Simultaneous determination of atropine and scopolamine in buckwheat and related products using modified QuEChERS and liquid chromatography tandem mass spectrometry

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Abstract

A method was developed for the determination of atropine and scopolamine in buckwheat and related products. A modified QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) extraction procedure was evaluated. Dispersive solid phase extraction (d-SPE) was studied as clean-up step, using graphitized black carbon (GBC) and primary secondary amine (PSA). The extract was diluted with water (50:50, *v/v*) prior to chromatographic analysis. The method was validated and recoveries (except chia

samples spiked at 10 µg/kg) ranged from 75% to 92%. Intra and inter-day precision was lower than or equal to 17%. The limit of quantification of atropine and scopolamine was 0.4 and 2 µg/kg, respectively. Eight types of samples (buckwheat, wheat, soy, buckwheat flour, buckwheat noodle, amaranth grain, chia seeds and peeled millet) were analyzed. Target compounds were not found above the detection limits of the method, but three transformation products of scopolamine (norscopine, hidroscopolamine and dihidroxyscopolamine) were putative identified in the tested samples using high resolution mass spectrometry (Exactive-Orbitrap).

Keywords: Atropine, scopolamine, buckwheat and related food, modified QuEChERS, liquid chromatography tandem mass spectrometry, transformation products.

1. Introduction

Buckwheat (*Fagopyrum esculentum*) is a well-known pseudo cereal with pharmacologic action, such as antidiabetic, hypotension, hypocholesterolemic, and hypoglycemic effects (Christa & Soral-Śmietana, 2008; Lee, Shen, Lai & Wu, 2013). The crop was originated from China and nowadays it is widely cultivated over the world as an important raw material used for functional food because it becomes a dietary source of bioactive compounds, such as nutritionally valuable protein, phenolic compounds, starch and dietary fiber, essential minerals and trace elements (Giménez-Bastida, Piskuła & Zieliński, 2015; Choy, Morrison, Hughes, Marriott & Small, 2013). However, buckwheat can be contaminated by stramonium (*Datura stramonium)*, which can contain high concentration of tropane alkaloids, and therefore, these toxic substances can be found in buckwheat and related matrices at concentrations higher than 100 µg/kg (EFSA, 2013).

Tropane alkaloids are widely used with pharmaceutical purposes (Grynkiewicz & Gadzikowska, 2008). They are synthesized by the plants of the genus from *Solanaceae* family, as *Atropa*, *Hyoscyamus*, *Datura*, *Duboisia*, *Scopolia* and *Brugmansia,* although they are also produced in smaller quantities in plants from the *Erythroxylaceae, Proteaceae, Euphorbiaceae, Rhizophoraceae, Convolvulaceae* and *Cruciferae* families (Adamse et al, 2014). Although there are more than 200 tropane alkaloids, atropine and scopolamine are the most common compounds of this family.

Tropane alkaloids can affect human and animal health due to their high toxicity and they can be present in contaminated food or animal feed. An important case is the mass–poisoning incident of atropine and scopolamine involving 73 subjects, which occurred in Slovenia in 2003 (Perharic, Juvan & Stanovnik, 2013) or other cases in India (Campbell-Platt, 1997), USA, Greece, etc. (Adamse et al, 2014). Risk assessment of tropane alkaloids in food and feed was performed by the European Food Safety Authority (EFSA), and an Acute Reference Dose (ARfD) was established at 0.016 µg/kg body weight (b.w.), expressed as the sum of atropine and scopolamine (EFSA, 2013). According to the EFSA scientific opinion in 2008, more attention must be paid to the contaminated levels of atropine and scopolamine in food from plant origin, such as cereal products (BfR. No 035/2014. 2013), grain-based baby-food (Mulder, Pereboom-de Fauw, Hoogenboom, de Stopelaar & de Nijs, 2015), buckwheat grain and food products (Perharič et al, 2013) and tea and herbal infusions (Shimshoni, Duebeche, Mulder, Cuneah & Barel, 2015).

Therefore, the development of quick, easy and reliable analytical methods for the trace analysis of tropane alkaloids in food from plant origin is desirable. Basically, chromatography and capillary electrophoresis (CE) techniques are widely used for the analysis of tropane compounds (Aehle & Dräger, 2010), although chromatographic

techniques are more common than CE. These analytes are usually separated either by gas chromatography (GC) or liquid chromatography (LC) coupled with different detectors. When GC is used, good chromatographic resolution between tropane compounds and sample matrix can be achieved using non-polar capillary column such as DB-5 MS (Caligiani et al, 2011; Bazaoui, Bellimam & Soulaymani, 2011). However, tropane compounds should generally be derivatized and analyzed as trimethylsilyl derivatives because target compounds underwent dehydration in GC inlet at high temperature (Namera, Yashiki, Hirose, Yamaji, Tani, & Kojima, 2002). LC analysis of tropane compounds overcomes the problem related to thermolysis. LC coupled to tandem mass spectrometry (MS/MS) provides higher sensitivity and selectivity (Hogendoorn & Zoonen, 2000) than LC-UV light detector or diode array detector (LC-DAD) (Rancic & Spasic, 2009). Good chromatographic resolution, higher than 1.5 among tropane compounds (except enantiomers), could be obtained without extra optimization of LC-MS/MS conditions (Jakabová, Vincze, Farkas, Kilár, Boros, & Felinger, 2012) and concentrations at ng/g levels in crops (Jandrić et al, 2011), animal feeds (Mulder, von Holst, Nivarlet & van Egmond, 2014) or biological samples (Ricard et al., 2012; John, Binder, Hochstetter & Thiermann, 2010; Ng, Ching, Chan & Mak, 2013), could be monitored. Therefore, LC-MS/MS is widely used for identification and analysis of tropane alkaloids in plants, for therapeutic tropane monitoring and pharmacokinetic investigation, as well as to evaluate tropane intoxications (John, 2012).

