Elsevier Editorial System(tm) for Food Chemistry

Manuscript Draft

Manuscript Number:

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

Article Type: Research Article (max 7,500 words)

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

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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 during 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 analysis. The method was validated and recoveries (except chia samples spiked at 10 µg/kg) ranged from 75%-92%. Intra and inter-day precision was ≤17%. The quantification limit of atropine and scopolamine was 0.4 and 2 µg/kg, respectively. Different 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).

Cover Letter

UNIVERSIDAD DE ALMERÍA
Department of Chemistry and Physics

Almería, 12th April 2016

Dear Editor,

Please find enclosed the manuscript entitled "*Simultaneous determination of atropine and scopolamine in buckwheat and related products using modified QuEChERS and liquid chromatography tandem mass spectrometry*" by Chen et al., for consideration of publication in Food Chemistry, as an original research paper.

Buckwheat is widely used as an important raw material for functional food development. However, health risk of tropane alkaloids for human may occur due to the intake of contaminated buckwheat and related products. Therefore, the development of a quick, easy, reliable analytical method for the trace analysis of tropane alkaloids in buckwheat and related products is desirable, as it has been indicated by the European Food Safety Agency (EFSA). Here, we described a simple and fast analytical method for the determination of atropine and scopolamine in buckwheat and related products using LC-MS/MS. During the extraction step, a modified QuEChERS extraction procedure was used, optimizing the sorbents used during the clean-up step. Therefore, the proposed method provided a fast and non-laborious method, which reduces the time employed to extraction, clean-up and ensure the high selectivity required for complex analysis using mass spectrometry as detection technique. Good performance characteristics such as linearity, trueness, inter-day and intra-day precision, quantification limits and confirmation limits were obtained. Finally, the proposed method was applied to quantify atropine and scopolamine in several matrices, and it could be applied in routine analysis. Furthermore, the analyzed samples were re-injected into an LC-Orbitrap-MS system and transformation products of the target compounds were detected.

Finally, we would like to indicate that this is the first application focused on the determination of these type of compounds in buckwheat and similar matrices, and it tries to cover the gap highlighted by EFSA in relation to the analysis of these toxic compounds in the matrices included in this study. Moreover, the use of Exactive-Orbitrap allows the

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identification of transformation products despite the target compounds were not detected in the tested samples.

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I hope that the reviewing process finds the manuscript acceptable for publication in the journal.

Yours Sincerely,
Prof. Antonia Garrido Frenich

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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

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 during 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 analysis. The method was validated and recoveries (except chia samples spiked at 10 µg/kg) ranged from 75%-92%. Intra and inter-day precision was ≤17%. The quantification limit of atropine and scopolamine was 0.4 and 2 µg/kg, respectively. Different 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 healthy pseudo cereal well-known because its pharmacologic action, such as antidiabetic, hypotension, hypocholesterolemic, and hypoglycemic effects (Christa & Soral-Śmietana, 2008). The crop was 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 (Grynkiewica & 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, van Egmond, Noordam, Mulder & de Nijs, 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 intake of contaminated food or animal feed. An important case is the mass–poisoning incident of atropine and scopolamine involving 73 subjects, occurred in Slovenia in 2003 (Perharic, Juvan & Stanovnik, 2013) or other cases in India (Campbell-Platt, 1997), USA, Greece, etc. (Adamse, van Egmond, Noordam,

63 Mulder & de Nijs, 2014). Risk assessment of tropane alkaloids in food and feed was performed by
64 the European Food Safety Authority (EFSA), and an Acute Reference Dose (ARfD) was established
65 at 0.016 µg/kg body weight (b.w.), expressed as the sum of atropine and scopolamine (EFSA, 2013).
66 According to the EFSA scientific opinion in 2008, more attention must be focused on the
67 contaminated levels of atropine and scopolamine in food from plant origin, such as cereal products
68 (BfR. No 035/2014. 2013), grain-based baby-food (Mulder, Fauw, Hoogenboom, Stopelaar & Nijs,
69 2015) and buckwheat grain and food products (Perharič, Kozelj, Druzina & Stanovnik, 2013).
70 Therefore, the development of quick, easy and reliable analytical methods for the trace analysis
71 of tropane alkaloids in food from plant origin is desirable. Basically, chromatography and capillary
72 electrophoresis (CE) techniques are widely used for the analysis of tropane compounds (Aehle &
73 Dräger, 2010), although chromatographic techniques are more common than CE. These analytes are
74 usually separated either by gas chromatography (GC) or liquid chromatography (LC) coupled with
75 different detectors. In GC, non-polar capillary column, such as DB-5 MS, could provide good
76 chromatographic resolution between tropane compounds and sample matrix (Caligiani, Palla,
77 Bonzanini, Bianchi & Bruni, 2011; Bazaoui, Bellimam & Soulaymani, 2011). However, tropane
78 compounds should generally be derivatized and analyzed as trimethylsilyl derivatives because target
79 compounds underwent dehydration in GC inlet at high temperature (Namera et al., 2002). LC
80 analysis of tropane compounds overcomes the problem related to thermolysis. Compared with
81 LC-UV light detector or diode array detector (LC-DAD) (Rancic & Spasic, 2009), LC coupled to
82 tandem mass spectrometry (MS/MS) provides higher sensitivity and selectivity (Hogendoorn &
83 Zoonen, 2000). Good chromatographic resolution, higher than 1.5 among tropane compounds
84 (except enantiomers) could be obtained without extra optimization of LC-MS/MS conditions

