Jankovics and others 2013; Fejos and others 2014; Zhang and others 2014). HPLC-UV has also been used in semipreparative chromatography for unknown compounds isolation purposes (Lee and others 2011). When using HPLC-DAD, the screening and detection of compounds is based on information of UV spectra and retention time compared to those obtained for pure standards. Thus, although being simpler and cost-effective compared to other techniques, HPLC-DAD has limited applicability, especially in the case of new analogs/designer drugs, for which no standards are available. Nevertheless, in some cases, this technique can still assist researchers in the preliminary detection of such adulterants, namely those analogs structurally similar to the original compound, as they will present different retention times but identical UV spectra (Hou and others 2006).

In the last decade, LC coupled to MS detection has become a primary tool in the analysis of adulterants in PFS, since it combines the separation capacity of LC with the sensitivity and selectivity of MS detectors allowing for molecular identification (Patterson and others 2012; Vaclavik and others 2014a, 2014b). When standards are available, the use of LC preceding MS analysis also contributes to the identification of compounds based on its retention time. The use of LC-MS for the elucidation of adulterant structures in food supplements, detailing different MS detectors (enabling low or high resolution mass measurements), different data acquisition modes, and potential application in posttargeted and nontargeted screening approaches, has been recently reviewed by Vaclavik and others (2014a, 2014b). Currently, LC-MS is considered by most researchers as being the method of choice to detect pharmaceutical adulterants in PFS, with several works available in the literature concerning the analysis of dietary supplements for weight loss and sexual performance enhancement, as described in Table 1 and 3, respectively. Regarding muscle building/sport enhancement food supplements, GC-MS has been intensively used over the last decades for the analysis of nondeclared doping sub-

stances added to those products (Becue and others 2011). However, more recently, several laboratories are adopting LC-MS as a valuable tool for this type of analysis since it generally requires a reduced sample treatment compared to GC-MS and allows detecting thermolabile compounds (Becue and others 2011). Nevertheless, a major disadvantage for LC-MS is that a general searchable mass spectral library, such as the Natl. Inst. for Standards and Testing (NIST) for GC-MS, is not available for LC-MS (Becue and others 2011; Peters and others 2010). Still, since accurate high-resolution mass measurements are specific for every compound regardless of the instrumentation used, potentially these data could enable the use of accurate mass databases for compound identification (Peters and others 2010).

Gas chromatography (GC). Despite being a sensitive, reproducible, accurate, and quantitative technique, well suited for the analysis of mixtures, and of lower cost compared to LC-MS, GC methodologies are rarely used for the analysis of the most frequently found pharmaceutical adulterants in dietary supplements, with a general exception being the detection of anabolic steroids in muscle building supplements, which is frequently accomplished using GC-MS. Other examples of GC applications are the works of Marchei and others (2006) and Man and others (2009). The main criterion that should be considered when using GC methodologies is the volatility and thermal stability of the compounds. This can explain the low usage of GC methods in the analysis of PDE-5 inhibitors and their analogs, as they are considered to be heat-labile and difficult to derivatize using standard silylation reagents (Patel and others 2014). Nevertheless, in certain cases where the LC-MS technique does not provide sufficient information for compound identification, the analysis of chemical reaction products (such as hydrolysis products) by GC-MS has been described as a useful complementary tool for structure elucidation and identification of designer drugs as, for example, new PDE-5 inhibitors analogs (Patel and others 2014; Vaclavik and others 2014a, 2014b).

Despite being a sensitive, reproducible, accurate, and quantitative technique, well suited for the analysis of mixtures, and of lower cost compared to LC-MS, GC methodologies are rarely used for the analysis of the most frequently found pharmaceutical adulterants in dietary supplements, with a general exception being the detection of anabolic steroids in muscle building supplements, which is frequently accomplished using GC-MS. Other examples of GC applications are the works of Marchei and others (2006) and Man and others (2009). The main criterion that should be considered when using GC methodologies is the volatility and thermal stability of the compounds. This can explain the low usage of GC methods in the analysis of PDE-5 inhibitors and their analogs, as they are considered to be heat-labile and difficult to derivatize using standard silylation reagents (Patel and others 2014). Nevertheless, in certain cases where the LC-MS technique does not provide sufficient information for compound identification, the analysis of chemical reaction products (such as hydrolysis products) by GC-MS has been described as a useful complementary tool for structure elucidation and identification of designer drugs as, for example, new PDE-5 inhibitors analogs (Patel and others 2014; Vaclavik and others 2014a, 2014b).