Analysis of tropane alkaloids was early focused on the determination of the target compounds in different plants and their organs, which usually contained high concentrations of tropane alkaloids as Datura species (Jakabová et al., 2012; Temerdashev, Kolychev & Kiseleva, 2012; Adams et al, 2006; Boros, Farkas, Jakabová, Bacskay, Kilár, & Felinger, 2010). Nowadays, the development of a suitable sample

preparation procedure is a difficult task to determine these type of compounds in complex matrices. An ideal sample preparation method should be simple, quick, easy, and environmentally friendly because it is often the bottleneck in analysis and there is a need to minimize the number of steps to reduce both time and sources of error (Ridgway, Lalljie & Smith, 2007). The QuEChERS (acronym of Quick, Easy, Cheap, Effective, Rugged and Safe) methodology was introduced as a green, user-friendly, and cheap approach to meet the challenge of trace residue analysis of various organic compounds in food from plant and animal origin (Anastassiades et al, 2003; Wilkowska & Biziuk, 2011; Bruzzoniti et al., 2014).

Among the reported analytical methods, tropane alkaloids were usually extracted with methanol or acetonitrile and analyzed by LC-MS/MS without clean-up (Shimshoni, Duebeche, Mulder, Cuneah & Barel, 2015; Jakabová, Vincze, Farkas, Kilár, Boros, & Felinger, 2012; Ng et al, 2013; Adams et al, 2006). It is well known that matrix effect has serious impact on method accuracy and sensitivity due to the presence of co-eluting substances (Taylor, 2005). High matrix effect and huge variation of tropane alkaloids analyzed by LC-MS/MS were observed when clean-up steps are avoided (Jandrić et al, 2011). Therefore, it is necessary to remove or minimize the matrix effect by modifying the sample preparation methodology, such as by the use of a clean-up step.

The presence of transformation products of the target compounds in the tested samples was also evaluated using high resolution mass spectrometry, utilizing several analyzers as Exactive-Orbitrap (Mol et al, 2011).

Although there are few methods that applied the QuEChERS approach (Jandrić et al, 2011; Mol et al, 2011), the aim of this study is the development and validation of a modified QuEChERS method for the determination of atropine and scopolamine residue in buckwheat and related products using ultra high performance liquid chromatography

(UHPLC)-MS/MS, utilizing triple quadrupole (QqQ) as analyzer. Moreover, the clean-up step was also optimized, evaluating the effect of several sorbents during this stage. Finally an Exactive-Orbitrap analyzer is also used for the identification of transformation products.

2. Material and methods

2.1. Reagents and chemicals

Atropine and scopolamine reference standards were obtained from Sigma-Aldrich (St. Louis, MO, USA). Stock standard solutions of atropine and scopolamine (200 mg/L) were individually prepared by exact weighing of the solid substances and dissolved in 50 mL of methanol (HPLC grade, Sigma), and they were stored at -20 °C in the dark. A mixture working standard solution was prepared at 5 mg/L with methanol and stored in screw-capped glass tubes at -20 °C in the dark.

HPLC grade acetonitrile was obtained from Sigma-Aldrich. Formic acid (Optima LC-MS) and ammonium formate were supplied from Fisher Scientific (Geel, Belgium). Graphitized black carbon (GBC), primary secondary amine (PSA) and florisil were purchased from Scharlab (Barcelona, Spain). Zirconia-coated silica (Z-Sep$^+$) was obtained from Supelco (Bellefonte, PA, USA). Anhydrous magnesium sulfate and ammonium acetate were purchased from Panreac (Barcelona, Spain). Ultrapure water was obtained by a gradient system Milli-Q water (Millipore, Bedford, MA, USA).

2.2. Instrument and Apparatus

Detection of atropine and scopolamine was performed with an Agilent series 1290 RRLC instrument (Agilent, Santa Clara, CA, USA) equipped with a binary pump (G4220A), an autosampler thermostat (G1330B) and a column compartment thermostat (G1316C). The RRLC system was coupled to an Agilent triple quadrupole mass

spectrometer (6460 A) with a Jet Stream electronic spray ionization (ESI) source (G1958-65138). A Zorbax plus C18 column (100 × 2.1 mm, 1.8 µm particle size) from Agilent (San Jose, CA, USA) was employed for separation. The MassHunter (Agilent) software was used for optimization and quantification.