(Jakabová et al., 2012) and concentrations at ng/g levels in crops (Jandrić et al, 2011), animal feeds (Mulder, Holst, Nivarlet & Egmond, 2014) or biological samples (Ng, Ching, Chan & Mak, 2013), could be monitored. Therefore, LC-MS/MS is widely used for identification and analysis of tropane alkaloids in plants, 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 concentration of tropane alkaloids as Datura species (Jakabová et al., 2012; Temerdashev, Kolychev & Kiseleva, 2012; Adams, Wiedenmann, Tittel & Bauer, 2006; Boros et al., 2010). In this sense, the most challenge of trace analysis is the development of a sample preparation procedure. An ideal sample preparation method for trace analysis 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. 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 of plant and animal origin (Anastassiades, Lehotay, Štajnaaher, Schenck, 2003; Wilkowska & Biziuk, 2011).

Among the reported analytical methods, tropane alkaloids were usually extracted with methanol or acetonitrile and analyzed by LC-MS/MS without clean-up (Jakabová et al., 2012; Ng, Ching, Chan & Mak, 2013; Adams, Wiedenmann, Tittel, Bauer, 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 clean-up.

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, Van Dam, Zomer & Mulder, 2011).

Although there are few methods that applied the QuEChERS approach (Jandrić et al, 2011; Mol, Van Dam, Zomer & Mulder, 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 was also optimized, evaluating the effect of sorbents used during this stage, and 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

129 (GBC), primary secondary amine (PSA) and florisil were purchased from Scharlab (Barcelona,
130 Spain). Zirconia-coated silica (Z-Sep^{+}) was obtained from Supelco (Bellefonte, PA, USA).
131 Anhydrous magnesium sulfate and ammonium acetate were purchased from Panreac (Barcelona,
132 Spain). Ultrapure water was obtained by a gradient system Milli-Q water (Millipore, Bedford, MA,
133 USA).
134
135 *2.2. Instrument and Apparatus*
136 Detection of atropine and scopolamine was performed with an Agilent series 1290 RRLC
137 instrument (Agilent, Santa Clara, CA, USA) equipped with a binary pump (G4220A), an
138 autosampler thermostat (G1330B) and a column compartment thermostat (G1316C). The RRLC
139 system was coupled to an Agilent triple quadrupole mass spectrometer (6460 A) with a Jet Stream
140 electronic spray ionization (ESI) source (G1958-65138). A Zorbax plus C18 column (100 × 2.1 mm,
141 1.8 µm particle size) from Agilent (San Jose, CA, USA) was employed for separation. The
142 MassHunter (Agilent) software was used for optimization and quantification.
143 Detection of atropine and scopolamine transformation products was performed with a Thermo
144 Fisher Scientific HPLC instrument (Thermo Scientific Transcend^{TM}, Thermo Fisher Scientific , San
145 Jose, CA, EE.UU.), coupled to Thermo Fisher Scientific Orbitrap mass spectrometer (Exactive^{TM},
146 Thermo Fisher Scientific, Bremen, Germany), with a heated electrospray ionization source (HESI
147 –II). The same column used in QqQ analyses was used. The Xcalibur^{TM} version 2.2.1 from Thermo
148 Fisher Scientific with Qualbrowser and Quanbrowser was used for identification purposes.
149 Samples were ground into powder using an automatic blender (Sammic S.L., Azkoitia, Spain). A
150 WX vortex from Velp Scientifica (Usmate, Italy) was used to homogenize the samples.

151 Centrifugation was carried out in a Consul 21 centrifuge from Orto Alresa (Madrid, Spain).

152

153 *2.3. Samples collection*

154 Eight different types of samples, free of atropine and scopolamine, were obtained local
155 supermarkets located in Almería (Spain). The analyzed samples were buckwheat, buckwheat flour
156 and buckwheat pasta (*Fagopyrum esculentum)*, soy (*Glycine max)*, wheat *(Triticum)*, amaranthus
157 grain *(Amaranthus)*, chia seeds *(Salvia hispanica)* and peeled millet *(Panicum miliaceum)*. These
158 samples were selected according to EFSA`s concern regarding food control of tropane alkaloids (Ng,
159 Ching, Chan & Mak, 2013). Samples were ground into powder prior to analysis. Blank samples were
160 used for the preparation of fortified samples during the optimization of sample extraction procedure
161 and method validation.

