



Analytical Methods

Multi-class methodology to determine pesticides and mycotoxins in green tea and royal jelly supplements by liquid chromatography coupled to Orbitrap high resolution mass spectrometry



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ABSTRACT

A multi-class methodology was developed to determine pesticides and mycotoxins in food supplements. The extraction was performed using acetonitrile acidified with formic acid (1%, v/v). Different clean-up sorbents were tested, and the best results were obtained using C₁₈ and zirconium oxide for green tea and royal jelly, respectively. The compounds were determined using ultra high performance liquid chromatography (UHPLC) coupled to Exactive–Orbitrap high resolution mass spectrometry (HRMS). The recovery rates obtained were between 70% and 120% for most of the compounds studied with a relative standard deviation <25%, at three different concentration levels. The calculated limits of quantification (LOQ) were <10 µg/kg. The method was applied to green tea (10) and royal jelly (8) samples. Nine (eight of green tea and one of royal jelly) samples were found to be positive for pesticides at concentrations ranging from 10.6 (cinosulfuron) to 47.9 µg/kg (paclobutrazol). The aflatoxin B1 (5.4 µg/kg) was also found in one of the green tea samples.

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1. Introduction

Green tea is one of the most consumed beverages in the world, used to prevent and treat some diseases, contributing to weight loss, lowering cholesterol levels, mental alertness, and due to the presence of polyphenols at high concentrations (The World's Healthiest Foods, 2014), it is also taken because of its antioxidant properties. On the other hand, royal jelly is one of the most important beehive product together with honey and propolis (Karazafiris, Menkissoglu, & Thrasyvoulou, 2008) and it has a range of functionalities, such as antioxidant, anti-inflammatory, antiviral, anti-ulcerous and antibacterial (Morita et al., 2012; Viuda, Ruiz, Fernández, & Pérez, 2008). These products can be found in different presentation formats, such as infusions, extracts and even capsules or tablets known as nutraceutical products.

This type of products is becoming of considerable interest to society and they have been acquiring importance over the last decade because of their nutritional potential and therapeutic effects. Nutraceutical can be defined as "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). Such products may vary from isolated nutrients, dietary supplements and specific diets to genetically engineered foods, herbal products, and processed foods such as cereals, soups and beverages (News-Medical, 2014). The Transparency Market Research in the United States of America (USA) indicated that, in 2011, these products generated worldwide sales of \$142.1 billion, and they are estimated to grow by 6.3% by 2017, reaching \$204.8 billion (Nutraceuticals World, 2014). This market success is based on the rising health concerns, the improving economic conditions and the growth of key demographics among consumers (Nutraceuticals World, 2014).

A nutraceutical product may be contaminated with toxic substances from the raw material, such as pesticides or mycotoxins. Previous studies have found pesticides in green tea samples, such as cypermethrin (26–325 µg/kg) (Cajka et al., 2012), acetamiprid (found in 11 samples) (Zhang et al., 2010) and carbendazim (56.6% of the samples) (Chen, Cao, & Liu, 2011). In the case of royal jelly, the most detected pesticides were organochlorines, organophosphorus and carbamates (Bogdanov, 2006). Regarding to mycotoxins, previous studies have been focused on tea obtained from *Camellia sinensis*-Kuntze. The mycotoxins most frequently detected in these studies were fumonisins (Monbaliu, Wu, Zhang, Van Peteghem, & De Saeger, 2010; Storari, Dennert, Bigler,

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Gessler, & Brogini, 2012) ochratoxin A (Storari et al., 2012; Haas et al., 2013) and T-2 toxin (Tan, Kuang, Zhao, Chen, & Wu, 2011). None of the articles determined mycotoxins in royal jelly, although two of the studies reported different mycotoxins found in honey and bee pollen respectively, such as citrinin (Kačaniová et al., 2011) and nivalenol (Rodríguez, Font, Mañes, & Berrada, 2013). However, it is important to highlight that the aforementioned studies determined these substances in the raw material but not in the nutraceutical product. There are only three articles that evaluated the presence of pesticides in dietary supplements from *Scutellaria baicalensis*-Giorgi and *Acacia catechu*-Köhler (Jeong, Zahn, Trinh, Brooke, & Ma, 2008), where 41 pesticides were analysed by gas chromatography coupled to simple quadrupole mass spectrometry (GC-Q-MS). Furthermore, up to 146 pesticides were determined in ginseng and dandelion using GC coupled to triple quadrupole mass spectrometry (QqQ-MS/MS) (Mastovska & Wylie, 2012) and time of flight (TOF-MS) (Kowalski, Misselwitz, Thomas, & Cochran, 2011) respectively. On the other hand, mycotoxins were only determined in dietary supplement made of green coffee beans (Vaclavik, Vaclavikova, Begley, Krynitsky, & Rader, 2013), where 34 mycotoxins were investigated with ultra high performance liquid chromatography (UHPLC) coupled to QqQ-MS/MS. These studies revealed the presence of some toxic substances like endosulfan (Jeong et al., 2008) and ochratoxin A (Vaclavik et al., 2013) in the studied samples.

The Regulation EC 396/2005 (Council Regulation, 2005) and EC 1881/2006 (Council Regulation, 2006) set maximum residue limits (MRLs) for pesticides in every food and feed as well as mycotoxins in foodstuffs, respectively. It is important to mention that the MRLs cited in these regulations only concern the raw material and not the nutraceutical presentations. There are only two European regulations that involve nutraceuticals, Directive 46/2002 (Council Directive, 2002), which covers food supplements, and Directive 27/2004 (Council Directive, 2004), which includes herbal medicinal products. However, these directives do not consider the potential presence of toxic substances, such as pesticides and mycotoxins. Therefore, analytical methods that ensure reliable determination of these substances in nutraceuticals are necessary.