Detection of atropine and scopolamine transformation products was performed with a Thermo Fisher Scientific HPLC instrument (Thermo Scientific Transcend™, Thermo Fisher Scientific , San Jose, CA, EE.UU.), coupled to Thermo Fisher Scientific Orbitrap mass spectrometer (Exactive™, Thermo Fisher Scientific, Bremen, Germany), with a heated electrospray ionization source (HESI–II). The same column used in QqQ analyses was used. The Xcalibur™ version 2.2.1 from Thermo Fisher Scientific with Qualbrowser was used for identification purposes.

Samples were ground into powder using an automatic blender (Sammic S.L., Azkoitia, Spain). A WX vortex from Velp Scientifica (Usmate, Italy) was used to homogenize the samples. Centrifugation was carried out in a Consul 21 centrifuge from Orto Alresa (Madrid, Spain).

2.3. Samples collection

Eight different types of samples were obtained from local supermarkets located in Almería (Spain). The analyzed samples were buckwheat, buckwheat flour and buckwheat pasta (*Fagopyrum esculentum)*, soy (*Glycine max)*, wheat *(Triticum aestivum)*, amaranthus grain *(Amaranthus caudatus)*, chia seeds *(Salvia hispanica)* and peeled millet *(Panicum miliaceum)*. These samples were selected according to EFSA`s concern regarding food control of tropane alkaloids (Ng et al, 2013). Samples were ground into powder prior to analysis. Blank samples were used for the preparation of fortified samples during the optimization of sample extraction procedure and method

validation.

2.4. Sample Preparation

Five g of sample was weighed in a 50 mL polypropylene centrifuge tube, and soaked with 10 mL of Milli-Q water by vortex for 1 min, and then, 10 mL of acetonitrile solution containing 1% (*v/v*) of formic acid was added. The mixture was homogenized for 2 min in a vortex. Then, 4 g of anhydrous sodium sulfate and 1 g of ammonium acetate were added and the mixture was homogenized by vortex for 2 min. Afterwards, the mixture was centrifuged at 5,000 rpm (4,136 ×*g*) for 5 min. One mL of the supernatant (acetonitrile phase) was transferred to a 15 mL centrifuge tube containing 25 mg of PSA and 25 mg of GBC. The tube was homogenized by vortex for 1 min and then centrifuged at 5,000 rpm (4,136 ×*g*) for 5 min. Finally, the supernatant was diluted with Milli-Q water (50:50, *v/v*), and filtered through 0.22 µm nylon syringe filter prior chromatographic analysis.

2.5. UHPLC-MS/MS Analyses

The chromatographic determination of atropine and scopolomine was carried out employing a binary mobile phase with acetonitrile (A) and an aqueous solution of formic acid (0.1%, *v/v*) (B). A gradient elution started at 90% of B and held 2 min at flow rate of 0.3 mL/min. This composition was reduced to 50% B in 5 min, and then to 0% B at 6 min and held for 1 min. The composition of the mobile phase returned to the initial conditions in 1 min and the stationary phase was equilibrated during 2 min. The total running time was 10 min. Injection volume was 5 µL and column temperature was kept at 25°C.

Atropine and scopolamine were ionized at positive ESI mode and detected using

selected reaction monitoring (SRM) mode. Source gas temperature and sheath gas temperature were 325 °C and 400°C, respectively. Source gas flow and sheath gas flow were 5 L/min and 11 L/min, respectively. Nebulizer was 45 psi. Capillary and nozzle voltage were 3500 V and 500 V, respectively. Retention time and MS/MS parameters are shown in Table 1.

In relation to the identification of transformation products a generic chromatographic method previously developed in our research group (Gómez-Pérez, Plaza-Bolaños, Romero-González, Martínez-Vidal & Garrido-Frenich, 2012) was used.

3. Results and Discussion

3.1. Optimization of UHPLC-QqQ-MS/MS

The optimization of UHPLC-QqQ-MS/MS parameters was performed by direct infusion of individual standard solutions of the target compounds at 5 mg/L in methanol:water (50:50, *v/v*) at a flow rate of 0.15 mL/min. Both compounds were analyzed using ESI+. Full scan and MS/MS spectra were performed to acquire the most sensitive transitions. The protonated molecule $[M+H]^+$ was the most intense ion for both compounds. Figure 1 shows the mass spectra and MS/MS transitions obtained after fragmentation of the protonated molecule.

Fragmentation of the protonated molecular ion at *m/z* 290.2 of atropine yielded 7 product ions at *m/z* 142.1, 124.0, 103.0, 93.0, 91.0, 77.0, and 67.0. The most abundant product ions at *m/z* 124.0 and 93.0 were obtained by loss of tropic acid ($C_9H_{10}O_3$, 166 Da) and by loss of NH_2CH_3 (31 Da), respectively. Fragmentation of the protonated molecular ion of scopolamine yielded 7 product ions at *m/z* 156.0, 138.0, 121.0, 110.0, 103.0, 98.0, and 79.0. The most abundant product ions at *m/z* 156.0 and 138.0 were formed by loss of $C_9H_8O_2$ (148 Da) and tropic acid ($C_9H_{10}O_3$, 166 Da) from protonated

molecular at m/z 304.1.