In the case of muscle building/sport performance dietary supplements, the substances most probably used as adulterants are prohormones and steroids. GC-MS has long been routinely used in the analysis of such compounds as doping agents in sports, being also suited for PFS analysis. Since GC-MS uses standardized and universal electron impact ionization (EI) conditions, similar spectra are obtained for the same compound, even when using

different equipment, thus allowing the use of standard reference databases such as NIST (Becue and others 2011). Nevertheless, this technique requires a derivatization step, generally trimethylsilylation, which makes sample preparation more cumbersome and time-consuming. Recently, a method based on comprehensive 2-dimensional (2D) gas chromatography (GC \times GC) with TOF-MS detection for the determination of 25 anabolic steroids in food supplements was proposed by Stepan and others (2008). Contrarily to most other GC-MS techniques, this technique does not require derivatization. Moreover, it allows obtaining lower limits of detection, achieving enhanced chromatographic resolution, and complete separation of target steroids as a result of separation being performed in 2 capillary columns with different polarities, thus reducing the risk of false positives (Stepan and others 2008).

Mass spectrometry. MS is the most applied detection method for the identification and structural elucidation of adulterants in dietary supplements as it fulfills the requirements of selectivity and sensitivity needed for such analyses. With technology evolution, there are currently several different types of MS detectors available, which are frequently used in hyphenated techniques, including single quadrupole, triple quadrupole, linear trap, Orbitrap (Fourier transform MS), Fourier transform ion cyclotron resonance MS (FT-ICR-MS) and time of flight (TOF), among others. MS can be rearranged to a tandem (MS/MS) or a multistage (MSⁿ) MS, improving its accuracy and resolution (Patel and others 2014).

Apart from its extensive use in hyphenated techniques, direct-infusion MS has also been reported in several works, mainly for structural identification purposes applied to isolated and purified compounds. Direct-infusion MSⁿ and collision-induced dissociation (CID) MS experiments have been used to provide useful information on the structural elucidation of new analogs of vardenafil and sildenafil, namely piperidenafil and nor-acetildenafil (Reepmeyer and Woodruff 2006, 2007). Ahn and others (2009) proposed the use of high-resolution MS and fast-atom bombardment coupled to high-energy CID tandem MS (FAB-CID-MS/MS) to elucidate the structures of sildenafil and its analogs isolated from food supplements. Direct infusion or flow injection MS (FI-MS) has also been proposed as an analytical tool to detect adulterants in different samples, including those added to PFS. FI-MS allows complex mixtures to be resolved into components differing in ion mass and has the advantage of being a fast and high-throughput approach since it does not involve any prior time-consuming LC separation (Koulman and others 2007). Nevertheless, a higher extent of matrix effects is generally observed in FI-MS compared to hyphenated MS techniques (Vaclavik and others 2014a, 2014b). Song and others (2012) reported the use of FI-MS with a triple quadrupole operating in multiple reaction monitoring (MRM) mode to screen for PDE-5 inhibitors in food supplements. The authors reported some difficulties in identifying some similar analogs (homosildenafil, hydroxyhomosildenafil, and sulfoaildenafil) as they shared both precursor and products ions. Identical difficulties were reported for a FI tandem MS (FI-MS/MS) with MRM monitoring applied for the semiquantitative screening of weight-loss drugs, namely phenolphthalein, sibutramine, and its pharmacologically active metabolites *N*-desmethylsibutramine and *N*-didesmethylsibutramine, in 17 food supplements (Song and others 2014). The authors reported that FI-MS/MS offered an improved sensitivity, selectivity, and wider detection ranges, although *N*-formyldidesmethylsibutramine could be assigned mistakenly as sibutramine based on FI-MS/MS analysis alone, as they give similar product ion profiles and ion ratio for the 3 common MRM

transitions used. In this study, FI-MS/MS allowed the detection of 11 positive samples for sibutramine and 9 samples for phenolphthalein, which were further confirmed by LC-MS/MS. Other reported applications used a hierarchical approach in which ESI-MS fingerprinting is followed by the targeted analysis of specific *m/z* signals by high resolution methods (Draper and others 2013).