Concerning the sample treatment, the QuEChERS (acronym of quick, easy, cheap, effective, rugged and safe) (Cajka et al., 2012; Chen et al., 2011; Mastovska and Wylie, 2012; Kowalski et al., 2011; Vaclavik et al., 2013) procedure is the most used methodology for the extraction of pesticides and mycotoxins from green tea, using an additional clean-up step based on dispersive solid phase extraction (d-SPE) (Cunha, Lehotay, Mastovska, Fernandes, & Oliveira, 2007). The generic extraction procedure named “dilute and shoot” is also very effective because it has shown suitable performance when it is applied to the analysis of several matrices for extracting a large number of pesticides simultaneously (Misselwitz, Lupo, Kowalski, Lake, & Cochran, 2012). Also, a SPE procedure was also tested by Karazafiris et al. (2008) for the extraction of pesticides from royal jelly.

For the separation and detection of pesticides and mycotoxins, GC (Cajka et al., 2012; Jeong et al., 2008; Mastovska & Wylie, 2012; Kowalski et al., 2011) and liquid chromatography (LC) (Chen et al., 2011; Haas et al., 2013; Monbaliu et al., 2010; Storari et al., 2012; Tan et al., 2011; Vaclavik et al., 2013; Zhang et al., 2010) were widely used. For the detection of these toxic substances, QqQ analyser (Cajka et al., 2012; Chen et al., 2011; Jeong et al., 2008; Monbaliu et al., 2010; Storari et al., 2012; Tan et al., 2011; Zhang et al., 2010; Mastovska & Wylie, 2012; Vaclavik et al., 2013) is commonly applied, detecting from 2 (Tan et al., 2011) to 236 (Chen et al., 2011) compounds.

Nowadays, simultaneous trace analysis of hundreds of compounds from different classes is required, preferably in one single run, but the analyser most commonly used (QqQ) has certain

limitations when it is applied to the determination of a large number of compounds (Gómez, Ferrer, Malato, Agüera, & Fernández, 2013). High resolution mass spectrometry (HRMS) analysers offer a powerful alternative. For multi-class analysis, the use of these systems, such as time of flight (TOF) and Orbitrap, has several advantages, such as full-scan operation mode, which can provide high specificity without limiting the number of detected compounds. For example, Gómez-Pérez, Plaza-Bolaños, Romero-González, Martínez-Vidal, and Garrido-Frenich (2012) applied Orbitrap mass spectrometry (Orbitrap-MS) to detect more than 350 compounds including pesticides and veterinary drugs in honey, and Thurman, Ferrer, Malato, and Fernández-Alba (2006) identified 350 pesticides in apple and orange using QTOF. Vaclavik, Krynitsky, and Rader (2014) also proposed the use of Orbitrap-MS to determine multiple pharmaceuticals, plant toxins and other secondary metabolites in herbal dietary supplements, determining approximately 100 compounds. Therefore, the use of HRMS is recommended to determine a large number of compounds in complex matrices, such as nutraceuticals.

Bearing in mind the advantages provided by HRMS in terms of sensitivity, number of compounds determined and precision, the objective of this work was the development of a multi-class methodology to determine and quantify more than 260 compounds (including pesticides and mycotoxins) in nutraceutical products (green tea and royal jelly). For that purpose, a “dilute and shoot” procedure was tested for extraction, and LC-Orbitrap-MS was used to separate and quantify the target compounds, increasing sample throughput.

2. Materials and methods

2.1. Reagents and chemicals

Pesticide reference standards (purity higher than 99%) were purchased from Dr. Ehrenstofer (Augsburg, Germany) and Riedel-de-Haën (Seelze-Hannover, Germany). Mycotoxin reference standards were purchased from LGC Standards (Wesel, Germany). Individual stock solutions of 200 mg/L were prepared by weighing out the powder or liquid, and dissolving them in 50 mL of HPLC-grade acetone (Sigma-Aldrich, Madrid, Spain) for the pesticides or 50 mL of acetonitrile LC-MS (Scharlab, Barcelona, Spain) for the mycotoxins. Multicomponent working standard solutions for pesticides and mycotoxins (2 mg/L concentration of each compound) were prepared by appropriate dilution of the stock solutions with acetone or acetonitrile, respectively, and stored at 4 °C. Anhydrous magnesium sulphate, sodium acetate, Z-Sep⁺ (zirconium oxide) and methanol LC-MS were obtained from Sigma-Aldrich. 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). Bondesil-C₁₈ was obtained from Agilent Technologies (Santa Clara, CA, USA). Ammonium formate was obtained from Fluka (St. Gallen, Switzerland). For accurate mass calibration of the Exactive-Orbitrap instrument, a mixture of caffeine, Met-Arg-Phe-Ala acetate salt (MRFA) and Ultramark 1621 (Proteo Mass LTQ/FT-Hybrid ESI positive and negative mode calibration mix) from Sigma-Aldrich was used in the Orbitrap analyser. All solvents were pesticide residue grade. Water LC-MS (Scharlab) was used throughout for the preparation of buffers and other solutions.

2.2. Instrument and apparatus

Centrifugation was carried out in a Consul21 centrifuge from Orto Alresa (Madrid, Spain). A WX vortex from Velp Scientifica

(Usmate, Italy) was used to homogenize the samples. A Reax 2 overhead shaker from Heidolph (Schwabach, Germany) was used for end-over-end agitation.

Chromatographic analysis was carried out using 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 ionization mode (ESI−). A mixture of an aqueous solution of ammonium formate 4 mM and formic acid 0.01% (v/v) (eluent A) and methanol with ammonium formate 4 mM and formic acid 0.01% (v/v) (eluent B) was used as mobile phase at a constant flow rate of 0.3 mL/min. The column used for the separation of the compounds was a Hypersil GOLD aQ column (100 × 2.1 mm, 1.9 μm particle size) from Thermo Scientific (San Jose, CA, USA). Column temperature was set at 30 °C and the injection volume was 10 μL.

Data was processed with the Xcalibur™ program version 2.2.1 from Thermo Fisher Scientific (Les Ulis, France) with Qual and Quanbrowser. Genesis peak detection was applied. The ToxID™ program 2.1.1, also from Thermo Scientific, was used for screening and the LCQuan™ 2.6 software from the same company was used for quantification of the target compounds.

2.3. Samples

Green tea (*C. sinensis*) tablets and royal jelly (liquid presentation) were obtained from a local store. After checking that no toxic substances were detected, they were used as blanks, for preparing fortified samples for recovery assays and matrix-matched standards for calibration purposes. For the analysis of real samples, 10 different green tea products (2 capsules and 8 tablets presentations) and 8 different royal jelly products (2 capsules and 6 liquid presentations) were also obtained from local supermarkets.