Further optimization was performed to evaluate the intensity of each product ion obtained from different fragmentor voltages (from 50 to 110 V) and collision energies (CE, from 10 to 50 eV). Finally, the most intense transitions for atropine m/z 290.2 > 124.0 (fragmentor voltage 90 V and CE 20 eV) and scopolamine 304.1> 156.0 (fragmentor voltage 70 V and CE 12 eV) were selected for quantification, whereas other three transitions were used for confirmation purposes (see Table 1).

The optimization of chromatographic conditions was carried out to obtain the best peak shape and reduce the analysis time. First, methanol and acetonitrile, as well as a mixture of methanol:acetonitrile (50:50,*v/v*), were tested as organic phase, and acetonitrile was selected because better peak shape was obtained. Several aqueous solutions of formic acid (0.01, 0.1 and 0.5%, *v/v*), or ammonium formate (0.01 M) were evaluated, utilizing acetonitrile as organic solvent. The results showed that the best recoveries were achieved using acetonitrile and an aqueous solution of formic acid (0.1%, *v/v*) as mobile phase. Other parameters, such as flow rate (0.1-1.0 mL/min) and gradient profile were evaluated, setting as optimal conditions those indicated in Section 2.4.

3.2. Optimization of the extraction procedure Using water for dry samples prior to acetonitrile extraction was utilized to improve the extraction efficiency of the polar atropine and scopolamine due to their high water solubility (Cajka et al., 2012; Walorczyk, 2007), which is 2.2 mg/mL and 100 mg/mL, respectively. In this study, all samples were soaked by the addition of 10 mL of water. The partition behavior of tropane alkaloids between organic solvent and water was pH-dependent (Miyazaki, Yomota & Okada, 1993), which may have an important effect on extraction efficiency.

In order to evaluate it, buckwheat was used as matrix, and it was spiked with the target compounds at 50 µg/kg. Recoveries were compared when acetonitrile, acetonitrile with acetic acid (1%, *v/v*), and acetonitrile with 0.01 M ammonium formate were used as extraction solvents. According to the results shown in Figure 2, acetonitrile containing acetic acid (1%, *v/v*) was selected as extraction solvent because it provides the best recovery values for the target compounds.

Plenty of matrix compounds from buckwheat and related products were leached into the raw extracts when water and acetonitrile were used during the extraction of the target compounds. The co-extracted substances could interfere the target compounds and provoke serious matrix effects. Several previous studies used PSA, C18, florisil and GBC as sorbents when dispersive solid phase extraction (d-SPE) was used to remove the co-extractants substances from cereals (Herrmann & Poulsen, 2015; González-Curbelo & Herrera-Herrera, 2012; Grande-Martínez, Arrebola-Liébanas, Martínez-Vidal, Hernández-Torres & Garrido-Frenich, 2015; He, Wang, Peng, Luo, Wang, & Liu, 2015). In this study, PSA, GBC, Z-Sep$^+$ and florisil were investigated through recovery studies, spiking blank buckwheat samples at 10 µg/kg. As shown in Figure 3A, recoveries were lower than 70% when Z-Sep$^+$ and florisil were used during the clean-up stage, indicating that they were not suitable for this step because of their strong absorption affinity of target compounds. Recoveries of atropine and scopolamine ranged from 84% to 86% when PSA (50 mg) was used, whereas recoveries of both compounds were in the range of 76%-77% using GBC (50 mg) as sorbent. The extraction was colorless and transparent when GBC (50 mg) was used, indicating that GBC was useful to remove matrix components. Therefore, because PSA and GBC provided good recovery, the amount of these sorbents were optimized since their combination could provide an excellent clean-up removing a variety of matrix

compounds (Anastassiades et al, 2003). The amount of PSA (5-50 mg) had little effect on recoveries of atropine (82-84%) and scopolamine (77-81%) (Figure 3B). As it is shown in Figure 3C, recoveries were reduced about 11% when the added amount of GBC increased from 25 mg to 50 mg, indicating that 25 mg of GBC was the best choice to obtain good recovery and clean-up effect. Finally, the effect of the combination of PSA (25 mg) and GBC (25 mg) was tested. Recoveries of scopolamine and atropine were 76±4% and 83±5% (n=3), respectively. The extracts after the clean-up stage was nearly colorless and transparent and this combination was used for further experiments.

Several matrix standard solutions at 5µg/L, diluted with water at several ratios (10:90, 25:75, 50:50, 75:25, 90:10 (*v/v*)) and without dilution, were individually analyzed by UHPLC-QqQ-MS/MS. Suitable peak symmetry and sensitivity for both compounds were obtained when the extracts were diluted with water at 50:50 or 25:75 (v/v). Therefore, the extract obtained after the clean-up step was diluted with 50:50 (*v/v*) to obtain better chromatographic peaks, and lower limit of detection than matrix:water dilution 25:75 (*v/v*).

3.3. Method Validation

Calibration curves were performed by injecting scopolamine and atropine in a matrix matched standard mixture with concentrations at 0.1, 0.5, 1, 2.5, 5, 10, 25, 50 and 100 µg/L. Suitable calibration curves were obtained for scopolamine and atropine with coefficients of determination (R^2) higher than 0.996.

