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Almería, May 24th 2017

Dear Editor,

Please find enclosed the manuscript entitled "Multi-analysis determination of tropane alkaloids in cereals and *Solanaceaes* seeds by liquid chromatography coupled to Exactive-Orbitrap" by Marín-Sáez et al., for consideration of publication in Journal of Chromatography A, as a full length article.

Tropane alkaloids produced mainly by *Solanaceaes* can contaminate cereal samples as buckwheat, millet, soy and linseed. These compounds have anticholinergic activity and the effect is higher in humans than in animals. Atropine and scopolamine are the most studied tropane alkaloids but there are more compounds belonging to this family, and the main aim of this study is the development of a method that allow for the multi-analysis of principal tropane alkaloids.

Herein, we described a simple and fast analytical method for the determination of the main tropane alkaloids (anisodamine, scopolamine, atropine, littorine, aposcopolamine, homatropine, apoatropine, cuscohygrine, scopoline, tropine, physoperuvine, pseudotropine, tropinone and tropane) in buckwheat, millet, soy and linseed, seeds and flours. Moreover contaminated buckwheat with *Stramonium* and *Brugmansia* seeds were analysed in order to check the reliability of the proposed procedure. This is based on solid-liquid extraction and in order to increase the sensitivity of the method, a solid phase extraction has been applied. A two coupled columns (Hilic and reverse phase) HPLC-MS-Orbitrap method was used to quantify the different samples. Therefore, the proposed method provided a fast method, which allow for the determination of a large range of tropane alkaloids. The addition of SPE as preconcentration and clean-up stage offer high sensitivity if necessary. The method provides good performance characteristics such as recoveries, linearity, intraday precision, interday precision, detection, identification and quantification limits and the Orbitrap analyzer has proved to be a powerful tool with a high reliability in the identification of these toxic compounds in complex matrix. Following this methodology, the routine analysis of samples is available, and in addition to well-known tropane alkaloids as atropine and scopolamine, other compounds were also determined.

Finally, we would like to indicate that this work tries to cover the gap highlighted by EFSA in relation to the analysis of these toxic compounds in these type of matrices.

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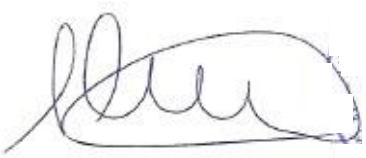

Yours Sincerely,

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Highlights

- Simultaneous analysis of tropane alkaloids using LC-Oribtrap, coupling two LC columns
- Extraction of target compounds using SLE and SPE as clean-up and purification stage
- Determination of tropane alkaloids in buckwheat, millet, soy and linseed samples
- Contaminated buckwheat sample with *Datura Stramonium* and *Brugmansia Arborea* seeds

MULTI-ANALYSIS DETERMINATION OF TROPANE ALKALOIDS IN CEREALS AND SOLANACEAES SEEDS BY LIQUID CHROMATOGRAPHY COUPLED TO EXACTIVE-ORBITRAP

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ABSTRACT

Tropane alkaloids are a wide group of substances that comprises more than 200 compounds occurring especially in the *Solanaceae* family. The main aim of this study is the development of a method for the analysis of the principal tropane alkaloids as atropine, scopolamine, anisodamine, tropane, tropine, littorine, homatropine, apoatropine, aposcopolamine, scopoline, tropinone, physoperuvine, pseudotropine and cuscohygrine. For that, a simple solid-liquid extraction was optimized and a liquid chromatographic method coupled to Exactive-Orbitrap was developed. The method was validated obtaining recoveries in the range of 60-109% (except for some compounds in soy), precision values (expressed as relative standard deviation) lower than 20% and detection and quantification limits equal to or lower than 2 and 3 µg/kg respectively. Finally, the method was applied to the analysis of different type of samples as buckwheat, linseed, soy and millet, obtaining positives for anisodamine, scopolamine, atropine, littorine and tropinone in a millet flour sample above the quantification limits, whereas atropine and scopolamine were detected in a buckwheat sample, below the quantification limit. Contaminated samples with *Solanaceaes* seeds (*Datura Stramonium* and *Brugmansia Arborea*) were also analysed, obtaining concentrations up to 693.5 µg/kg (scopolamine) for contaminated samples with *Brugmansia* seeds and 1846.9 µg/kg (atropine) when samples were contaminated with *Stramonium* seeds.

Keywords: tropane alkaloids, LC-MS-Orbitrap, cereals, *Solanaceaes* seeds

1. INTRODUCTION

Tropane alkaloids are large number of compounds which can be detected in numerous species of plants as *Erythroxylaceae, Proteaceae, Euphorbiaceae, Rhizophoraceae, Convolvulaceae* and *Cruciferae* [1], being more abundant in *Solanaceaes* plant family [2]. Although there are many cases of intended ingestions of parts of these plants for recreative [3] or medicinal use at low doses [4], normal ingestions occur when toxic plant parts (the highest concentration are usually detected in seeds [5]) are accidentally mixed with crops like grains, cereals, legumes and pseudocereals during harvest or processing [1,6], or ingested in herbal tea [7,8].

The most abundant tropane alkaloids are (-)-hyoscyamine, that racemizes to atropine under the extraction conditions used in pharmaceutical process [2,9], and scopolamine, although there are more than 200 compounds present at lower concentrations [2,10,11], mainly produced by *Solanaceaes* plants (**Figure 1**) [12,13]. The principal studied compounds are atropine and scopolamine that have pharmacological effects because the inhibition of muscarinic acetylcholine receptors in the central nervous system (CNS) and the autonomic nervous system (ANS) [2]. They can produce dryness of the mucosa in the upper digestive and respiratory tract, pupil dilation (mydriasis), alterations in the heart rate, restlessness, irritability, ataxia, seizures, and respiratory depression [14].

