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Multi-class determination of pesticides and mycotoxins in isoflavones supplements obtained from soy by liquid chromatography coupled to Orbitrap high resolution mass spectrometry

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ABSTRACT

A methodology has been developed to identify and quantify 257 toxic substances (including pesticides and mycotoxins) in diverse isoflavones supplements obtained from soy. Two different extraction procedures were compared, QuEChERS and "dilute and shoot". The best results were observed when the "dilute and shoot" methodology was applied using acetonitrile acidified with formic acid (1% v/v) as extraction solvent followed by a clean-up step with Florisil cartridges. Validation of the method was carried out evaluating trueness, repeatability and intermediate precision, obtaining recoveries between 70 and 120% with relative standard deviation (RSD) values lower than 20%. Limits of detection and quantification were below 5 and 10 μg kg^{-1} respectively. The validated methodology was applied to the analysis of real samples, finding pesticides such as flutolanil (12.2 μg kg^{-1}) and etofenprox (48.2 μg kg^{-1}). Regarding mycotoxins, aflatoxin B1 (8.2–17.1 μg kg^{-1}) and aflatoxin G2 (6.4 μg kg^{-1}) were detected.

1. Introduction

Today, lifestyle diseases have been increasing all over the world mainly because of inadequate nutrition. In fact, 40% of worldwide mortality has been attributed to nutrition-related factors in developing nations (Frost and Sullivan, 2007). For this reason, and because of the high costs of healthcare, consumers have been driven to consume dietary supplements and functional foods, also known as nutraceutical products. This market has emerged to provide health benefits to society, including prevention or treatment of diseases (Frost and Sullivan, 2007).

Nutraceutical is defined as "a term used to describe a medicinal or nutritional component that includes a food, plant or naturally occurring material, which may have been purified or concentrated, and that is used for the improvement of health, by preventing or treating a disease" (Lockwood, 2007). Many different products can be considered nutraceuticals, such as green tea, royal jelly or soy. Among them, soy-based nutraceutical products are very popular because of the high level of isoflavones (Higdon, 2009). They are considered polyphenolic compounds, which can be obtained from legumes, especially soybeans. Scientific studies have attributed different health benefits to them, such as lowering the cholesterol levels, cardiovascular diseases, osteoporosis prevention and a treatment for menopausal symptoms (Higdon, 2009). Nowadays, soy products can be found in many different presentations, including beans, meat, milk, protein and tofu. Additionally, supplements and infant formulas can be found in the market (Higdon, 2009), and these products could also be considered as nutraceuticals.

Keeping in mind that a nutraceutical product obtained from soy is a concentrated form of this product and that the soy grains can be exposed to different toxic substances involved in the agricultural practices, pesticides and/or mycotoxins could be detected in this type of samples. Thus, pesticides like glyphosate (400–8800 μg kg^{-1}) (Bohn et al., 2014; Gonçalves de Abreu, Rizzo da Matta, & Montagner, 2008), chlorpyrifos (10–102 μg kg^{-1}) (Marchis, Ferro, Brizio, Squadrone, & Abete, 2012) and pirimiphos methyl (1.1 μg kg^{-1}) (Marchis et al., 2012) have already been detected in soy-related

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products, while mycotoxins as deoxynivalenol (11–260 μg kg^{-1}) (Schollenberger et al., 2007; Warth et al., 2012; Zhao, Wang, Zou, & Zhao, 2013), zearalenone (2–214 μg kg^{-1}) (Schollenberger et al., 2007; Warth et al., 2012), aflatoxins (3.1–19.1 μg kg^{-1}) (Warth et al., 2012), ochratoxin A (5.7–13.8 μg kg^{-1}) (Warth et al., 2012) and fumonisins (28.2–73.8 μg kg^{-1}) (Warth et al., 2012) have also been found. It is important to mention that none of these articles report the detection of toxic substances in nutraceutical products but only in the raw material. However, there are some articles that determine pesticides in dietary supplements obtained from *Scutellaria baicalensis* and *Acacia catechu* (Lee, Zahn, Trinh, Brooke, & Ma, 2008), or in ginseng and dandelion (Kowalski, Misselwitz, Thomas, & Cochran, 2011; Mastovska & Wylie, 2012), while mycotoxins have been detected in soy isoflavones (Di Mavungu et al., 2009) and in supplements from green coffee beans (Vaclavik, Vaclavikova, Begley, Krynitsky, & Rader, 2013), ginger (Whitaker, Trucksess, Weaver, & Slate, 2009), herbs (Vaclavik, Krynitsky, & Rader, 2014), wheat and oat (Vidal, Marín, Ramos, Cano, & Sanchis, 2013). These two types of toxic substances can be found in this kind of products; therefore methodologies that allow a multi-class analysis are necessary in order to ensure food safety in nutraceutical products.

There are some regulations in the European Union (EU) defining maximum residue limits (MRLs) for several types of contaminants. Thus, the Regulation EC 396/2005 (Council regulation no 396/2005) assigns MRLs for pesticides in every food and feed, while the Regulation EC 1881/2006 (Council regulation no 1881/2006) establishes MRLs for mycotoxins in foodstuffs. Nevertheless, these MRLs are set in the raw material and they are not applied to nutraceutical products. Keeping in mind the high consumption of this type of products, legislation involving MRLs for toxic substances in nutraceutical products would be discussed in the near future. Therefore, analytical methods that can identify and quantify these toxic substances in this kind of products are necessary.