Spectroscopic methods. Even though hyphenated methodologies using MS detection are undoubtedly considered as the most used and adequate methodologies for the determination of pharmaceutical drugs and their analogs illegally added in food supplements, recently, there has been an increased interest on the use of spectroscopic methods as they are rapid, have high throughput, are simple to use, require little or no sample preparation, and can be possibly adapted for on-field screening. For those reasons, spectroscopic methods have been suggested as feasible and interesting screening tools to be used by inspectors and customs, which are frequently confronted with a large number of products suspected of being adulterated that must be confiscated and sent to a laboratory for analysis inspection (Deconinck and others 2014).

Among spectroscopic methods, vibrational spectroscopic methods, such as infrared (IR), near infrared (NIR) or Raman spectroscopy have already been used to detect counterfeit pharmaceuticals (Sacré and others 2010; Deconinck and others 2012b) and have shown usefulness for the authentication of different food matrices (Rodriguez-Saona and Allendorf 2011; Liu and others 2013; Rohman and others 2014). Fourier transform IR spectroscopy (FTIR) is frequently associated with attenuated total reflectance (ATR) sampling enabling samples to be analyzed directly with no need for sample preparation. Champagne and Emmel (2011) proposed the use of FTIR-ATR operating in the mid-infrared region (4000 to 650 cm⁻¹) to screen adulterations in raw materials used in the formulation and manufacture of food supplements. With this aim, the authors selected 84 raw ingredients (including different botanical materials, amino acids, proteins, polysaccharides, among others) used in food supplements formulations, which were analyzed before and after being spiked with appropriate adulterants (sildenafil, vardenafil, tadalafil) or adulterant surrogates (progesterone, a metabolite of DHEA, glutamine, niacinamide, tyrosine, and melamine). The Compare Function in AssureID software from PerkinElmer was used for spectral comparisons and further calculating correlation thresholds, disregarding band intensity or amplitude and considering shape only, thus allowing automation by providing a "pass" or "fail" report. The lowest level of adulteration needed to create a "fail" report was set by serially adulterating 4 selected raw materials with increasing amounts of adulterant. The proposed method proved to be fast, easy to do, and not requiring expertise technicians; it is thus suited for on-field screening. Still, criticism of this work concerns the extrapolation of results since, with the exception of PDE-5 inhibitors, surrogates with similar structure were used instead of the adulterant substances themselves. Additionally, the proposed method has a short applicability since it only allows testing raw materials, because, as mentioned by the authors, the obtained detection limits would be unacceptable for finished product screening. More recently, Deconinck and others (2014) suggested the use of ATR-IR spectra combined with *k*-nearest neighbors chemometrics for the detection of sibutramine in adulterated food supplements. The authors analyzed a set of 125 food supplements suspected of being adulterated (products were previously evaluated for the presence of sibutramine) and concluded that FTIR-ATR was able to detect all the adulterated products, with a minimum of false positives. The authors suggested its possible

use as a screening tool since no adulterated samples would pass the customs inspection and the possible cases of false positives, although resulting in products being retained, would be clarified after laboratory analysis.

Vibrational spectroscopy methodologies have also been used in conjunction with other techniques, such as accurate MS, X-ray crystallography, and nuclear magnetic resonance (NMR) spectroscopy, for the identification and structural elucidation of new adulterants in PFS. The use of FTIR has also been described as an additional tool in the identification of different analogs isolated from food supplements such as thiosildenafil (Vaysse and others 2012), nitroso-prodenafil (Venhuis and others 2011b), propoxyphenyl sildenafil, and methiosildenafil (Kee and others 2012), while NIR spectroscopy and Raman spectroscopy proved useful in detecting Rimobant polymorphs (Venhuis and others 2011b).