162

163 *2.4. Sample Preparation*

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

173 *2.5. UHPLC-MS/MS Analyses*

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

181 Atropine and scopolamine were ionized at positive ESI mode and detected using selected
182 reaction monitoring (SRM) mode. Source gas temperature and sheath gas temperature were 325 °C
183 and 400°C, respectively. Source gas flow and sheath gas flow were 5 L/min and 11 L/min,
184 respectively. Nebulizer was 45 psi. Capillary and nozzle voltage were 3500 V and 500 V, respectively.
185 Retention time and MS/MS parameters are shown in Table 1.

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

189

190 **3. Results and Discussion**

191 *3.1. Optimization of UHPLC-QqQ-MS/MS*

192 The optimization of UHPLC-QqQ-MS/MS parameters was performed by direct infusion of
193 individual standard solutions of the target compounds at 5 mg/L in methanol:water (50:50, *v/v*) at a
194 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 $[M+H]^+$.

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 transition 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. Second, 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 sensitivity was 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

217 gradient profile were evaluated, setting as optimal conditions those indicated in Section 2.4.

218

219 *3.2. Optimization of the extraction procedure* Using water for dry samples prior to acetonitrile
220 extraction was used to improve the extraction efficiency of the polar atropine and scopolamine due to
221 their high water solubility (Cajka et al., 2012), which is 2.2 mg/mL and 100 mg/mL, respectively. In
222 this study, all samples were soaked by the addition of 10 mL of water because some samples, *e.g.*
223 buckwheat noodle, can be completely soaked when 10 mL of water was used. The partition behavior
224 of tropane alkaloids between organic solvent and water was pH-dependent (Miyazaki, Yomota &
225 Okada, 1993), which may have an important effect on method trueness when extraction solvents with
226 different pH values were used. Buckwheat was used as matrix, and it was spiked with the target
227 compounds at 50 µg/kg. Recoveries were compared when acetonitrile, acetonitrile with acetic acid
228 (1%, *v/v*), and acetonitrile with 0.01 M ammonium formate were used as extraction solvents.
229 According to the results shown in Figure 2, acetonitrile containing acetic acid (1%, *v/v*) was selected
230 as extraction solvent because it provides the best recovery values for the target compounds.

231 Plenty of matrix compounds from buckwheat and related products were leached into the raw
232 extracts when water and acetonitrile were used during the extraction of the target compounds. The
233 co-extracted substances could interfere the target compounds and provoke serious matrix effects.
234 Several previous studies used PSA, C18, florisil and GBC as sorbents when dispersive solid phase
235 extraction (d-SPE) was used to remove the co-extractants substances from cereals (Herrmann &
236 Poulsen, 2015; Grande-Martínez, Arrebola-Liébanas, Martínez-Vidal, Hernández-Torres &
237 Garrido-Frenich, 2015; He et al., 2015). In this study, PSA, GBC, Z-Sep^{+} and florisil were
238 investigated through recovery studies, spiking blank buckwheat samples at 10 µg/kg. As shown in

239 Figure 3A, recoveries were lower than 70% when Z-Sep$^+$ and florisil were used during the clean-up
240 stage, indicating that they were not suitable for this step because of their strong absorption affinity of
241 target compounds. Recoveries of atropine and scopolamine ranged from 84% to 86% when PSA (50
242 mg) was used, whereas recoveries of both compounds were in the range of 76%-77% using GBC (50
243 mg) as sorbent. The extraction was colorless and transparent when GBC (50 mg) was used,
244 indicating that GBC was useful to remove matrix components. Therefore, because PSA and GBC
245 provided good recovery, the amount of these sorbents were optimized since their combination could
246 provide an excellent clean-up removing a variety of matrix compounds (Anastassiades, Lehotay,
247 Štajnaaher, Schenck, 2003). The amount of PSA (5-50 mg) had little effect on recoveries of atropine
248 (82-84%) and scopolamine (77-81%) (Figure 3B). As it is shown in Figure 3C, recoveries were
249 reduced about 11% when the added amount of GBC increased from 25 mg to 50 mg, indicating that
250 25 mg of GBC was the best choice to obtain good recovery and clean-up effect. Finally, the
251 recoveries and cleaning up effect using the combination of 25 mg PSA and 25 mg GBC were tested.
252 Recoveries of scopolamine and atropine were 76±4% and 83±5% (n=3), respectively. The extracts
253 after the clean-up stage using the proposed sorbents was nearly colorless and transparent and this
254 combination was used for further experiments.