Matrix effect was evaluated in buckwheat and related matrices as pasta obtained from buckwheat, amaranthus, millet, chia and soy. In order to check the influence of the matrix on the response, several concentrations (from 0.1 to 100 µg/kg) were added to blank extracted samples, including two types of buckwheat and solvent. The slopes of

the calibration curves are shown in Table S1 (see Supplementary Material). First, it can be noted that there is a remarkable difference between the slope obtained in solvent and in matrices, observing matrix enhancement despite the dilution step discussed above. In order to check if the slopes obtained from the tested matrices are different, an analysis of covariance (ANCOVA) was used to compare the slopes of the calibration curves (Garcia-Campaña, Cuadros-Rodríguez, Aybar-Muñoz & Alés-Barrero, 1997). First, it can be indicated that when the slopes obtained from all the matrices were compared (excluding the solvent), a significant difference (p-value lower than 0.05) was obtained for scopolamine and atropine. Therefore, the use of a representative matrix is not possible for the quantification of these two compounds in the selected matrices. However, it can be observed that for scopolamine, the slopes obtained in both types of buckwheat, amaranthus and soy are not statistically different (p-value > 0.05), as well as millet and chia (p-value = 0.36). For atropine, it is important to indicate that the two buckwheat tested samples as well as the pasta produced from buckwheat and amaranthus provided similar slopes (p value = 0.25), as well as millet and chia (p value = 0.14) are not statistically different. Therefore, buckwheat could be used as representative matrix for the quantification of these two compounds in different types of buckwheat and derived matrices as pasta, although for other matrices (chia and millet), calibrations using these matrices should be used in order to perform a reliable quantification of both analytes. On the other hand, the use of isotopically labelled internal standards could also be utilized as another alternative to overcome this problem, although the price of these standards could increase the cost of the analysis.

Trueness was investigated through recovery trials, spiking blank samples at four levels (10, 25, 50 and 100 µg/kg) for buckwheat, whereas for millet, soy and chia only two levels (10 and 50 µg/kg) were evaluated. The tested concentrations were selected

bearing in mind that several effects were observed in animals at 60 µg/kg of atropine and scopolamine in feed (Piva & Piva, 1995). Each fortified concentration was repeated five times and the results are shown in Table 2. Recoveries of scopolamine and atropine spiked in buckwheat samples were in the range of 77%-80% and 80%-88%, respectively. Similar results were obtained for the other matrices, and recoveries of scopolamine and atropine in millet, soy and chia ranged from 76%-88% and 75%-92%, respectively. Only, results below 70 % were obtained for chia at low levels (10 µg/kg). Thus, recovery was 50% and 66% for scopolamine and atropine, respectively. Although these recoveries are not close to 100%, they can be considered acceptable since they were repetitive (see Table 2) and correction factor could be used for the quantification of these two compounds in this matrix at low concentrations. It can be highlighted that recoveries obtained in this work, except chia spiked at 10 µg/kg, could be acceptable according to European Commission SANTE/11945/2015 (SANTE, 2015), which indicated that the recoveries should be in the range of 70%-120%. Recoveries obtained in this study were similar to those obtained in previous studies (89%-109% for atropine and scopolamine) (Caligiani et al, 2011; Jandrić et al, 2011), but simpler extraction procedure was applied, and the use of internal standards was avoided (Caligiani et al, 2011), although it could be a good option in order to improve the recovery when chia samples are analyzed.

Precision, expressed as relative standard deviation (RSD), was evaluated in terms of repeatability (intra-day precision) and intermediate precision (inter-day precision). As it is shown in Table 2, good precision was obtained with all values lower than or equal to 17%, observing that most of them are below 10 %.

Limits of detection (LODs) and quantification (LOQs) were defined as the lowest concentration for which area values were three and ten times the ratio of signal-to-noise,

respectively. For that, low concentrations of the target compounds (from 0.01 to 5 µg/kg) were added to the samples. LODs for atropine and scopolamine were 0.04 and 0.2 µg/kg, respectively, while LOQs of atropine and scopolamine were 0.4 and 2 µg/kg. These limits were checked in the other matrices evaluated, obtaining similar results. The developed methodology offered lower LOQs than other studies (Perharič et al, 2013; Caligiani et al, 2011; Jandrić et al, 2011), which were in the range of 2.2-30 µg/kg for both compounds.

3.4. Analysis of real samples

The developed method was applied for the determination of atropine and scopolamine in 8 samples (buckwheat, wheat, soy, buckwheat flour, buckwheat noodle, amaranth grain, chia seeds, and peeled millet). Quality control was carried out to ensure the reliability of the results. Quality control implies the analysis of blank samples, matrix-matched standard solutions from 0.1 to 100 µg/kg, and fortified samples at 10 and 50 µg/kg. Furthermore, the retention time and the relative intensities of monitored transitions in real samples were compared to those obtained by matrix-matched standard solutions according to SANTE/11945/2015 (SANTE, 2015). The compounds were not detected in the 8 samples analyzed above the established LODs.