Moreover, there are other tropane alkaloids (**Figure 1**) as anisodamine [11,12,15-18], which has the same effects but has less potency and toxicity than atropine and scopolamine, so it is more interesting for its use as a medicine, littorine [12,15,18,19], homatropine [15,17,19,20], apoatropine [11,15], tropine [12,15,20] and tropinone [15] were evaluated previously. However there are scarce studies [11,12,15,17-19] focused on the multi-analyte analysis of tropane alkaloids as it has been required by European Food Safety Agency (EFSA) [2], and there are not any method that enables the analysis

of a large number of alkaloids in food and feed, where tropane alkaloids can be found at concentrations higher than 100 µg/kg [2], except the recent study requested by EFSA [15].

The extraction procedures will depend on the chemical properties of the compounds to be analysed and specifically on the tropane alkaloids solubility [21] that is related to the pH conditions, being more soluble in acid solutions [2]. The most common extraction techniques are solid-liquid extraction (SLE), liquid-liquid extraction (LLE) or solid-phase extraction (SPE). LLE was usually used to extract biological samples like plasma and urine [19,22,23], being the samples mixed with acetonitrile for deproteinization and an internal standard was utilized. The SPE extraction was used when the sample is liquid (blood and urine), and the compounds retained in the SPE cartridge were eluted with a mixture of ammonia (25%)/methanol (1:19, *v/v*) [24] or dichloromethane [25], whereas for the extraction from plant material, compounds were eluted with 0.2% trifluoroacetic acid (TFA), followed by 7.5 mL of methanol (0.2% in TFA):water (98:2, *v/v*) [11]. Moreover, SPE is used during clean-up stage [14,15]. SLE is the most widely extraction technique used for plant material or matrices like buckwheat. Due to the polar nature of tropane alkaloids, the target compounds were extracted using alcoholic solutions [26,27], mixtures of ethanol or methanol and water [8,15] or mixtures of water and acetonitrile [15,20]. In general, when these mixtures were used, the pH of the water is $\geq$ 7, since most of the tropane alkaloids, except apoatropine, cocaine and the truxillines, are fairly water soluble at acidic pH [21].

The chromatographic analysis can be performed by electrophoretic techniques [28] or gas chromatography [21,28-30], although liquid chromatography (LC) is the most widely used [15,19,26]. Triple quadrupole (QqQ) is mostly utilized [12,17], because it provides lower detection and quantification limits than other analysers, which were less

frequently used as time of flight-mass spectrometry (TOF-MS) [31], quadrupole-time of flight (QTOF) [10] or quadrupole-Orbitrap (Q-Orbitrap) [20,32]. These high resolution mass spectrometry (HRMS) analysers are commonly used in profiling studies of *Solanaceaes* species, although they were also used for the multi-analysis of a huge number of toxins [33] and some of this including few tropane alkaloids [20]. Several parameters such as retention time windows (RTW), accurate mass error (<5ppm) and isotopic pattern can be used when HRMS is utilized for the identification of target compounds. Furthermore, they can provide the identification of unknown compounds [34].

Due to there are few studies focused on multi-analyte tropane alkaloids determination, the objective of this manuscript is the development of a new method by LC-MS-Orbitrap that allows for the determination of different tropane alkaloids present naturally in *Solanaceaes* seeds and, therefore, they can be detected in cereals and oilseeds, studying most of the tropane alkaloids indicated by EFSA [2]. In addition, the developed method has been applied to the analysis of samples belonging to the principal seeds matrices that can be contaminated as buckwheat, linseed, soy and millet as well as intentionally contaminated matrices with *Datura Stramonium* and *Brugmansia Arborea* seeds were also determined.

2. MATERIALS AND METHODS

2.1. Reagents and chemicals

Scopolamine, atropine, anisodamine, tropane, tropine, homatropine and tropinone reference standards were purchased from Sigma-Aldrich (St. Louis, MO, USA); littorine, apoatropine, cuscohygrine and aposcopolamine reference standards were obtained from Santa Cruz Biotechnology (Heidelberg, Germany) and scopoline,

112 physoperuvine and pseudotropine were purchased from Chemfaces (Wuhan, China). All
113 compounds present a purity ≥ 98% except tropine (≥ 97%).
114 Stock standard solutions of the compounds (200 mg/L) were individually prepared by
115 exact weighing of the solid substances and dissolved in 50 mL of methanol (HPLC LC-
116 MS grade, J.T. Baker, Deventer, Holland), and they were stored at -20 °C in the dark.
117 Then a mixture working standard solution was prepared at 5 mg/L with methanol and
118 stored in screw-capped glass tubes at -20 °C in the dark. The stock standard solutions
119 were stable up to one year, whereas working standard solutions were prepared every
120 two months.
121 LC-MS grade acetonitrile was obtained from VWR Chemicals (Radnor, PE, USA)
122 and water from J.T. Baker. Formic acid (Optima LC-MS) was supplied from Fisher
123 Scientific (Geel, Belgium) while ammonium hydroxide 25% solution was obtained from
124 Fluka (Steinheim, Suiza). Graphitized black carbon (GBC) and primary secondary
125 amine (PSA) were purchased from Scharlab (Barcelona, Spain). Anhydrous magnesium
126 sulphate, ammonium acetate and acetic acid were purchased from Panreac (Barcelona,
127 Spain).
128 For the SPE preconcentration and clean-up stage OASIS MCX 150 mg/6 mL (Waters,
129 Milford, MA, USA) and a Strata-X-C 200 mg/3 mL (Phenomenex, Torrance, CA, USA)
130 cartridges were used.
131 A 0.22 µm nylon syringe filters provided by Agilent Technologies (Santa Clara, CA,
132 USA) were used.
133
134 *2.2. Instrument and apparatus*
135 The samples were ground into powder using an automatic blender (Sammic S.L.,
136 Azkoitia, Spain). To homogenize the samples, a WX vortex from Velp Scientifica

(Usmate, Italy) was used and for the extraction, a Reax 2 rotatory shaker from Heidolph (Schwabach, Germany) was utilized. A Consul 21 centrifuge from Orto Alresa (Madrid, Spain) was used for centrifugation.