255 Injection solvent for chromatographic analysis was also investigated. Several matrix standard
256 solutions at 5µg/L, diluted with water at several ratios (10:90, 25:75, 50:50, 75:25, 90:10 (*v/v*)) and
257 without dilution, were individually analyzed by UHPLC-QqQ-MS/MS. As it can be observed in
258 Figure S1 (see Supplementary Material), bad peak shapes were obtained when matrix standard
259 solution was not diluted with water, whilst symmetrical peaks for both compounds were obtained
260 when dilution of matrix:water 50:50 and 25:75 (*v/v*) were used. Therefore, the extract obtained after

261 the clean-up step was diluted with 50% water (*v/v*) to obtain good chromatographic peaks, and lower
262 limit of detection compared to matrix:water dilution 25:75 (*v/v*).
263
264 *3.3. Method Validation*
265 Calibration curves were performed by injecting scopolamine and atropine in a matrix matched
266 standard mixture with concentrations at 0.1, 0.5, 1, 2.5, 5, 10, 25, 50 and 100 µg/L. Suitable
267 calibration curves were obtained for scopolamine and atropine with coefficients of determination (R^2)
268 higher than 0.996.
269 Matrix effect was evaluated in buckwheat and related matrices as pasta obtained from buckwheat,
270 amaranthus, millet, chia and soy. In order to check the influence of the matrix on the response,
271 several concentrations (from 0.1 to 100 µg/kg) were added to blank extracted samples, including two
272 types of buckwheat and solvent. The slopes of the calibration curves are shown in Table S1 (see
273 Supplementary Material). First, it can be noted that there is a remarkable difference between the
274 slope obtained in solvent and in matrices, observing matrix enhancement despite the dilution step
275 discussed above. Moreover similar slope values were obtained for the different matrices evaluated in
276 this study. In order to check if the slopes obtained from the tested matrices are different, an analysis
277 of covariance (ANCOVA) was used to compare the slopes of the calibration curves
278 (Garcia-Campaña, Cuadros-Rodríguez, Aybar-Muñoz & Alés-Barrero, 1997). First, it can be
279 indicated that when the slopes obtained from all the matrices were compared (excluding the solvent),
280 a significant difference (p-value lower than 0.05) was obtained for scopolamine and atropine.
281 Therefore, the use of a representative matrix is not possible for the quantification of these two
282 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 provided similar slopes (p value = 0.25), whereas the slopes obtained in amaranthus and soy (p value = 0.08), as well as millet and chia (p value = 0.14) are not statistically different. Therefore, it can be indicated that 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, millet, amaranthus), calibrations using these matrices should be used in order to perform a reliable quantification of both analytes.

Trueness was investigated through recovery trials, spiking blank samples at four levels (10, 25, 50 and 100 µg/kg) for buckwheat, as well as two levels (10 and 50 µg/kg) for millet, soy and chia. 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, Palla, Bonzanini, Bianchi & Bruni, 2011; Jandrić et al, 2011), but simpler extraction procedure was applied, and the use of internal standards was avoided (Caligiani, Palla, Bonzanini, Bianchi & Bruni, 2011).

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 at 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, Kozelj, Druzina & Stanovnik, 2013; Caligiani, Palla, Bonzanini, Bianchi & Bruni, 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

327 control implies the analysis of blank samples, matrix-matched standard solutions from 0.1 to 100
328 µg/kg, and fortified samples at 10 and 50 µg/kg. Furthermore, the retention time and the relative
329 intensities of monitored transitions in real samples were compared to those obtained by
330 matrix-matched standard solutions according to SANTE/11945/2015 (SANTE, 2015). The
331 compounds were not detected in the 8 samples analyzed above the established LODs.

332 Bearing in mind that the studied tropane alkaloids can suffer transformations due to the
333 metabolism and the environment, a total of 23 transformation products (Chen et al., 2006, Huaixia et
334 al., 2005) (shown in Table S2 (see Supplementary Material)) of atropine and scopolamine were
335 studied using high resolution Orbitrap mass spectrometry. For that, a generic method previously
336 developed was used (Gómez-Pérez et al., 2012), and the samples analyzed by UHPLC-QqQ-MS/MS,
337 were injected in the LC-Orbitrap-MS system and they were analyzed by full scan mode. The
338 identification of these transformation products was performed knowing its molecular formula and
339 molecular weight (see Table S2), and they were monitored in positive and negative ionization mode.