Different green tea compositions and formats were presented in the studied samples, including powdered green tea leaves and extracts. They were used by consumers to help weight loss in special diets and because of their antioxidants properties. The royal jelly samples were mostly claimed to be pure royal jelly without any other component, except two particular samples that were formulated as a mixture of royal jelly and pollen. They are recommended as an energetic and vitamin supplements.

For green tea, in order to eliminate variations between single tablets or capsules, the samples were prepared by homogenizing the contents of twenty capsules or tablets with a coffee grinder and stored with a desiccant at 5 °C. Royal jelly samples were also stored at this temperature before the experiments.

2.4. Extraction procedure

2.4.1. Procedure I: Acetate QuEChERS

Acetate QuEChERS (Lehotay et al., 2007) was applied as follows: (1) 2 g of sample were weighed in a 50 mL centrifuge tube; (2) the sample was fortified with 50 μL (from a multicomponent standard solution of 2 mg/L) to achieve a final concentration of 50 μg/kg of the target compounds and let stand it for 30 min; (3) 8 mL of water were added to the mixture, which was shaken vigorously for 30 s and let stand it for 15 min; (4) 10 mL of a mixture of acetonitrile and acetic acid at 1% (v/v) were added to the mixture and shaken vigorously for 1 min; (5) 4 g of magnesium sulphate and 1 g of sodium acetate were added to the mixture and shaken vigorously for 1 min, and (6) the mixture was centrifuged at 3700 rpm (2755g) during 10 min and 1 mL of the organic phase was transferred to a vial for chromatographic analysis.

2.4.2. Procedure II: Dilute and shoot

A generic “dilute and shoot” method tested in a previous work (Gómez-Pérez et al., 2012) was applied as follows: (1) 2.5 g of sample were weighed in a 50 mL centrifuge tube; (2) the sample was fortified with 63 μL (from a multicomponent standard solution of 2 mg/L) to achieve a final concentration of 50 μg/kg; (3) 2.5 mL of water were added to the mixture and it was shaken vigorously for 30 s; (4) 7.5 mL of a mixture of acetonitrile and formic acid at 1% (v/v) were added to the mixture and the tube was shaken end-over-end for 2 h, and (5) the mixture was centrifuged at 3700 rpm (2755g) for 10 min and 1 mL of the extract was transferred to a vial for chromatographic analysis.

2.4.3. Procedure III: Dilute and shoot + clean up

In order to improve recoveries provided by Procedure II, different sorbents (PSA, GBC, C₁₈, Florisil and Z-Sep⁺) were tested during the clean up step.

PSA, GBC, C₁₈ and Z-Sep⁺: Applying Procedure II, after centrifugation, step 5, 1.5 mL of the extract was transferred to an Eppendorf micro tube containing 200 mg of magnesium sulphate and 50 mg of PSA, 100 mg of GBC, 100 mg of C₁₈ or 100 mg of Z-Sep⁺, respectively and shake it by vortex for 1 min. Then, the tube was centrifuged at 3700 rpm (1438g) for 10 min and 1 mL of the organic phase was transferred to a vial for chromatographic analysis.

Florisil: Applying Procedure II, after centrifugation, 2 mL of the extract was slowly transferred through a Florisil cartridge and then 1 mL of the extract was used for chromatographic analysis.

2.5. UHPLC–Orbitrap-MS

A method tested in a previous work (Gómez-Pérez et al., 2012) for pesticides analysis was used. For the analysis of the compounds, a gradient was applied as follows: the analysis started with 95% of eluent A; after 1 min, this percentage was linearly decreased to 0% in 7 min; this composition was held during 4 min and increased again up to 95% in 0.5 min, followed by a re-equilibration time of 1.5 min. The total running time was 14 min.

The Orbitrap mass spectrometer parameters were: spray voltage, 4 kV; sheath gas (N₂, >95%), 35 (dimensionless); auxiliary gas (N₂, >95%), 10 (dimensionless); skimmer voltage, 18 V (−18 V in ESI−); capillary voltage, 35 V (−35 V in ESI−); tube lens voltage, 95 V (−95 V in ESI−); heater temperature, 305 °C; and capillary temperature, 300 °C. Mass range in the full scan experiments was set at *m/z* 100–1000. All the analyses were performed without lock mass. Mass accuracy was carefully monitored as follows: checked every day with multi-compound standard solution; evaluated (once a week) and calibrated when necessary (at least every two weeks) with mass accuracy standards. The automatic gain control (AGC) was set at a target value of 1 × 10⁶. The mass spectra were acquired using four alternating acquisition functions: (1) full MS, ESI+, without fragmentation (the higher collisional dissociation (HCD) collision cell was switched off), mass resolving power = 25,000 FWHM; scan time = 0.25 s; (2) full MS, ESI− using the aforementioned settings; (3) all ion fragmentation (AIF), ESI+, (HCD on, and collision energy = 30 eV, mass resolving power = 10,000 FWHM; scan time = 0.10 s; and (4) AIF, ESI− using the settings explained for (3). Considering the scan time of the four acquisition functions, and the polarity switching (approx. 0.27 s) an overall scan rate of 0.56 Hz was obtained.

2.6. Validation procedure

The methodology was validated in order to ensure that the obtained results were reliable. The parameters were chosen keeping in mind international guidelines (SANCO guideline, 2013). For

linearity, matrix matched standard calibration was used by the analysis of nutraceutical blank samples. These samples were spiked after the extraction procedure at five different concentration levels (2, 5, 10, 25 and 50 $\mu\text{g/L}$). For trueness, recovery studies were performed by spiking blank samples with the corresponding volume of the multi-compound working standard solutions (pesticides and mycotoxins). Three concentrations were evaluated (10, 50 and 100 $\mu\text{g/kg}$) by spiking five blank samples at each concentration level.

Precision was evaluated studying intraday (repeatability) and interday precision, and they were expressed as relative standard deviation (RSD). For intraday precision, results were obtained from the injection of five spiked samples at concentration levels of 10, 50 and 100 $\mu\text{g/kg}$, while for interday precision, three spiked samples, at the same aforementioned concentration levels, were analysed in five different days.