Detection of the compounds was performed with a Thermo Fisher Scientific Transcend 600 LC (Thermo Scientific Transcend™, Thermo Fisher Scientific, San Jose, CA, USA).

A Zorbax Eclipse Plus C_{18} column (100 × 2.1 mm, 1.8 µm particle size) and a Zorbax Eclipse Plus HILIC (100 × 2.1 mm, 3.5 µm particle size) were used for separation (Agilent Technologies).

A mass spectrometer Orbitrap Thermo Fisher Scientific (Exactive™, Thermo Fisher Scientific, Bremen, Germany) with electrospray interface (ESI) (HESI-II, Thermo Fisher Scientific, San Jose, CA, USA) in positive mode was used for the detection, identification and quantification purposes. The following parameters were applied: spray voltage, 4 kV; sheath gas (N_2, > 95%), 35 (adimensional); auxiliary gas (N_2, > 95%), 10 (adimensional); skimmer voltage, 18 V; capillary voltage, 35 V; tube lens voltage, 95 V; heater temperature, 305 °C; capillary temperature, 300 °C.

For the mass spectra acquisition, first a full-scan MS, (ESI^+), without fragmentation (higher collisional dissociation (HCD) collision cell was switched off), at mass resolving power = 25,000 FWHM, was used to obtain the molecular ion of each compound. Then, fragments were obtained using all-ion fragmentation (AIF) mode, ESI^+, with fragmentation (HCD on, collision energy = 30 eV), at mass resolving power = 10,000 FWHM.

Finally, the chromatograms were processed using Xcalibur™ version 2.2, with Quanbrowser and Qualbrowser, and Mass Frontier™ 6.0 (Thermo Fisher Scientific, Les Ulis, France).

162

163 *2.3. Samples collection*

164 Samples were obtained from local supermarkets located in Almería (Spain). The
165 analysed samples were buckwheat, buckwheat flour and buckwheat pasta (*Fagopyrum*
166 *esculentum)*, soy and soy flour (*Glycine max)*, peeled millet and millet flour *(Panicum*
167 *miliaceum)* and linseed and linseed flour (*Linum usitatissimum*), which were selected
168 according to EFSA`s concern regarding food control of tropane alkaloids [2] and
169 previous studies [35]. Before extraction, samples were ground into powder. Blank
170 samples were used for the preparation of fortified samples.

171 Two *Solanaceaes* seeds, *Datura Stramonium* [30] and *Brugmansia arborea* [36] plant
172 seeds, used to contaminate cereal samples, were collected from Huelva (Spain). For the
173 contamination of the samples, two methods were applied: 1 g of *Stramonium* and
174 *Brugmansia* seeds was mixed with 1 kg of sample, bearing in mind previous studies
175 [30,35], and 0.1 g for *Stramonium* seeds and 0.5 g for *Brugmansia* seeds were mixed
176 with 1 kg of sample (approximately 1 seed of each plant in 100 g of sample). The
177 mixture was homogenized during 2 hours in a rotatory agitator before aliquots were
178 collected to perform the extraction of the target compounds.

179

180 *2.4. Sample preparation*

181 A simple solid-liquid extraction procedure, based on previous method [8], was
182 employed. Briefly, one gram of sample was mixed with 10 mL of a solution containing
183 water (0.5% acetic acid):methanol (1:2 *v/v*) for 1 minute in a vortex. Then, it was mixed
184 for 30 minutes in a rotatory agitator and after that, the mixture was centrifuged at 5000
185 rpm for 10 min. The supernatant was passed through a strong cationic exchange SPE
186 cartridge, previously conditioned with methanol and methanol:water 1% acetic acid (2:1

187 *v/v*), washed with methanol:water 1% acetic acid (2:1 *v/v*) and the compounds were
188 eluted with methanol containing 3% of ammonium hydroxide solution (25%). The
189 extract was evaporated under nitrogen flow and reconstituted in 500 µL of
190 methanol:water 0.1% formic acid (50:50 *v/v*). Finally, the sample was filtered into a
191 0.22 µm nylon syringe filter and injected into the LC system.
192
193 *2.5. LC-Orbitrap-MS analyses*
194 The chromatographic separation was carried out employing a binary mobile phase
195 with acetonitrile (A) and an aqueous solution of formic acid (0.1%, *v/v*) (B) at flow rate
196 of 0.2 mL/min. The gradient elution started at 90% of A and held 1 min at flow rate of
197 0.2 mL/min. This composition was reduced to 70% A in 4 min, and then to 50% A in 2
198 min and finally to 5% A at 9 min. This composition was held for 1 min. The
199 composition was increased to 50% A in 2 min and was returned to the initial conditions
200 in 2 min, equilibrating the stationary phase during 4 min. The total running time was 18
201 min. Injection volume was 10 µL and column temperature was kept at 25°C (room
202 temperature).
203 The compounds were ionized with electrospray ionization (ESI) in positive mode.
204 Retention time and MS/MS parameters are shown in **Table 1**.
205
206 *2.6. Method validation*
207 The method was validated following the indications of the SANTE guidelines [37]
208 and the AOAC Guidelines for Dietary Supplements and Botanicals [38]. Parameters
209 evaluated were: linearity, matrix effect, trueness in term of recoveries, precision intra
210 and interday and the detection (LOD), identification (LOI) and quantification (LOQ)
211 limits.