Besides being considered a crucial tool for structural identification of new analogs (Zou and others 2008; Balayssac and others 2012) or other new compounds used as PDE-5 inhibitors (Ge and others 2008), the use of NMR spectroscopy has also been described for the detection and quantification of adulterants in PFS. NMR is considered a robust technique, highly reproducible, requiring minimum sample preparation, and with no need for reference standards (Martino and others 2010; Johansson and others 2014). Other referred advantages concern the improvement of sensitivity in recent NMR equipment and the possibility of using ^1H NMR for quantitative purposes (Balayssac and others 2009; Johansson and others 2014). Moreover, the use of advanced techniques that are able to provide global information, such as 2D diffusion ordered spectroscopy ^1H NMR (2D DOSY ^1H NMR), or 3-dimensional (3D) DOSY-COSY ^1H NMR, enables the analysis of various compounds in complex matrices in a single run, since those methods are nonselective and do not require prior knowledge of the components present in the mixture (Balayssac and others 2009; Vaysse and others 2012). Balayssac and others (2009) used conventional ^1H NMR, 2D DOSY ^1H NMR and 3D DOSY-COSY ^1H NMR to analyze 17 commercial herbal drugs or food supplements marketed for sexual dysfunction and found 8 of those samples to be illegally augmented with PDE-5 inhibitors or respective analogs, namely sildenafil, tadalafil, vardenafil, hydroxyhomosildenafil, thiosildenafil, and the newly identified adulterant thiomethiosildenafil. Most recently, Gilard and others (2015) analyzed a total of 150 food supplements advertised as being natural products for sexual performance enhancement and concluded that 92 of those samples (corresponding to 61%) were adulterated with PDE-5 inhibitors or its analogs. The authors also reported the presence of flibanserin, an experimental drug for the treatment of hypoactive sexual desire disorder in women, in 2 samples and steroidal hormones (testosterone and DHEA) in 5 samples. Besides PDE-5 inhibitor drugs and/or its analogs, detection of the anorexic drug sibutramine, the stimulant synephrine and the laxative phenolphthalein have also been detected in food supplements by using NMR (Vaysse and others 2010). Recently, the use of NMR spectroscopy in combination with LC-QTOF-MS also allowed detecting sibutramine, orlistat, sildenafil, fluoxetine and/or yohimbine in 21 weight-loss food supplements (Johansson and others 2014).

Other methods. The use of bioassays, such as the estrogen and androgen mammalian reporter gene assays (RGAs) and the yeast androgen bioassay, have been suggested as a general screening procedure to detect steroids in sports dietary supplements (Rijk and others 2009; Plotan and others 2012). These bioassays present

several advantages, such as high sensitivity and lower cost, compared to methods that require the use of advanced and expensive equipment, as well as high throughput as they screen samples for a large group of compounds presenting similar biological effects. However, they generally are comprised of lengthy analysis, they can give inconclusive results due to toxic effects generated by the extracts, and, above all, they are unable to identify steroid compound(s), thus requiring confirmation analyses by using techniques that allow for identification (Peters and others 2010; Becue and others 2011). In this regard, Peters and others (2010) proposed the use of a bioassay-guided fractionation based on a recombinant yeast androgen bioassay and further analysis by UHPLC/TOF-MS of the positive fractions to confirm and identify unknown androgens in food supplements. The authors also tested the possibility of directly analyzing the samples by UHPLC/TOF-MS without previous bioassay-guided fractionation, and they concluded that, although it allowed to tentatively identify androgens and their derivatives in samples of sport food supplements, the method was much more laborious since many compounds are identified and have to be screened as potential adulterants (Peters and others 2010). More recently, Aqai and others (2013) proposed a novel bioaffinity liquid chromatography-MS (BioMS) method for screening and identification of designer anabolic steroids in dietary supplements. The bioaffinity assay was developed for molecules binding to the recombinant human sex hormone-binding globulin (rhSHBG), with the possibility of using the same biopurified extract for subsequent compound identification using chip-ultra-performance-LC(NanoTile)-quadrupole-time-of-flight MS (chip-UPLC-Q-ToF-MS) with full scan accurate mass measurement. The authors suggested that this would be a powerful tool for early detection of emerging unknown designer steroids in food supplements, thus contributing to fight doping in sports (Aqai and others 2013).

Conclusions

Following the current legislation in EU countries and the U.S., dietary supplements (including PFS) are not subjected to any specific regulatory preapproval requirements or safety assessments prior to commercialization. This allows for unscrupulous manufacturers and distributors to deliberately adulterate supplements through the addition of pharmaceutical drugs or analogue substances (designer drugs, often not characterized for their efficacy or toxicity) in order to increase product effectiveness. However, consumers are not aware of such possibilities and cases of drug interactions and deleterious health effects can occur. Thus, with the growing consumption of these products, with special emphasis on PFS, and market globalization, there is also an increased need for more effective control by competent authorities in order to detect possible adulterations and, in such cases, take enforcement measures to safeguard public health. Therefore, the development of new and improved analytical methodologies for the detection and structural identification of adulterants from different pharmacological classes (including new/unknown analogs) is critically important to protect public health and ensure the quality of dietary supplements.

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Author Contributions

Tiago Rocha and Joana Amaral compiled the sources and wrote the manuscript; Beatriz Oliveira supervised the work.

Conflicts of Interest

The authors declare that they do not have any conflicts of interest.

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