Finally, limits of detection (LODs) and quantification (LOQs) were determined fortifying blank samples at lower concentration levels (0.5, 1, 2, 5 and 10 $\mu\text{g/kg}$). To specify these lower limits, the LOD was defined as the minimum concentration at which the molecular ion has been detected (mass error < 5 ppm), and the LOQ as the minimum concentration where both the molecular ion and at least one fragment ion have been detected (mass error < 5 ppm) and the response was linear.

3. Results and discussion

3.1. UHPLC–Orbitrap-MS

Different families of pesticides were analysed by the Orbitrap system, including organophosphates, pyrethroids, carboxamides, carbamates, pyrroles and pyridines among others. For mycotoxins, aflatoxin B1, aflatoxin B2, aflatoxin G1, aflatoxin G2, deoxynivalenol, fumonisin B1, fumonisin B2, HT-2 toxin, ochratoxin A, T-2 toxin and zearalenone were included in the method because of their occurrence in tea (Haas et al., 2013; Monbaliu et al., 2010; Storari et al., 2012; Tan et al., 2011) and in bee products (Kačániová et al., 2011; Rodríguez et al., 2013). An in-house database previously developed (Gómez-Pérez et al., 2012) was updated, including these new pesticides and mycotoxins. For that, additional data were collected for each compound, including retention time (RT), ionization mode, characteristic ions, possible adducts as H^+ , NH_4^+ and Na^+ , and fragments. Aliquots of each individual standard solution for pesticides and mycotoxins were injected into the Orbitrap system following the conditions defined by Romero-González, Aguilera-Luiz, Plaza-Bolaños, Garrido-Frenich, and Martínez-Vidal (2011). Mass spectra acquisition was performed in positive and negative mode, with and without fragmentation in the collision cell using higher energy collisional dissociation (HCD). Fragments determination was achieved processing the data obtained from Orbitrap with the Xcalibur™ Qual Browser program. Firstly, the chemical formula for each compound was processed with the software to obtain the theoretical mass and molecular ion. Then, characteristic fragments were obtained using all ion fragmentation (AIF). Bearing in mind that no precursor ion was available using Exactive-Orbitrap, the identification of the fragments was performed by comparing the intensity of the ions in the MS and AIF spectra, taking into account that the fragments from the molecular ion should have a higher intensity after the application of AIF. An example of this can be seen in Fig. 1. In Fig. 1a, the chromatogram obtained for the molecular ion (experimental m/z 313.0704) and the corresponding mass spectra for aflatoxin B1 are presented. After the fragmentation, the spectrum was collected, obtaining different fragment ions presented in Fig. 1b. In this case, the selected fragment ion was m/z 285.0751, observing in

the chromatogram (Fig. 1b) a similar retention time and peak shape than the molecular ion, indicating that this is a fragment corresponding to aflatoxin B1.

Retention time windows (RTWs) were proposed to detect the target compounds. They were defined as the RT average plus/minus three times the standard deviation (SD). For that purpose, five spiked samples at 50 $\mu\text{g/L}$ were injected and analysed by the Exactive-Orbitrap system. Mass accuracy of the characteristic ion was also studied, using the same spiked samples. The results are shown in the Electronic Supplementary Material (ESM, Table S1). As it can be seen, the RTWs are between 1.53 (daminozide) and 12.91 min (fumonisin B1) for all the studied compounds. The mass accuracy was always below 3 ppm for all the compounds (except triallate, 5.52 ppm). These parameters were checked prior to every experiment, just before the sample injection. About the monitored ions, most of the spectra showed the $[\text{M}+\text{H}]^+$ adduct and fewer spectra presented the $[\text{M}+\text{NH}_4]^+$ one, such as achrinathrin and vinclozolin, or $[\text{M}+\text{Na}]^+$ adducts (atrazine, azinphos methyl and benfluralin) (see Table S1). Positive ionization was applied for most of the studied compounds, although some of them were more sensitive using negative ionization, as bentazone and teflubenzuron (see Table S1). Moreover, information regarding the fragments selected for every single compound is presented in Table S1.

3.2. Optimization of the extraction procedure

Initially, acetate QuEChERS (Lehotay et al., 2007) was applied using Procedure I. Three replicates were evaluated at 50 $\mu\text{g/kg}$. Suitable recoveries (between 70% and 120%) were only obtained for 18% of the compounds in green tea and 13% in royal jelly (see Fig. 2a). Therefore, a different extraction procedure was needed. A generic “dilute and shoot” method proposed in a previous study (Gómez-Pérez et al., 2012) was tested (Procedure II), and suitable recoveries (from 70% to 120%) were obtained for 46% of the compounds in green tea and 50% in royal jelly (see Figure 2a). This indicates that, for these particular matrices, the tested “dilute and shoot” procedure provided better results than the QuEChERS alternative. Therefore, the “dilute and shoot” methodology was used for further experiments.

During preliminary experiments, a strong matrix effect was observed. Therefore, and in order to minimize it, a clean-up step was added to the “dilute and shoot” methodology and different sorbents were tested using the Procedure III. For that, PSA, GBC, Florisil, C_{18} and Z-Sep⁺ were evaluated as clean-up sorbents for both matrices (three replicates at 50 $\mu\text{g/kg}$) and the results are shown in Fig. 2b. Better results were obtained when C_{18} was used for green tea and Z-Sep⁺ for royal jelly. When this clean-up step was used, 72% and 70% of the target compounds evidenced suitable recoveries (between 70% and 120%) in green tea and royal jelly, respectively. In the case of green tea, when other clean-up sorbents were tested, the compounds with suitable recoveries (70–120%) ranged from 63% to 69%, whereas for royal jelly, they ranged from 49% to 64%. These results indicate the relevance of the clean-up steps when this kind of matrices are analysed, observing that the extracted compounds increased from 46% to 72% (green tea) and from 50% to 70% (royal jelly) when this clean-up step was included in the method. However, it was observed that depending on the type of matrix, a different sorbent is needed, and it is difficult to find a sorbent that can efficiently work for both matrices. Thus, when C_{18} and Z-Sep⁺ were compared in both matrices, it can be noted that Z-Sep⁺ only extracted 63% of the compounds with good recoveries from green tea and C_{18} only extracted 60% from royal jelly. Therefore, a combination of two sorbents is needed to obtain good recoveries in both matrices. This can be explained because Z-Sep⁺ provided good results removing phospholipids and carboxylic acids and it is a mixture of two sorbents (C_{18} and silica)