212 Linearity was appraised with a matrix-matched calibration, spiking blank extracts
213 samples at the concentration of 0, 0.5, 1, 2.5, 5, 10, 25, 50 and 100 µg/kg.
214 To evaluate the matrix effect, matrix-matched calibrations of the different matrices
215 (buckwheat, millet soy and linseed) and in solvent were prepared at concentration
216 between 0.5-100 µg/kg and compared.
217 Trueness was investigated through recovery trials, spiking blank samples at three
218 levels (1, 5 and 50 µg/kg) for buckwheat, millet, soy and linseed. Each fortified
219 concentration was repeated five times.
220 Precision was performed in terms of repeatability (intra-day precision) and
221 reproducibility (inter-day precision), expressed as relative standard deviation (RSD, %).
222 For intra-day precision, five replicates at three concentration levels (1, 5 and 50 µg/kg)
223 were evaluated. For inter-day precision, spiked blank samples were tested at the same
224 concentration of the intra-day precision for buckwheat and at the concentration of 5
225 µg/kg for the other matrices, during 5 days.
226 Finally for the estimation of the LOD, LOI and LOQ, extracted blank samples before
227 and after the SPE process were spiked at low concentrations between 0.1 to 25 µg/kg.
228 The LOD was defined as the minimum concentration at which the characteristic ion is
229 monitored with a mass error lower than 5 ppm; the LOI as the minimum concentration
230 at which the characteristic ion and one fragment at least is monitored with a mass error
231 lower than 5 and 10 ppm respectively, and the LOQ as the minimum concentration at
232 which the characteristic ion and one fragment at least is monitored with a mass error
233 lower than 5 and 10 ppm respectively, providing good linearity and recoveries.
234

235 **3. Results and discussion**

236 *3.1. Optimization of LC-Orbitrap-MS*

First the MS conditions were optimized. Full-scan MS (ESI+) was applied to obtain the characteristic ion for each compound. For this purpose, the target compounds individually prepared at 1 mg/L were injected into the LC-Orbitrap system. The characteristic ions were in every case the protonated molecular ion (**Figure 2**). Then, fragments were obtained using all-ion fragmentation (AIF) in positive mode, setting the collision energy at 30 eV. The fragments obtained were picked out according to their sensitivity and their formulas were proposed by fragmentation of its weakest links, using the Massfrontier software in some cases. Then the fragments were proposed according to the parent structure of the compound (**Figure 2**).

The most frequent and common break of tropane alkaloids is produced by the oxygen of the ester of the structure, leaving only the tropane ring. Thus, and depending of the atoms tied to the tropane ring, the mass of the product ions obtained could be *m/z* 124.1121 (if nothing else is connected with the ring as atropine, littorine, homatropine, apoatropine and pseudotropine), *m/z* 138.0913 (when an epoxy ring is connected; scopolamine and aposcopolamine) or *m/z* 140.1070 (if there is a hydroxyl group; anisodamine). Other common fragmentations are the oxygen of the ester and the nitrogen of the tropane ring, that generates the fragment *m/z* 93.0699 (atropine, littorine, homatropine, apoatropine, tropine, physoperuvine and pseudotropine), the link of the ester with the α-carbon, that provides the fragment *m/z* 103.0542 (scopolamine, aposcopolamine and apoatropine) and finally the ester link, that gives the fragment *m/z* 156.1019 (scopolamine) or *m/z* 142.1226 (littorine and homatropine), (see **Table 1** and **Figure 2**). For compounds with the same characteristic ion, as atropine and littorine, which also have similar retention time (6.69-6.90 and 6.60-6.81 minutes respectively) this fragment (*m/z* 142.1226) allow their quantification separately.

Then different columns were tested: Hypersil Gold aQ (100x2.1 mm, 1.9µm) (Thermo Fisher Scientific, Bremen, Germany), Zorbax Eclipse Plus C8 (100x2.1 mm, 1.8µm) (Agilent Technologies), Hypersil Gold Phenyl (100x2.1 mm, 1.9µm) (Thermo Fisher Scientific, Bremen, Germany), Zorbax Hilic plus (100x2.1 mm, 3.5 µm) (Agilent Technologies) and Zorbax Eclipse Plus C18 (100x2.1 mm, 1.8µm) (Agilent Technologies). When these columns were used, the separation is good for high molecular weight compounds but for the low molecular weight substances (tropine, pseudotropine, cuscohygrine, physoperuvine and tropinone) the separation is bad and the retention time is too short (minute 1). This could make that the compounds eluted next to the compounds present in the matrices and strong matrix effect could be presented. For this reason, the combination of two columns, a Zorbax HILIC Plus and Eclipse Plus C_{18} column was used, obtaining the best peak forms and suitable retention times, ranging from 6.26 (apoatropine) to 11.49 (cuscohygrine) min.

3.2. Optimization of the extraction procedure

The first extraction procedure tested was a modified QuEChERS procedure previously developed [35], where the phase separation of water and acidified acetonitrile permits the extraction of atropine and scopolamine. While the recoveries obtained for the high molecular weight compounds were acceptable, for the low molecular weight and polar compounds, recoveries were lower than 40% (**Table 2**). Other extraction procedures based on the original QuEChERS methodology were checked. QuEChERS methodology with acetonitrile containing 1% formic acid and water without phases separation (no salts were added) was tested in order to avoid that the polar analytes kept in the aqueous phase, and the QuEChERS methodology with different percent of

285 water:acidified acetonitrile were checked, but the recoveries were lower than 60% for
286 most of the target compounds in both cases.

287 It is well-know that the majority of tropane alkaloids are very soluble in acid aqueous
288 solutions, especially low molecular weight compounds [21]. For this reason a possible
289 strategy to get the complete extraction of the target analytes is the use of basified
290 aqueous solutions for the migration of the compounds to the organic phase. Thus, a
291 modification of the original method employed in a previous study [35] was evaluated,
292 employing water containing 2% ammonium hydroxide solution (25%) and acetonitrile.
293 The recoveries of the low molecular weight compounds improved (20-48%) but they
294 decreased for high molecular weight compounds (**Table 2**), being lower than 63%. This
295 can be explained considering that compounds can be extracted from the solid sample to
296 the acidified aqueous phase, whereas if basified aqueous phase was used, the
297 compounds were kept in the sample.