Bearing in mind that the studied tropane alkaloids can suffer transformations due to the metabolism and the environment, a total of 23 transformation products (Chen, Chen, Du, Han, Wang, & Zhang, 2006, Chen, Chen, Wang, Du, Han & Zhang, 2005) (shown in Table S2 (see Supplementary Material)) of atropine and scopolamine were studied using high resolution Orbitrap mass spectrometry. For that, a generic method previously developed was used (Gómez-Pérez et al., 2012), and the samples analyzed by UHPLC-QqQ-MS/MS, were injected in the LC-Orbitrap-MS system and they were

analyzed by full scan mode. The identification of these transformation products was performed knowing its molecular formula and molecular weight (see Table S2), and they were monitored in positive and negative ionization mode.

These metabolites were searched in the samples and three scopolamine transformation products were putative identified. In all the cases, mass error was lower than 5 ppm. Norscopine was detected in amaranthus, whereas hydroscopolamine and dihydroxyscopolamine were detected in chia, as it can be observed in Figure 4, where the chromatogram, theoretical and experimental spectra of the detected transformation products are shown. Further investigation should be done in order to confirm these compounds using analytical standards. After that, if the compounds were identified, these compounds should be included in routine monitoring of these type of samples, bearing in mind that although parent compounds were not detected, transformation products could be present in the theoretical "blank" samples.

4. Conclusions

This study proposes a rapid and fast method for the determination of scopolamine and atropine in buckwheat and related products using a modified QuEChERS technique and UHPLC-QqQ-MS/MS. The modified QuEChERS technique is based on acetonitrile extraction and clean-up stage using a mixture of PSA and GBC, which is useful to remove matrix interferents. UHPLC-QqQ-MS/MS allows a fast detection of the target compounds in less than 10 minutes. The validation showed suitable linearity, trueness, precision and LOQs. A total of 8 real samples were analyzed using the developed method. Three scopolamine transformation products were putative identified, although neither of target compounds was found above the LODs of the method in the analyzed samples. Therefore, after further investigation the compounds could be included in routine analysis of scopolamine residue in buckwheat and related products.

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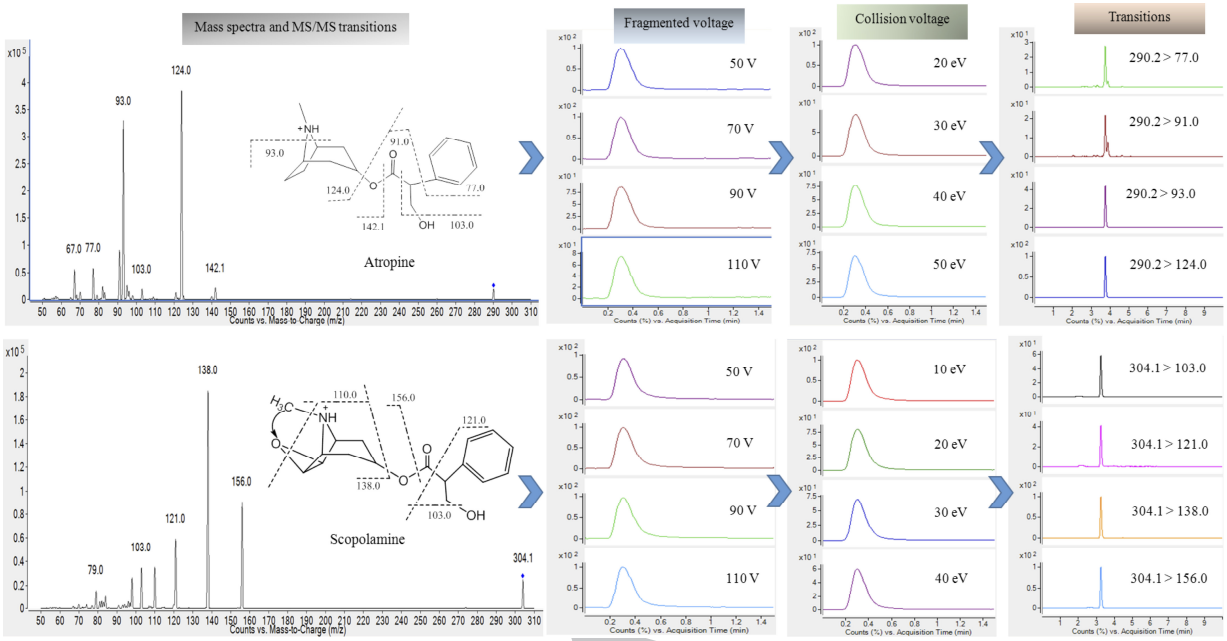
Figure Captions