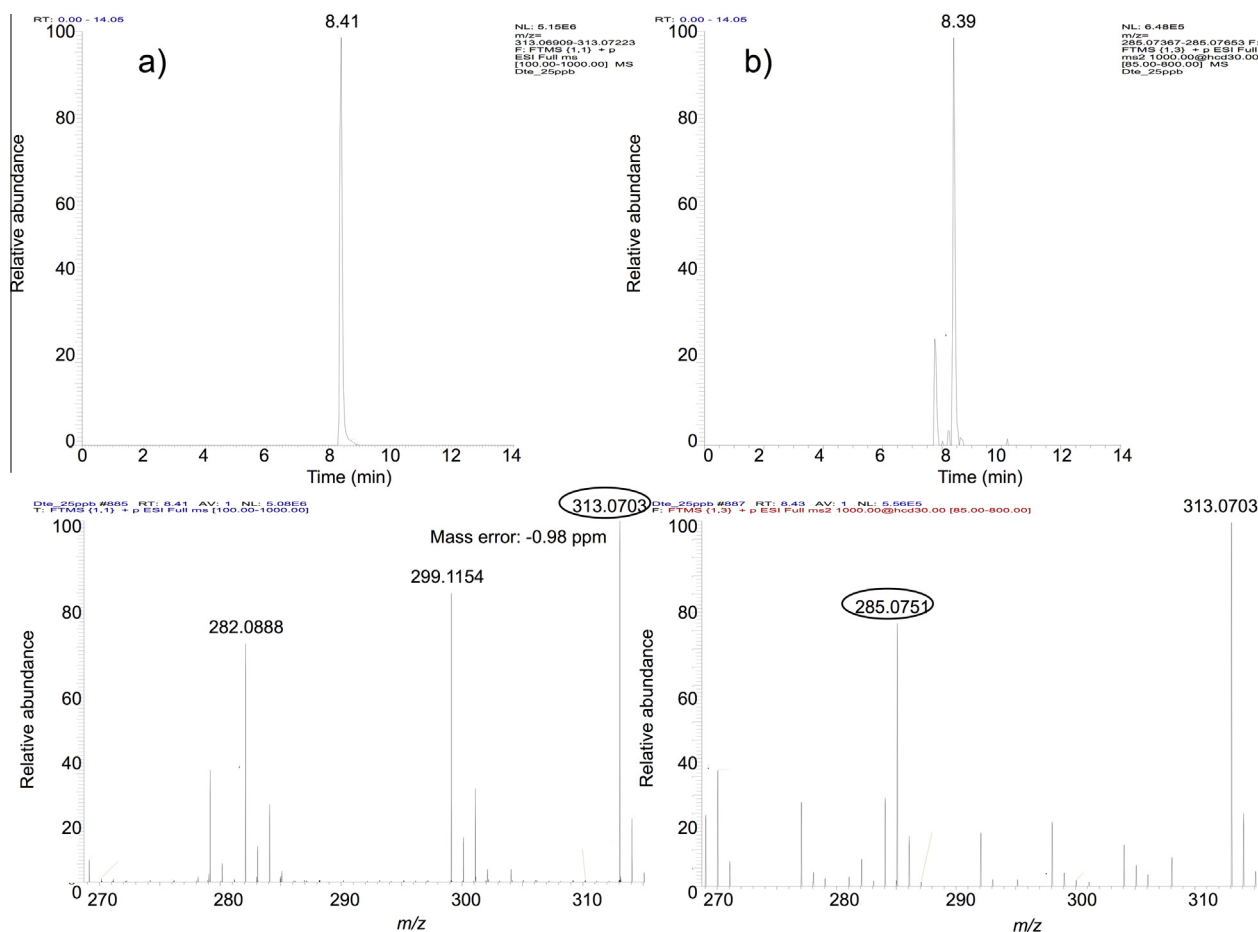


Fig. 1. Characterisation of aflatoxin B1 showing the: (a) chromatogram and mass spectra for the molecular ion (m/z 313.0704), and (b) the chromatogram and mass spectra for the ions after fragmentation (m/z 285.0751).

coated with zirconium dioxide. On the other hand, C_{18} is more effective removing pigments (Lozano et al., 2014). Therefore, for the validation procedure, a clean-up step was included using C_{18} for green tea and Z-Sep⁺ for royal jelly.

Finally, extraction time was tested to improve the recoveries obtained using the Procedure III. For this experiment, only the sorbents that provided better results (C_{18} and Z-Sep⁺ for green tea and royal jelly, respectively) were tested. The studied extraction times were 30 min, 1 h, 2 h and 4 h for each matrix (three replicates at 50 $\mu\text{g}/\text{kg}$) and Fig. 2c shows the obtained results. It can be seen that an extraction time of 2 h provided better results for the two matrices. In the case of green tea, suitable recoveries were obtained for 81% of the compounds, while for royal jelly, 80% of the compounds provided recoveries between 70% and 120%. Therefore, an extraction time of 2 h was used for further experiments.

3.3. Analytical method validation

The optimised methodology was validated in order to verify its applicability to the routine analysis of samples and to ensure the reliability of the results. To perform this study, only the compounds with suitable recoveries (272 compounds) were studied, including those with recoveries between 60% and 70%.