Concerning extraction procedures of pesticide residues from soy products, different methodologies as QuEChERS (acronym of quick, easy, cheap, effective, rugged and safe) (Li et al., 2013a; Marchis et al., 2012; Wang, Cheung, & Chow, 2013), "dilute and shoot" (Gonçalves de Abreu et al., 2008; Prasad, Upadhyay, & Kumar, 2013; Li, Wang, Yang, Fu, & Dai, 2013b), solid phase extraction (SPE) (Hernández, Rodríguez, García, & Cifuentes, 2005) and matrix solid phase dispersion (MSPD) (Maldaner, Santana, & Jardim, 2008) have been applied. QuEChERS has been proposed as a reference methodology for pesticide extraction because of its speed, cheapness and robustness. Regarding mycotoxins, solid–liquid extraction (SLE) (Schollenberger et al., 2007; Warth et al., 2012; Zhao et al., 2013) was mainly used to separate these substances from soy products.

Gas chromatography (GC) (Bohn et al., 2014; Gonçalves de Abreu et al., 2008; Li et al., 2013b; Marchis et al., 2012) and liquid chromatography (LC) (Li et al., 2013a; Maldaner et al., 2008; Prasad et al., 2013; Wang et al., 2013) were mainly used to determine pesticides in soy related products, as well as mycotoxins (Schollenberger et al., 2007; Warth et al., 2012; Zhao et al., 2013). For the detection of pesticides, different detectors were used, including electron capture detector (ECD) (Bohn et al., 2014), diode array detector (DAD) (Hernández et al., 2005; Maldaner et al., 2008) ultraviolet detection (UV) (Prasad et al., 2013), single quadrupole (Q) (Gonçalves de Abreu et al., 2008; Marchis et al., 2012; Li et al., 2013b) triple quadrupole (QqQ) (Li et al., 2013a) and Orbitrap (Wang et al., 2013; Farré, Picó, & Barceló, 2014). In the case of mycotoxins, Q (Schollenberger et al., 2007; Zhao et al. 2013), ion trap (IT) (Warth et al., 2012) and QqQ (Di Mavungu et al., 2009) were applied. The number of compounds determined by these techniques ranged between 3 (Bohn et al., 2014) and 151 (Wang et al., 2013). However, the use of high resolution mass spectrometers, like Orbitrap, allows the determination and quantification of a large number of compounds from different classes and families, bearing in mind that QqQ can present some limitations (sensitivity, running time, scan speed) when comprehensive analysis must be performed. Moreover, high resolving power, accurate mass measurement, high full scan sensitivity and retrospective analysis (Gómez, Ferrer, Malato, Agüera, & Fernández, 2013) are some of the advantages that high resolution mass spectrometry can offer.

In order to provide a comprehensive analysis of a large number of multi-class toxics in supplement products, the use of high resolution mass spectrometry is needed. Therefore, a methodology has been developed in this study to identify more than 250 toxic substances, including pesticides and mycotoxins in isoflavones supplements obtained from soy, evaluating different extraction procedures and ultra high performance liquid chromatography (UHPLC) coupled to Exactive-Orbitrap.

2. Material and methods

2.1. Reagents and chemicals

Pesticide standards (purity higher than 99%) were purchased from Dr. Ehrenstofer (Augsburg, Germany) and Riedel-de-Haën (Seelze-Hannover, Germany). Mycotoxins standards were purchased from LGC Standards (Wesel, Germany). Individual stock standard solutions of 200 mg L^{-1} (pesticides and mycotoxins) were prepared by exact weighing of powder or liquid and dissolved in 50 mL of HPLC-grade acetone (Sigma–Aldrich, Madrid, Spain) for pesticide solutions or 50 mL of acetonitrile LC-MS grade (Scharlab, Barcelona, Spain) for mycotoxins. Multi-compound working standard solutions (344 compounds) for pesticides and mycotoxins (2 mg L^{-1} concentration of each compound) were prepared by appropriate dilution of the stock solutions with acetone or acetonitrile respectively, and stored under refrigeration at 4 °C. Water LC-MS (Scharlab, Barcelona, Spain) was used throughout for the preparation of buffers and other solutions. Formic acid (Optima LC-MS) was obtained from Fisher Scientific (Geel, Belgium). Primary secondary amine (PSA), graphitized black carbon (GBC) and Florisil cartridges were obtained from Scharlab (Barcelona, Spain). Ammonium formate was obtained from Fluka (St. Gallen, Switzerland). Anhydrous magnesium sulfate, sodium acetate, zirconium oxide (Z-Sep^{+}) and methanol LC-MS were obtained from Sigma–Aldrich. Bondesil-C_{18} was obtained from Agilent Technologies (Santa Clara, CA, USA).

For an accurate mass calibration, a mixture of acetic acid, caffeine, Met–Arg–Phe–Ala–acetate salt and Ultramark 1621 (ProteoMass LTQ/FT-hybrid ESI positive), and a mixture of acetic acid, sodium dodecyl sulfate, taurocholic acid sodium salt hydrat and Ultramark 1621 (fluorinated phosphazines) (Proteo-Mass LTQ/FT-Hybrid ESI negative) from Thermo-Fisher (Waltham, MA, USA) were employed in the Orbitrap analyzer.

2.2. Instrument and apparatus

A WX vortex from Velp Scientifica (Usmate, Italy) was used to homogenize the samples. Centrifugation was carried out in a Consul21 centrifuge from Orto Alresa (Madrid, Spain). A Reax 2 overhead shaker from Heidolph (Schwabach, Germany) was used for end-over-end agitation.

A Transcend 600 LC (Thermo Scientific Transcend™, Thermo Fisher Scientific, San Jose, CA, USA) coupled to a single stage Orbitrap mass spectrometer (Exactive™, Thermo Fisher Scientific, Bremen, Germany) operating with a heated electrospray interface (HESI-II, Thermo Fisher Scientific, San Jose, CA, USA), in positive (ESI+) and negative (ESI−) ionization modes was used for chromatographic analysis. The column used for the compounds