340 These metabolites were searched in the samples and three scopolamine transformation products
341 were putative identified. In all the cases, mass error was lower than 5 ppm. Norscopine was detected
342 in amaranthus, whereas hydroscopolamine and dihydroxyscopolamine were detected in chia, as it
343 can be observed in Figure 4, where the chromatogram, theoretical and experimental spectra of the
344 detected transformation products are shown. Further investigation should be done in order to confirm
345 identification of these compounds using analytical standards. After that, if the compounds were
346 identified, these compounds should be included in routine monitoring of these type of samples,
347 bearing in mind that although parent compounds were not detected, transformation products could be
348 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.

Acknowledgments

The authors gratefully acknowledge the Spanish Ministry of Economy and Competitiveness (MINECO) and FEDER (project ref. CTQ2015-69899-R). HC acknowledges the earmarked fund for Innovative Research Team in Chinese Academy of Agricultural Sciences (CAAS-ASTIP-2014-TRICAAS).

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Figure Captions

Fig. 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.

Fig. 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.

Fig. 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).

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

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).

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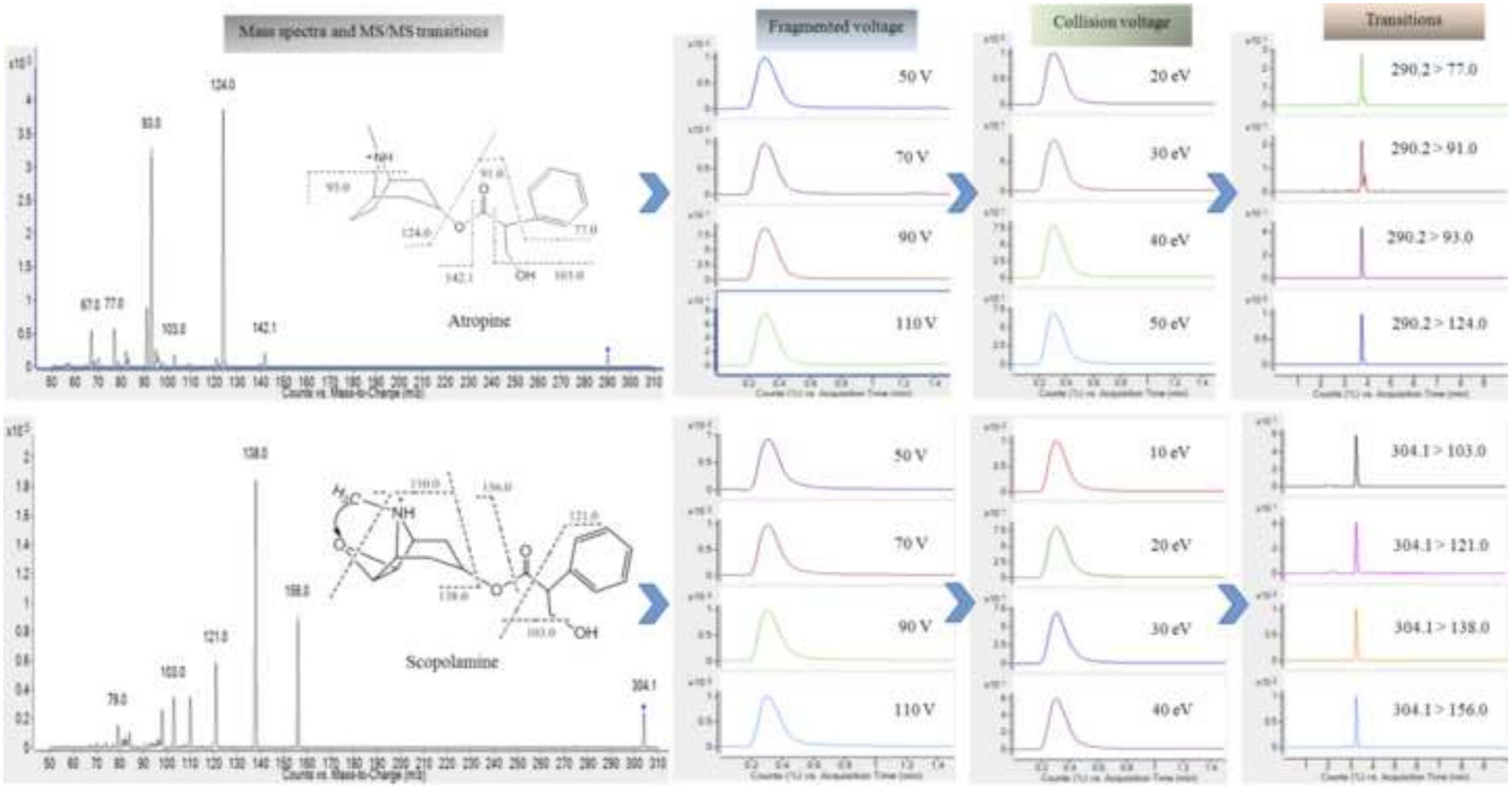