Firstly, matrix effect was evaluated for all the studied compounds using the ratio between the slope obtained for the calibration curves prepared uniquely with standards dissolved in solvent and the slope from the matrix-matched calibration curves. If

matrix effect is not presented, the ratio between slopes should be between 0.8 and 1.2. These experiment results are shown in Fig. 3, and it can be seen that for green tea, only 4% of the compounds did not present matrix effect (81% of the compounds provided values below 0.5% and 11% between 0.5 and 0.8). These values can be attributed to a strong matrix suppression effect. Also, 2% of the compounds provided values above 1.5% and 1% between 1.2 and 1.5. In the case of royal jelly, only 13% of the compounds did not present matrix effect, while 65% provided values below 0.5, and 20% between 0.5 and 0.8, evidencing matrix suppression. Moreover, 1% of the compounds obtained values above 1.5% and 1% between 1.2 and 1.5, presenting matrix enhancement. It can be indicated that despite a clean-up step was included, matrix effect was still high for most of the measured compounds.

Therefore, in order to avoid the mentioned matrix effects, matrix-matched calibration standards were used with concentration levels of 2, 5, 10, 25 and 50 $\mu\text{g}/\text{L}$. Linearity was evaluated by least-squares regression of peak area versus concentration of the calibration standards. All the obtained determination coefficients (R^2) were higher than 0.98 for the target compounds in both matrices, with deviation of each individual level from the calibration curve $\leq 20\%$.

Different parameters were measured in order to validate the method. These were trueness, repeatability, intermediate precision and LODs and LOQs following the validation procedure mentioned in Section 2.6. A summary of the results can be seen in Table 1 for both matrices. For more specific information, Tables S2 and S3 can be consulted for green tea and royal jelly, respectively.

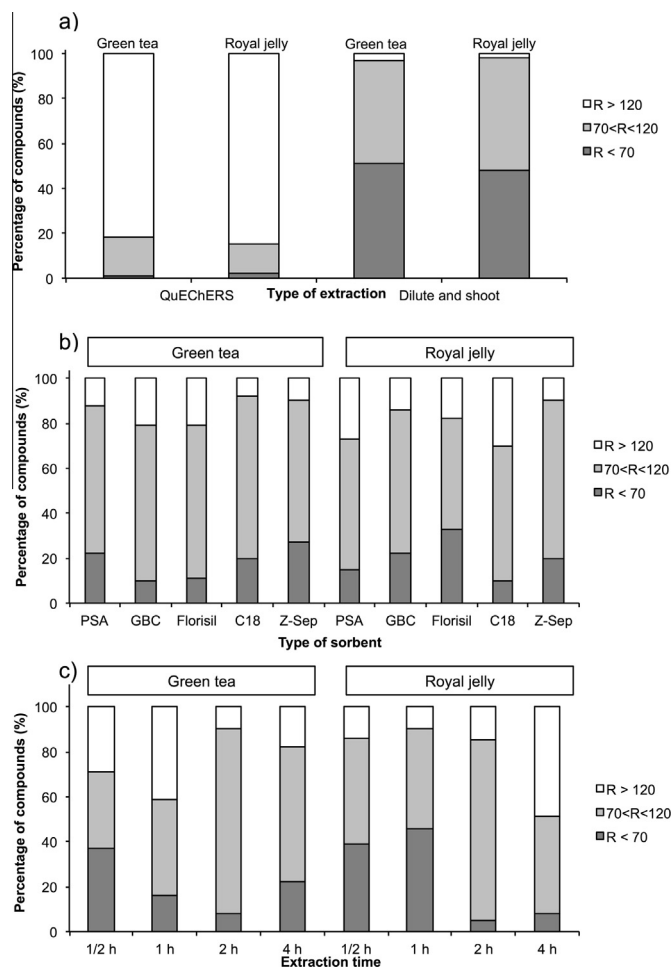


Fig. 2. Recoveries obtained for all the compounds studied: (a) using the QuEChERS (Procedure I) and the “dilute and shoot” methodology (Procedure II); (b) evaluating the effect of several sorbents during the clean-up step, and (c) studying the effect of the extraction time on the recovery of the compounds in green tea and royal jelly. All the samples were fortified at 50 $\mu\text{g}/\text{kg}$.

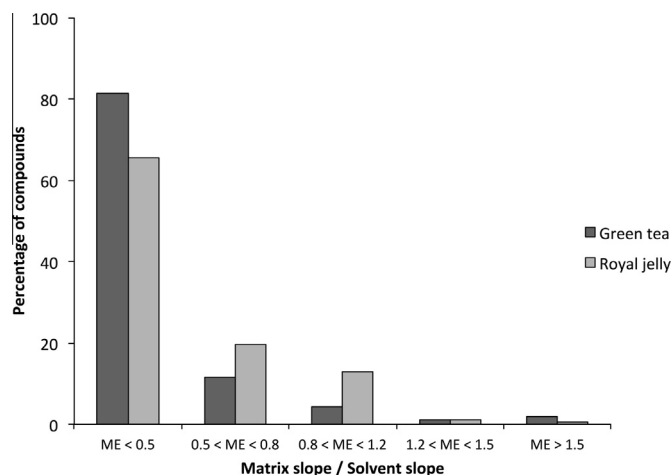


Fig. 3. Evaluation of matrix effect (ME) in the both matrices studied.

When trueness was evaluated, the recoveries obtained were between 70% and 120% for most of the compounds studied at three concentration levels (10, 50 and 100 $\mu\text{g}/\text{kg}$), except for cloridazon, cyfluthrin, desethyl atrazine, dimethomorph, diniconazole, diuron,

Table 1

Summary of the validation results for green tea and royal jelly.

	Concentration	Green tea	Royal jelly
Number of compounds with suitable recovery (70–120 %)	10 $\mu\text{g}/\text{kg}$	202	192
	50 $\mu\text{g}/\text{kg}$	257	262
	100 $\mu\text{g}/\text{kg}$	267	262
Repeatability		<25% ^a	
Intermediate precision		<25% ^a	
LOD ^b		0.5–10.0 $\mu\text{g}/\text{kg}$	
LOQ ^c		1.0–20.0 $\mu\text{g}/\text{kg}$	

^a RSD values ($n = 5$).

^b LOD: Limit of detection.