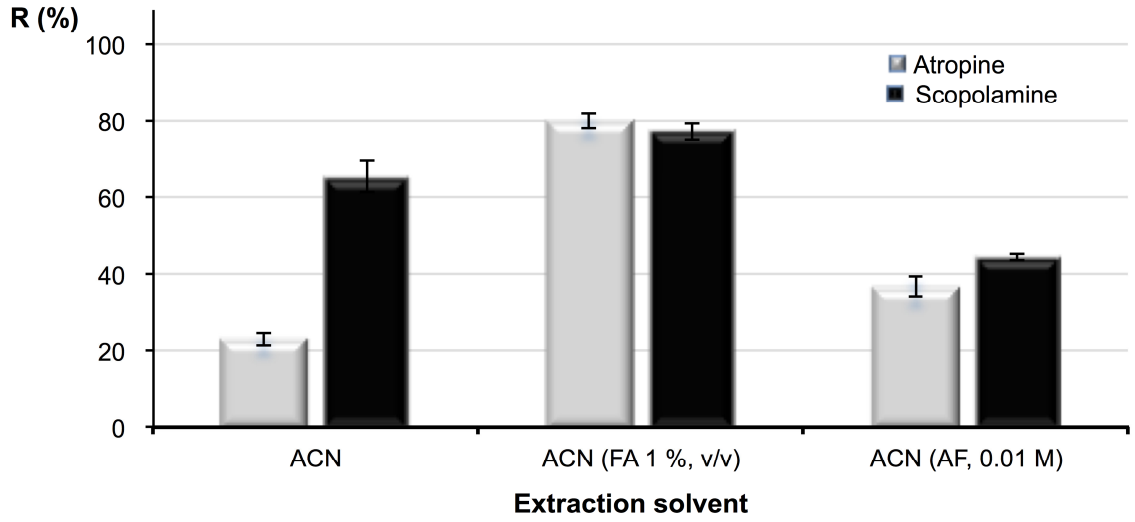
Figure 1. Mass spectra of the protonated molecule $[M+H]^+$ the target compounds, and chromatograms using the optimized fragmentor voltage, collision energy of the selected transitions. Note: Mass spectra and chromatograms obtained from standard solution at 5 mg/L.

Figure 2. Recoveries of atropine and scopolamine obtained from different extraction solvent. Buckwheat blank sample fortified at 10 µg/kg (n=3). Abbreviations: ACN: Acetonitrile; AF: Ammonium formate; FA: Formic acid.

Figure 3. Recoveries of atropine and scopolamine obtained from different sorbents at 50 mg (A), PSA (B) and GBC (C) at 5, 10, 25, and 50 mg. Buckwheat blank sample fortified at 10 µg/kg (n=3).

Figure 4. UHPLC-Orbitrap-MS chromatograms of scopolamine's transformation products: (A) norscopine in amaranthus, (B) hydroscopolamine in chia (C) dihydroxyscopolamine in chia.





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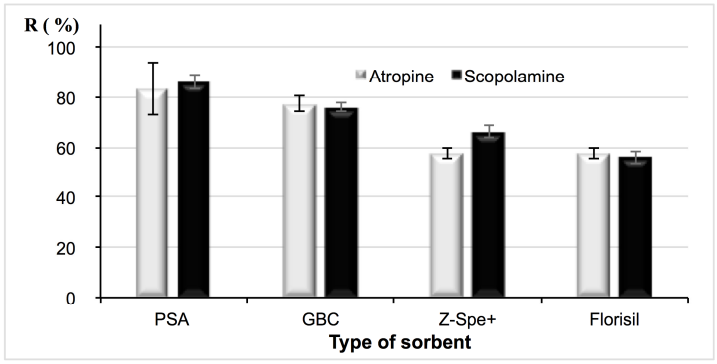

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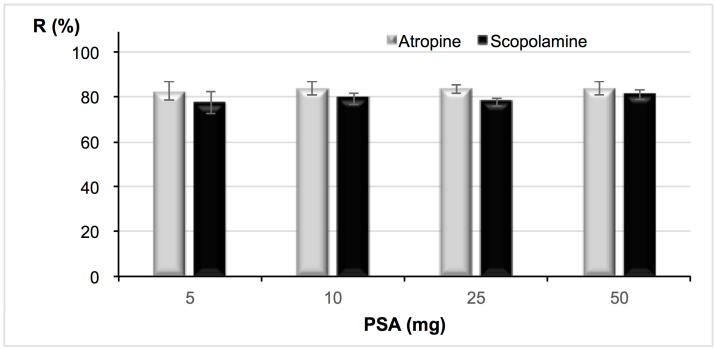

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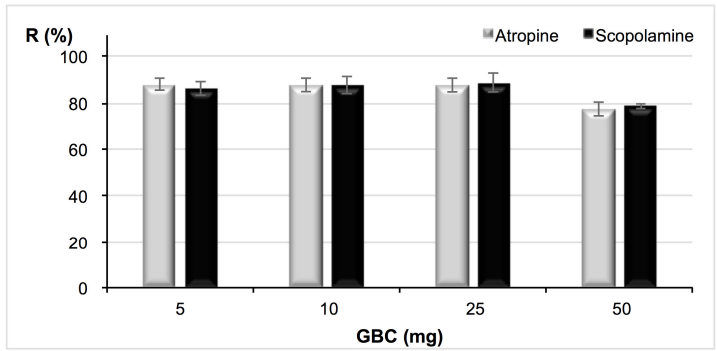