298 Moreover, the extraction without water was tested, employing 10 mL of methanol
299 containing 0.5% acetic acid (**Table 2**), but the recoveries remained low (23-70 %),
300 because the compounds are more soluble in acidified aqueous medium than in acidified
301 methanol.

302 Knowing that the compounds were kept in the aqueous phase, an extraction procedure
303 developed by Shimshoni et al (2015) [8], was employed, using a mixture of
304 methanol:water 0.5% acetic acid (2:1 *v/v*). Following this methodology the recoveries
305 improved (79-103%) (**Table 2**) but the dilution factor of the method provoked that the
306 LOD and LOQ values were higher than those recommended by EFSA [15]. In order to
307 improve the lower limits of the method, a mixture of methanol:water 0.5% acetic acid
308 (90:10 *v/v*) or methanol 0.5% acetic acid were tested, and 10 mL of extract were

309 evaporated and redissolved in 500 µL of mobile phase, but the recoveries are lower
310 than 70% in every case.

311 An extra step of preconcentration and clean-up stage with a SPE method could be
312 applied using strong cation exchange (SCE) cartridges like Oasis MCX [15,39,40],
313 StrataX [15], SCX [14] or Cleanert PCX [24]. The Oasis MCX and StrataX were tested,
314 obtaining better result with the StrataX cartridges. For low molecular weight
315 compounds, recovery ranged from 50-80% when Oasis MCX cartridges were used,
316 whereas if Strata X cartridges were utilized recovery ranged from 75 to 93%. The
317 conditioning of the cartridge was performed with methanol and methanol:water from
318 0% to 1% acetic acid (2:1 *v/v*), providing better results when methanol:water 1% acetic
319 acid (2:1 *v/v*) was used. The washing of the cartridges was performed with the same
320 mixture used for the conditioning step. The cartridges were dried 30, 60 and 120
321 minutes under vacuum stream, choosing 1 h. Finally the elution of the compounds was
322 tested utilizing methanol with different percentages of ammonium hydroxide solution
323 (25%) from 0.5 to 3%, or methanol containing sodium hydroxide (0.05 M). The best
324 result (recoveries between 68-93%) were obtained when methanol containing 3% of the
325 ammonium hydroxide solution (25%) was used.

326 After the nitrogen flow evaporation, the extracts were recomposed with different
327 mixtures of methanol:water: 100:0 (*v/v*), 50:50 (*v/v*), 50:50 with 0.1% formic acid (*v/v*),
328 10:90 (*v/v*) and 10:90 with 0.1% formic acid (*v/v*), providing the best results when the
329 mixture of methanol:water containing 0.1% formic acid (50:50 *v/v*) was used, obtaining
330 recoveries ranging from 63-109%.

331

332 *3.3. Method validation*

Linearity (**Table 3**) was evaluated through coefficients of determination (R^2), which were higher than 0.993, as well as residuals, which were always lower than 20%. Also, **Table 3** shows that there is a high difference between the slope obtained in solvent and in the tested matrix. It can be seen that the matrix effect was similar for the majority of compounds in the tested samples except in soy, so it is possible the use of a representative matrix for quantification purposes except for soy.

Recoveries ranged from 60-109% except for soy at 1 µg/kg for tropine, pseudotropine and tropane (34-53%), as it can be observed in **Table 4**.

The precision was evaluated as intra and interday precision (**Table 4**), expressed in terms of relative standard deviation (RSD) and good values were obtained, ranging from 1 to 20%. Although some recoveries were lower than 60%, a correction factor could be used for the quantification of these compounds in this matrix considering the repeatability of the proposed method.

Lower limits were calculated and they range from 0.1 to 2 µg/kg (LOD) and from 0.5 to 3 µg/kg (LOI and LOQ). Some compounds present a lower LOQ in millet than in buckwheat, due to its lower matrix effect. Applying the developed methodology, similar limits than the recent study developed by EFSA [15] were obtained for a large group of tropane alkaloids. It is important to highlight that there are not legal limits for these compounds in food and feed, except the recent normative of the European Union about atropine and scopolamine in cereal based products for children, which set a maximum allowable content of 1 µg/kg for atropine and scopolamine [41].

It can also be observed that without the application of the SPE, for some compounds as atropine, scopolamine, anisodamine and tropane, the limits were only slightly higher than those obtained with the SPE process, whereas the other compounds had higher limits without the SPE (5-25 µg/kg) (**Table 3**). However, if low limits are not required,

the method can be applied without SPE process, simplifying the analytical procedure in routine analysis.

3.4. Real samples analysis

Samples (15 in total) analysed were soy, linseed and millet, seeds and flour, and buckwheat seeds, flour and pasta. Target compounds were only detected in buckwheat and millet flour (**Figure 3**). For the buckwheat sample, atropine and scopolamine were detected but at concentrations below the LOQ and for the millet flour sample the compounds detected were anisodamine, scopolamine, atropine, littorine and tropinone, being the highest concentration of 23.4 µg/kg for scopolamine (**Table 5**).

In addition, contaminated samples with *Datura Stramonium* and *Brugmansia Arborea* according to previous studies [9,30] were evaluated (see section *2.3. Sample collection*). The concentration obtained (**Table 5**) shown that the largest concentrations were different for both seeds. For *Brugmansia* seeds, the highest concentrations were obtained for anisodamine (114.6 µg/kg), atropine (291.9 µg/kg), scopolamine (693.5 µg/kg) and homatropine (337.4 µg/kg) and all the compounds included in this study were detected except aposcopolamine, apoatropine, cuscohygrine, tropinone and tropane. When samples were contaminated with *Stramonium* seeds, the compounds detected at highest concentrations were scopolamine (89.7 µg/kg), littorine (139.0 µg/kg) and atropine (1846.9 µg/kg), whereas aposcopolamine, cuscohygrine and tropane were not detected. The highest concentration was obtained for scopolamine at the samples contaminated with *Brugmansia* and atropine (much higher than the rest of compounds) for *Stramonium*. Even with the small amount of *Solanaceaes* seeds plant added (10 and 50 mg per 100 g matriz ≈ 1 seeds of *Datura Stramonium* and *Brugmansia Arborea* respectively) the concentration in tropane alkaloids was higher than 100 µg/kg

for atropine (*Stramonium* and *Brugmansia*), scopolamine and homatropine (*Brugmansia*), which exceed the limits set by European Union [41].