^c LOQ: Limit of quantification.

ethiofencarb sulfoxide, ethion, fenamiphos sulfone, fenhexamid, flutriafol, methiocarb sulfoxide, quinmerac, quinoxifen and rimsulfuron in green tea, and kresoxim-methyl in royal jelly. For these compounds, recoveries between 40% and 65% were obtained at 10 $\mu\text{g}/\text{kg}$. Also, high recoveries were observed for azaconazole, procymidone and tepraloxym in green tea, and alachlor, bifenthrin, bromacil, clotiadinin, coumaphos, cycloate, difenoconazole, EPTC, ethofumesate, fenarimol, indoxacarb, lufenuron, phosalone, tepraloxym, tetramethrin and trifloxystrobin for royal jelly (between 129% and 166%) at this concentration level. When 50 $\mu\text{g}/\text{kg}$ was studied, low recoveries (between 44% and 67%) were obtained for some compounds, as carbosulfan, chlorantranilprole, diflubenzuron, ethiprole, etofenprox, flufenoxuron, haloxyfop, hexythiazox, isofenphos methyl, methiocarb sulfone and oxyfluorfen in green tea, and carbaryl, cyproconazole, lenalacil, protham and simazine in royal jelly. Moreover, at 50 $\mu\text{g}/\text{kg}$, some compounds presented high recoveries such as cyanofenphos, imidacloprid, pyrethrin I and tefluthrin in green tea. This was also observed in the case of royal jelly (aldicarb sulfoxide, fluralinate, mevinphos, monocrotophos and pimeprozin) with recoveries between 142% and 165%. Finally, at 100 $\mu\text{g}/\text{kg}$, low recoveries were observed (between 46% and 66%) for carbosulfan, etofenprox, fenazaquin and hexythiazox in green tea; and benfluralin, demeclocycline, monolinuron, thiophanate methyl and trifluralin in royal jelly. Furthermore, high recoveries were obtained for bifenthrin and cyanofenphos for green tea with 127% and 139% respectively; and diphenylamine, hexazinone, methomyl, monocrotophos, propachlor and pymetrozine for royal jelly (between 131% and 146%). Some compounds could not be detected at 10 $\mu\text{g}/\text{kg}$ (see Tables S2 and S3). Regarding mycotoxins, deoxynivalenol could not be detected in royal jelly and fumonisin B1, fumonisin B2 and HT-2 toxin, in both matrices. A more specific extraction parameters are probably needed to determine these particular compounds in such a matrices.

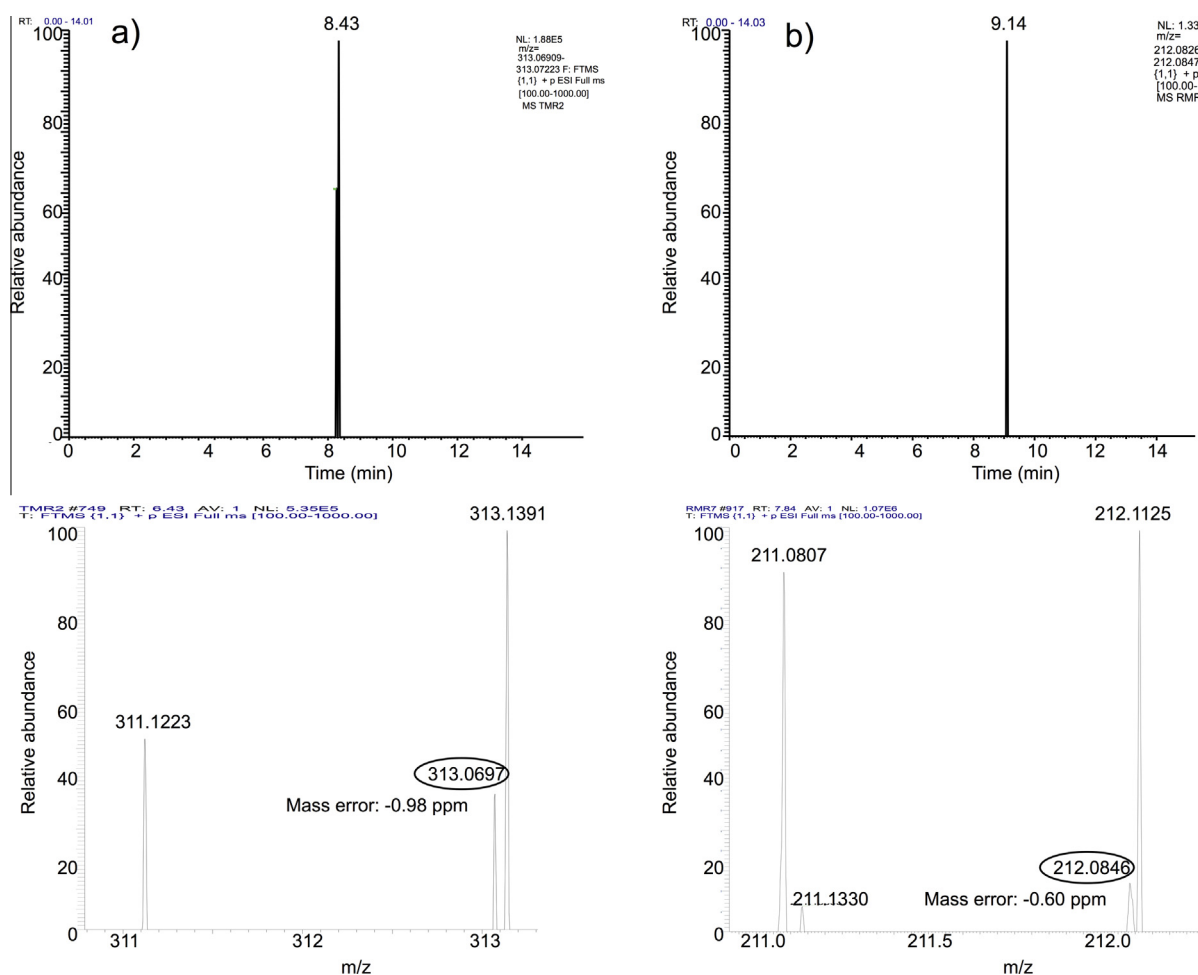
For repeatability assays, RSD values were below 20% for all the compounds studied in both matrices. In the case of intermediate precision, RSD values below 25% were obtained for all the compounds studied in both matrices (see Table 1). Regarding the lower limits of the method, the calculated LODs ranged from 0.5 to 5.0 $\mu\text{g}/\text{kg}$, whereas the LOQs ranged from 1 to 10 $\mu\text{g}/\text{kg}$ for most of the compounds studied in both matrices (see Table 1), except for those compounds that could not be detected at 10 $\mu\text{g}/\text{kg}$. For these compounds, the LODs and LOQs were 10 and 20 $\mu\text{g}/\text{kg}$ respectively.