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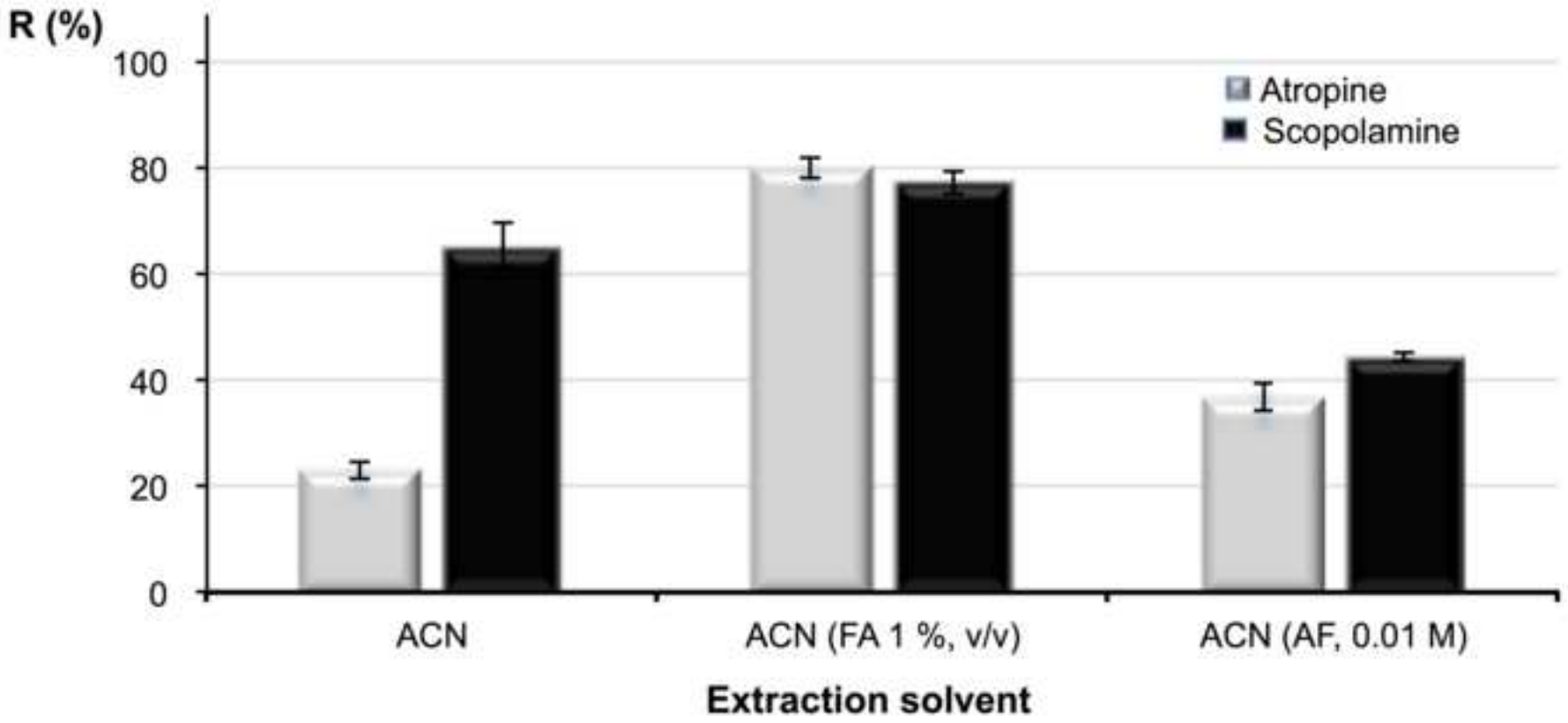