4. Conclusions

The proposed method provides a rapid and simple determination of a large number of tropane alkaloids present in the *Solenaceaes* plants, and it can be used to evaluate the possible contamination of different matrices with these compounds. For this, a solid-liquid extraction with a SPE purification step process and the LC-Orbitrap-MS analysis were used. The validation parameters were suitable for the compounds and matrices included in this study, except the recoveries obtained for some compounds in soy at low concentrations. The LOD and LOQ values allow for the determination at very low concentration of tropane alkaloids in real samples. A total of 15 real samples were analyzed using the developed method, detecting other tropane alkaloids in addition of atropine and scopolamine as anisodamine, littorine and tropinone. Finally the contaminated samples shown that the concentration obtained with a low quantity of *Solanaceaes* seeds added were higher than the established limits.

Acknowledgments

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Figure captions

Figure 1: Scheme of tropane alkaloid biosynthesis pathways (Adapted from references 12 and 13).

Figure 2: Characteristic and fragment ions mass spectrum of each target compound.

Figure 3: Chromatogram and mass spectrum of the positive millet flour sample: A) Extracted ion chromatogram of scopolamine (23.4 µg/kg); B) Extracted fragment ion chromatogram at *m/z* 156.1022; C) Experimental mass spectrum of scopolamine, and D) Theoretical mass spectrum of scopolamine

Figure

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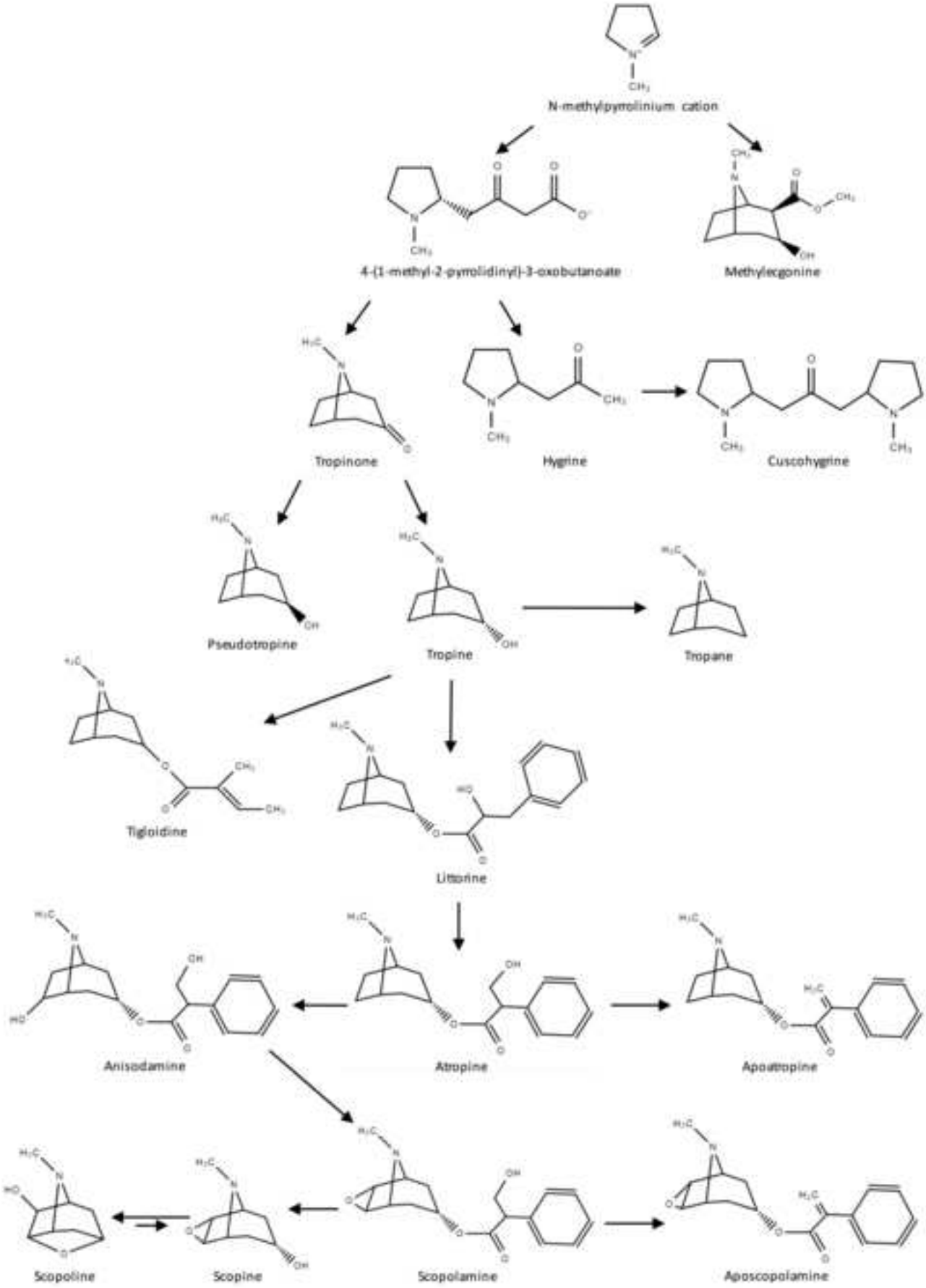