Comparing these results with a related study (Vaclavik et al., 2014), which determined a wide range of pharmaceuticals, plant toxins and other biologically active secondary metabolites in botanical dietary supplements using an Orbitrap system, it can be seen that both methods offer reliable results, although in this study, a large quantity of compounds was studied. Moreover, the LODs obtained in this work were lower than the values obtained

Table 2

Compounds found in nutraceuticals from green tea.

Compounds	Concentration ($\mu\text{g}/\text{kg}$)							
	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 9	Sample 10
Acetamiprid	43.3	–	42.0	–	–	–	–	–
Aflatoxin B1	–	5.4	–	–	–	–	–	–
Cadusafos	–	10.9	–	–	–	–	–	–
Carbosulfan	–	–	–	–	–	10.4	–	–
Cinosulfuron	10.1	–	10.6	10.2	–	–	–	–
Gamma-cyhalothrin	–	–	–	–	–	–	12.1	10.7
Imidacloprid	36.7	–	–	–	–	–	–	–
Kresoxim-methyl	–	–	–	–	18.5	–	–	–
Paclobutrazol	–	–	–	–	47.9	–	–	–
Thiophanox sulfone	–	–	–	–	–	–	13.0	13.5

**Fig. 4.** Chromatograms and mass spectra obtained for: (a) aflatoxin B1 ($5 \mu\text{g}/\text{kg}$) and (b) propachlor ($14.9 \mu\text{g}/\text{kg}$) in real samples from green tea and royal jelly respectively.

by Vaclavik et al. ($10 \mu\text{g}/\text{kg}$) (2014). Comparing the extraction methods, a dilute and shoot procedure can be more convenient than the QuEChERS (Vaclavik et al., 2014) because it consumes less solvents and salts. Both methods agree that clean-up steps are needed in order to suppress or minimize the possible matrix effect and that matrix match calibration standards is an adequate solution. In terms of number of studied toxic compounds, the proposed method determines a higher number of these substances in green tea than alternative methods, observing the determination of 25 (Liu et al., 2014), 50 (Zhu et al., 2014) or 135 pesticides (Cajka et al., 2012) in other studies. In the case of royal jelly, only one article has evaluated the presence of contaminants in such a matrix, as

mentioned before (Karazafiris et al., 2008) where only 9 pesticides were studied. None of the above mentioned works used HRMS. Additionally, lower LODs and LOQs were obtained applying the proposed method. Overall, keeping in mind the large quantity of compounds studied in this work and the LODs obtained in the validation procedure, it represents a suitable option to quantify different toxic substances in a short time of analysis.

3.4. Application to real samples

Eighteen nutraceutical products, including green tea supplements (2 capsules and 8 tablets presentations) and royal jelly (2

capsules and 6 liquid presentations), were analysed with the validated method in order to determine the presence of the toxic substances included in this study.

Table 2 shows the results obtained analysing green tea samples. Eight of them resulted to be contaminated with pesticides; cinosulfuron was determined in three samples (10.1, 10.2 and 10.6 µg/kg), whereas paclobutrazol was found at the highest concentration (47.9 µg/kg). Most of the pesticides found are used as insecticides, except kresoxim methyl and paclobutrazol, which are used as fungicide and plant growth regulator, respectively (Pesticides Properties DataBase PPDB, 2014), so they could be applied in the *C. sinensis* plant for pest control. Regarding mycotoxins, one sample was contaminated with aflatoxin B1 (5.4 µg/kg), which is considered the most toxic of the aflatoxins (Council Regulation, 2006). Fig. 4a shows the chromatogram and mass spectra obtained for this compound found in the sample. At the moment of this investigation, green tea is not regulated by the current European legislation for maximum levels of mycotoxins (Council Regulation, 2006) but there are some limits in other matrices, which are below 2 µg/kg. Moreover, most of the samples were contaminated with more than one toxic substance. This is the case of sample 1 (acetamiprid, cinosulfuron and imidacloprid), sample 2 (aflatoxin B1 and cadusafos), sample 3 (acetamiprid and cinosulfuron), sample 5 (kresoxim-methyl and paclobutrazol), sample 8 and 9 (gamma-cihalothrin and thiofanox sulfone). Therefore, the presence of compounds belonging to several families of contaminants and/or residues sustains the development of the current multi-class methodology.

In the case of nutraceuticals from royal jelly, only one sample was contaminated with propachlor (14.9 µg/kg), which is used as herbicide (PPDB, 2014), although the concentration is below the corresponding MRL for propachlor in royal jelly (20.0 µg/kg) (Council Regulation, 2005). Fig. 4b shows the chromatogram and mass spectra obtained for this compound in the real sample. The presence of this pesticide in royal jelly can be explained because the assayed sample is a mixture of royal jelly and pollen (capsule presentation). So, it is presumable that the pesticide could be present in the pollen. Regarding mycotoxins, none of the substances included in this study were found in the analysed products.

4. Conclusions

A multi-class method was developed to identify and quantify more than 260 toxic substances, including pesticides and mycotoxins, in nutraceutical products obtained from green tea and royal jelly. A “dilute and shoot” method was applied for the extraction, including a clean-up step with C₁₈ (green tea) and Z-Sep⁺ (royal jelly). The method was validated, obtaining good results in terms of trueness, reproducibility and repeatability, although significant matrix effect is still noted for most of the compounds. HRMS allowed the analysis of a large quantity of compounds (272) and LOQs were below the current MRLs for pesticides in royal jelly. The validated method was applied to real samples from green tea and royal jelly, detecting pesticides in some of the analysed samples. Regarding mycotoxins, aflatoxin B1 was found in a green tea sample. Different toxic substances were detected in the same samples, indicating that multi-residue and multi-class methods are needed to control the quality of these products and ensure health safety.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.foodchem.2015.11.070>.

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