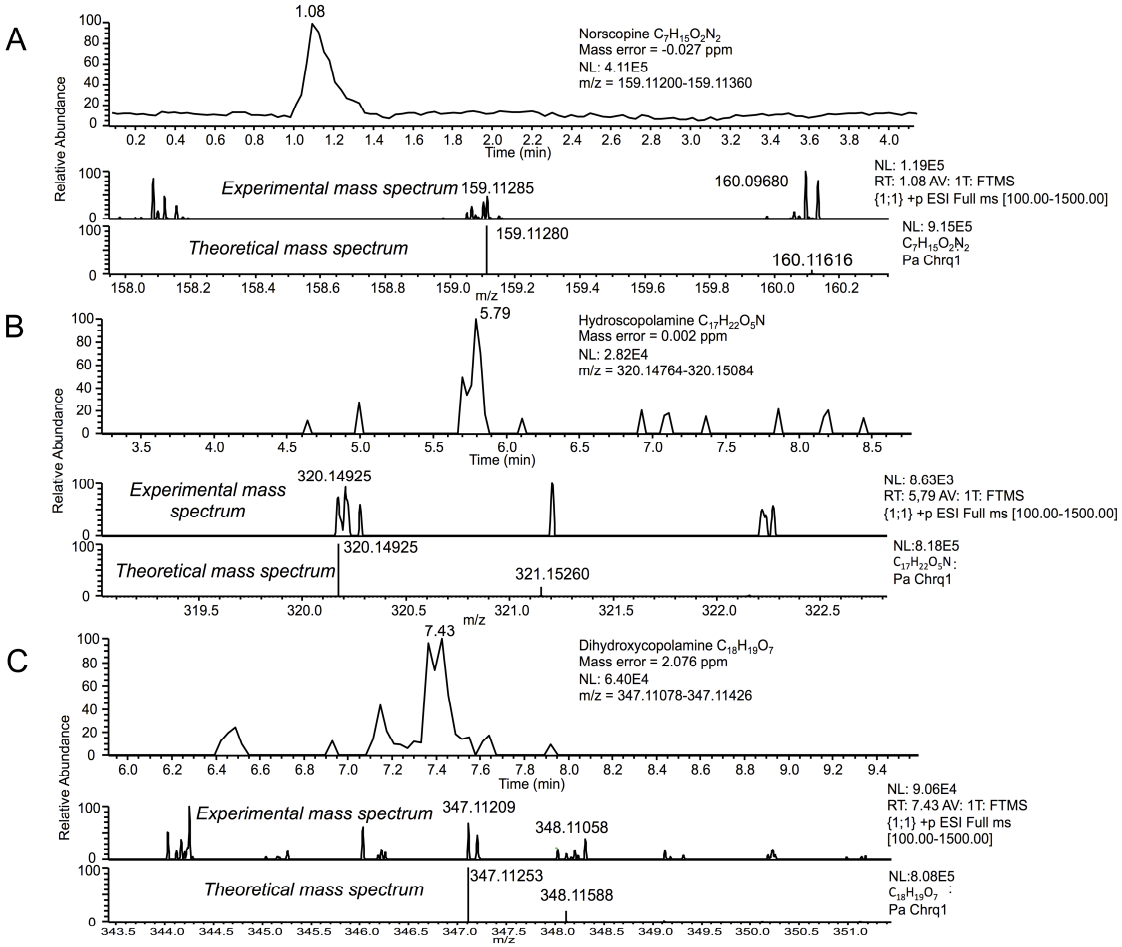

Table 1.

Retention time windows (RTWs) and MS/MS parameters of scopolamine and atropine.

Compound	RTW (min)	Precursor ion (*m/z*)	Product ion (m/z) [a]	Ion ratio (%)
Scopolamine	3.21-3.31	304.1 (90)[b]	**156.0** (12)[c]	
			138.0 (16)	98
			121.0 (16)	40
			103.0 (40)	56
Atropine	3.70-3.80	290.2 (70)	**124.0** (20)	
			93.0 (28)	42
			91.0 (48)	21
			77.0 (50)	26

[a] Transition in bold was used for quantification.

[b] Fragmentor voltage (V) is given in brackets.

[c] Collision energy (eV) is given in brackets.

Table 2. Performance characteristics of the optimized method.

Compound	Matrix	Recovery (%)[a]				Inter-day precision (n =5)				LOD	LOQ
		10 µg/kg	*25 µg/kg*	*50 µg/kg*	*100 µg/kg*	*10 µg/kg*	*25 µg/kg*	*50 µg/kg*	*100 µg/kg*	(µg/kg)	(µg/kg)
Scopolamine	Buckwheat	80 (4)	79 (3)	77 (4)	77 (2)	5	4	4	5	0.2	2.0
	Millet	88(4)	-	88(1)	-	-	-	-	-		
	Soy	76(3)	-	76(4)	-	-	-	-	-		
	Chia	50(11)	-	81(9)	-	-	-	-	-		
Atropine	Buckwheat	84 (9)	88 (3)	80 (4)	80 (3)	10	2	4	5	0.04	0.4
	Millet	92(4)	-	90(2)	-	-	-	-	-		
	Soy	80(6)	-	80(5)	-	-	-	-	-		
	Chia	66(17)	-	75(10)	-	-	-	-	-		

[a] Intra-day precision, expressed as RSD, is given in brackets (n = 5).

HIGHLIGHTS

- Development of a modified QuEChERS extraction procedure of atropine and scopolamine
- Suitable performance characteristics (sensitivity and recovery) were obtained
- Different samples as buckwheat and related matrices (soy, chia) were analyzed
- Qualitative identification of transformation products by Exactive-Orbitrap-MS was carried out