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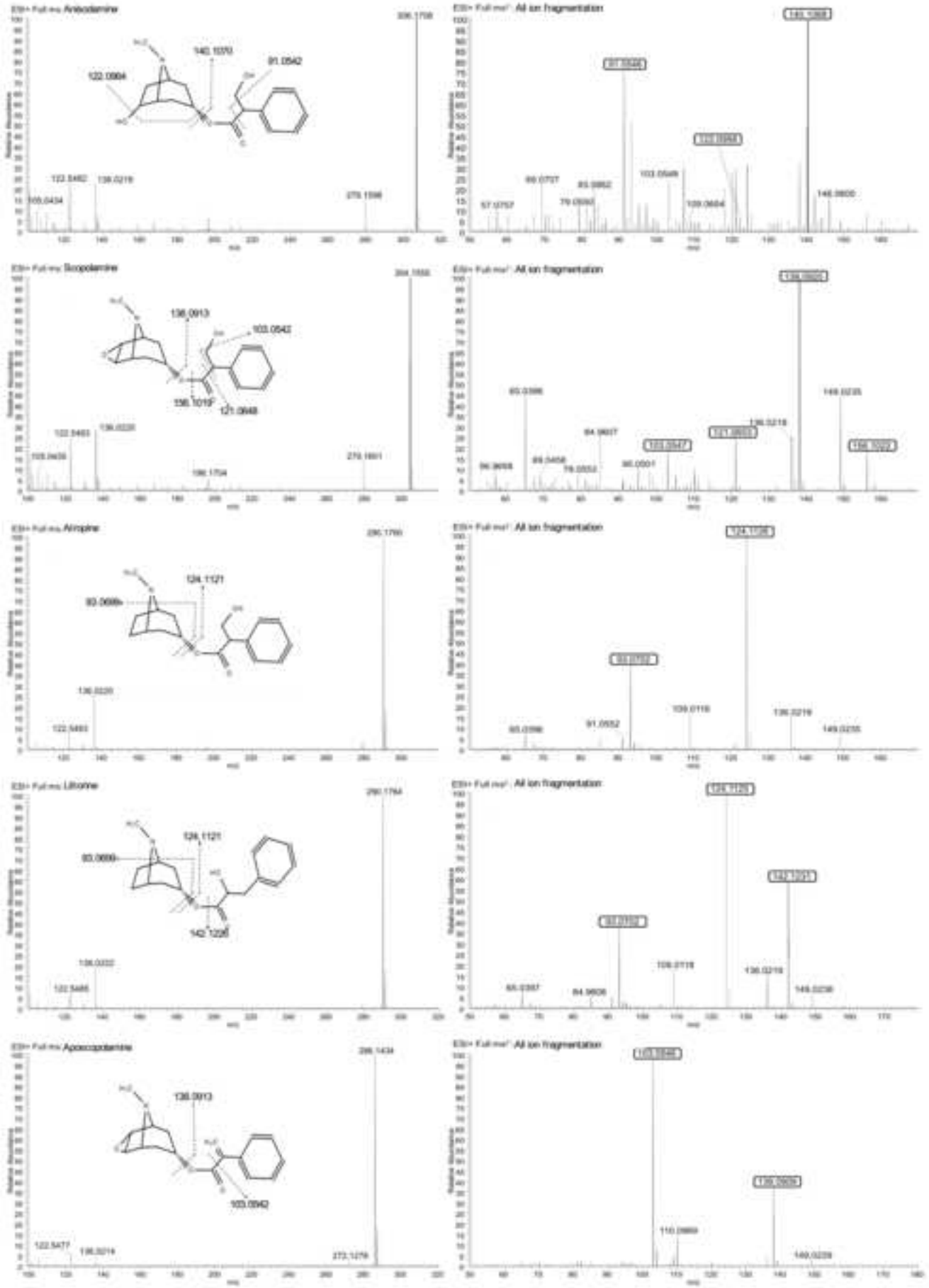