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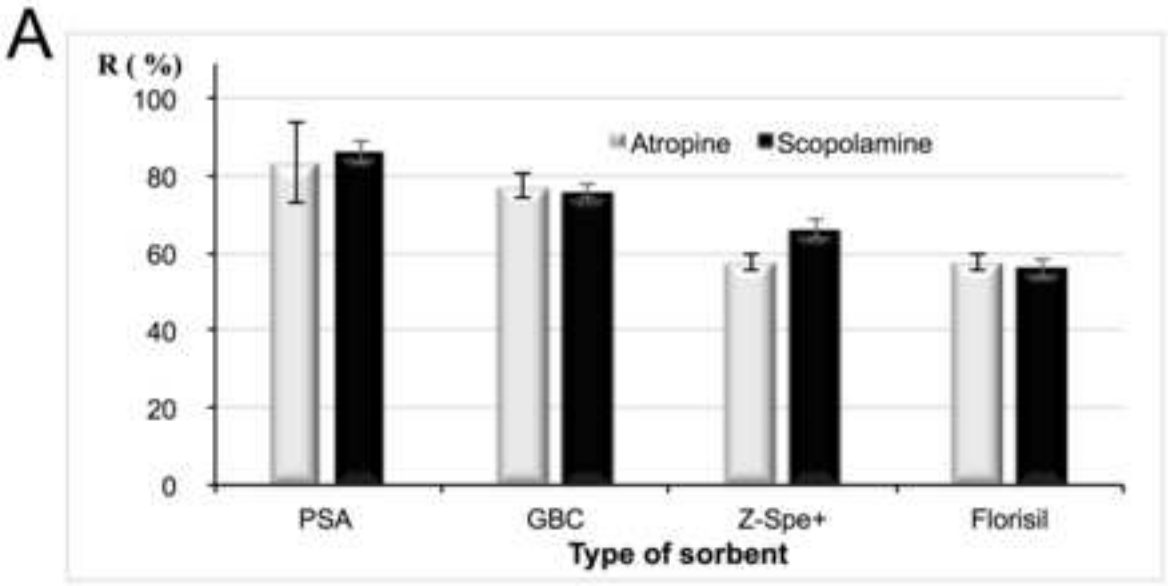

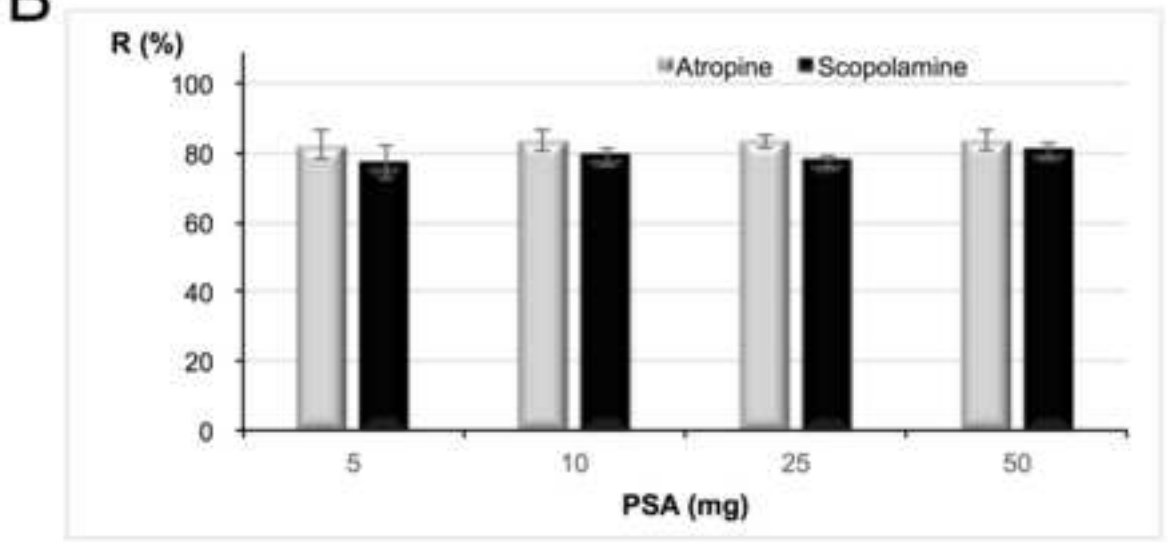

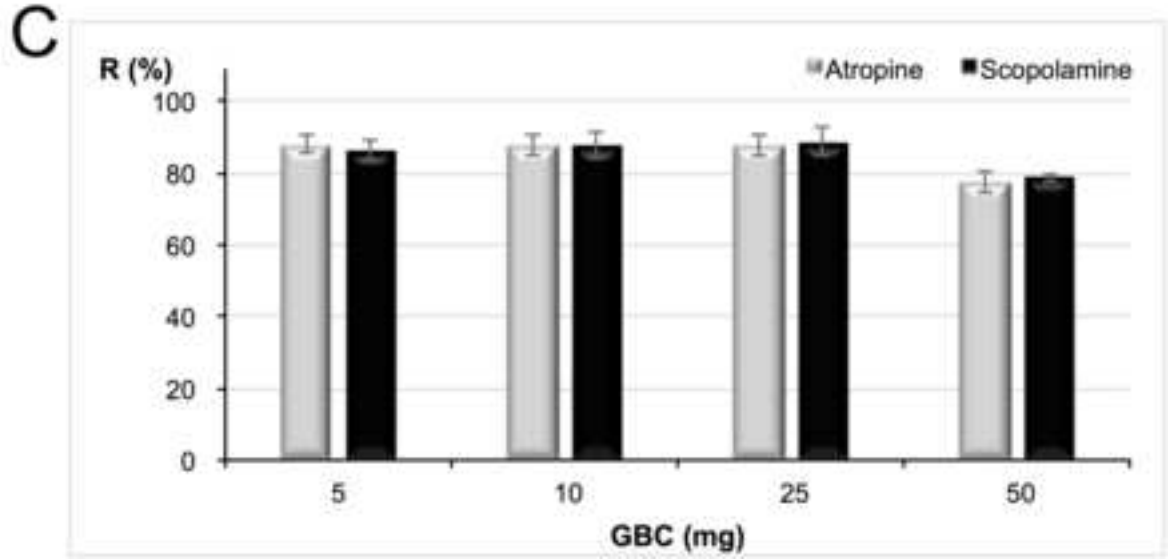

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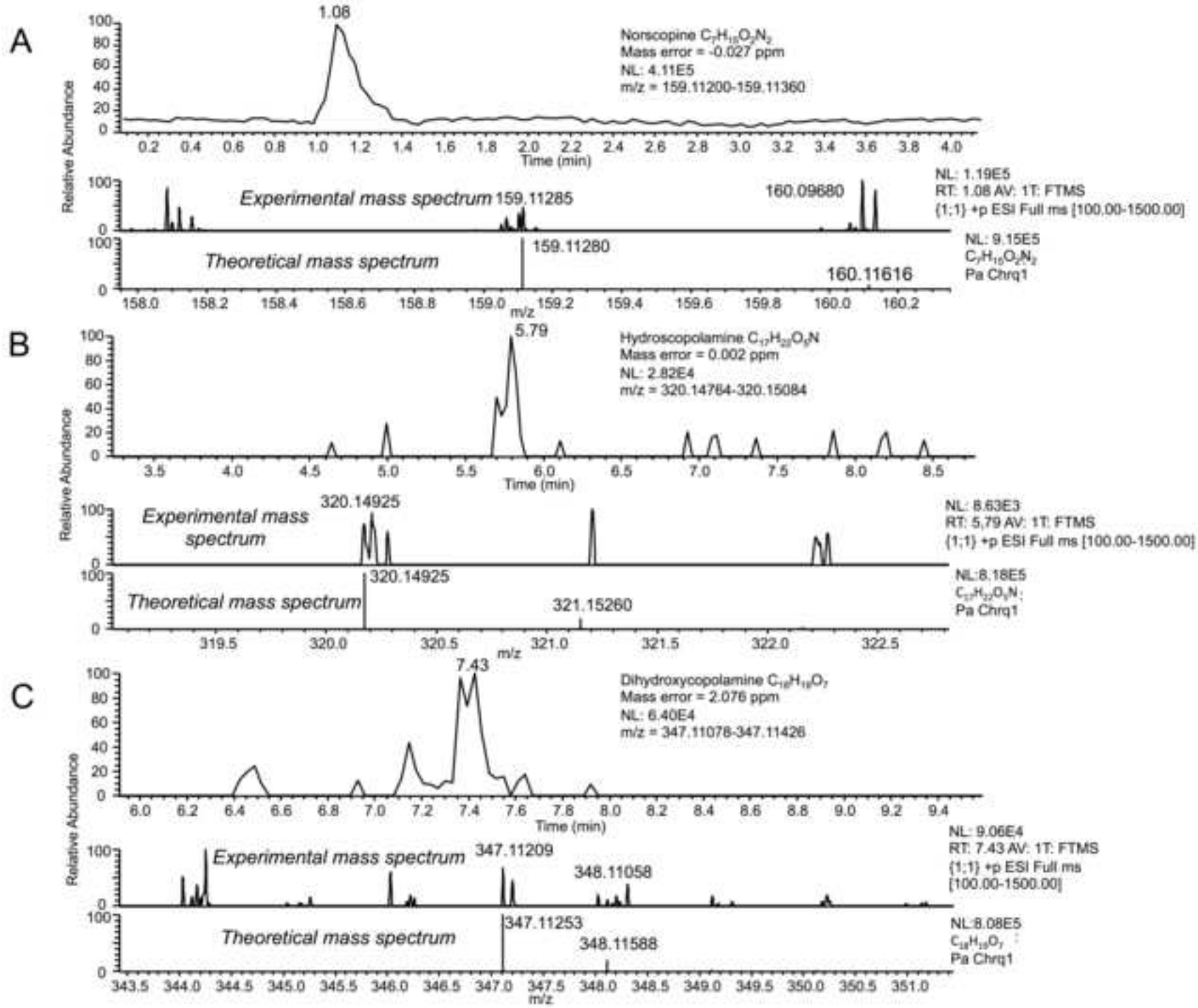

Supplementary Material

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