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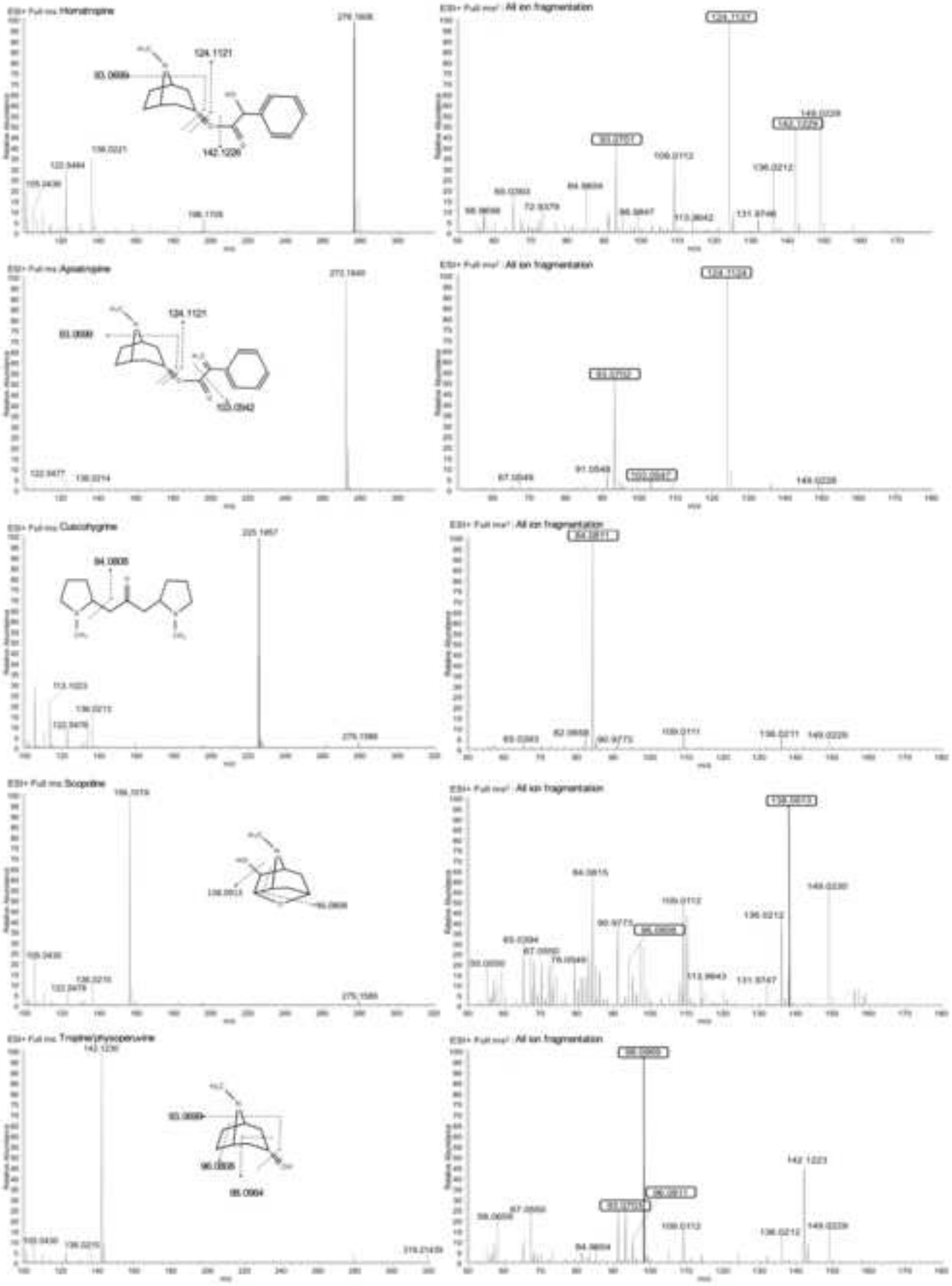

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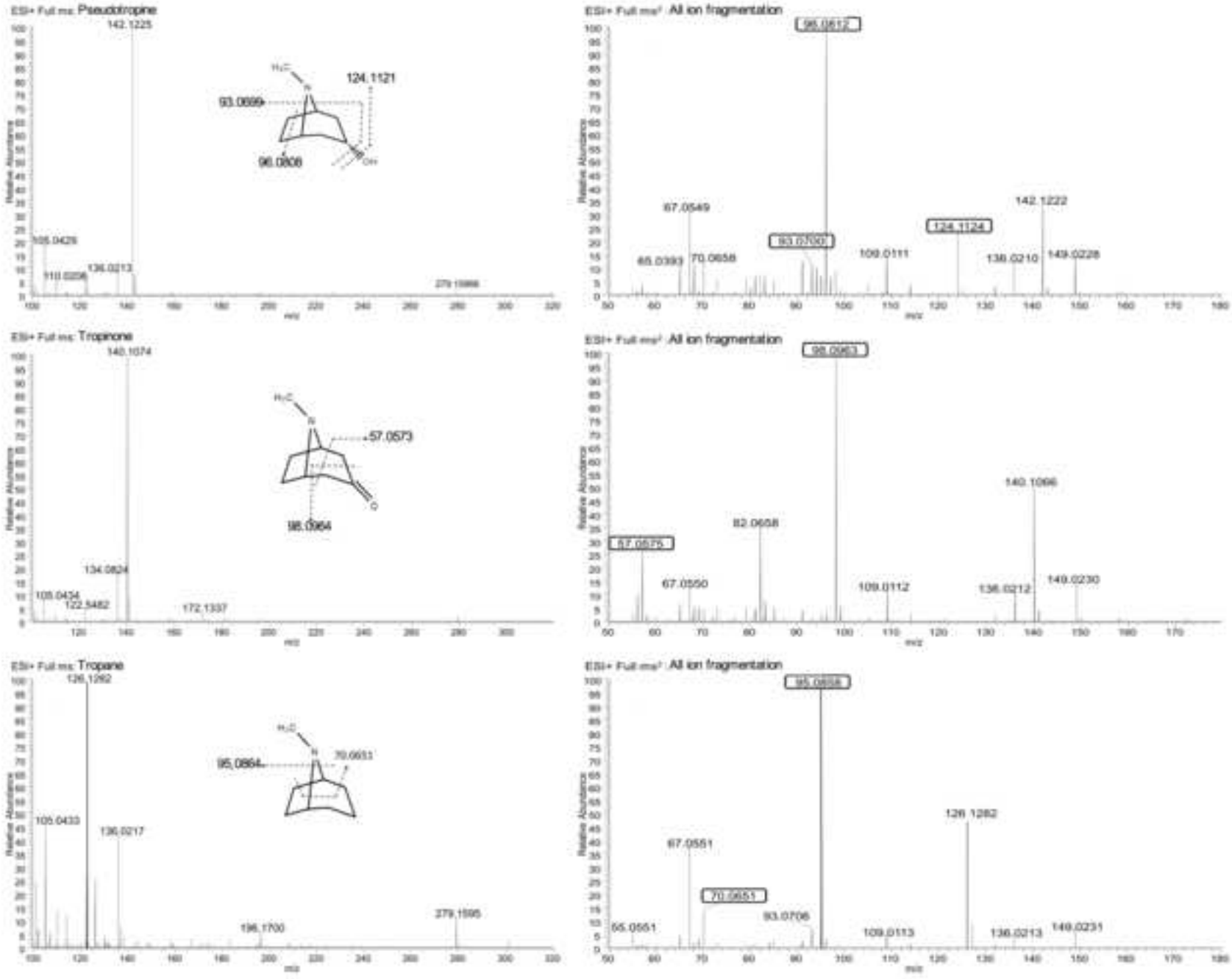

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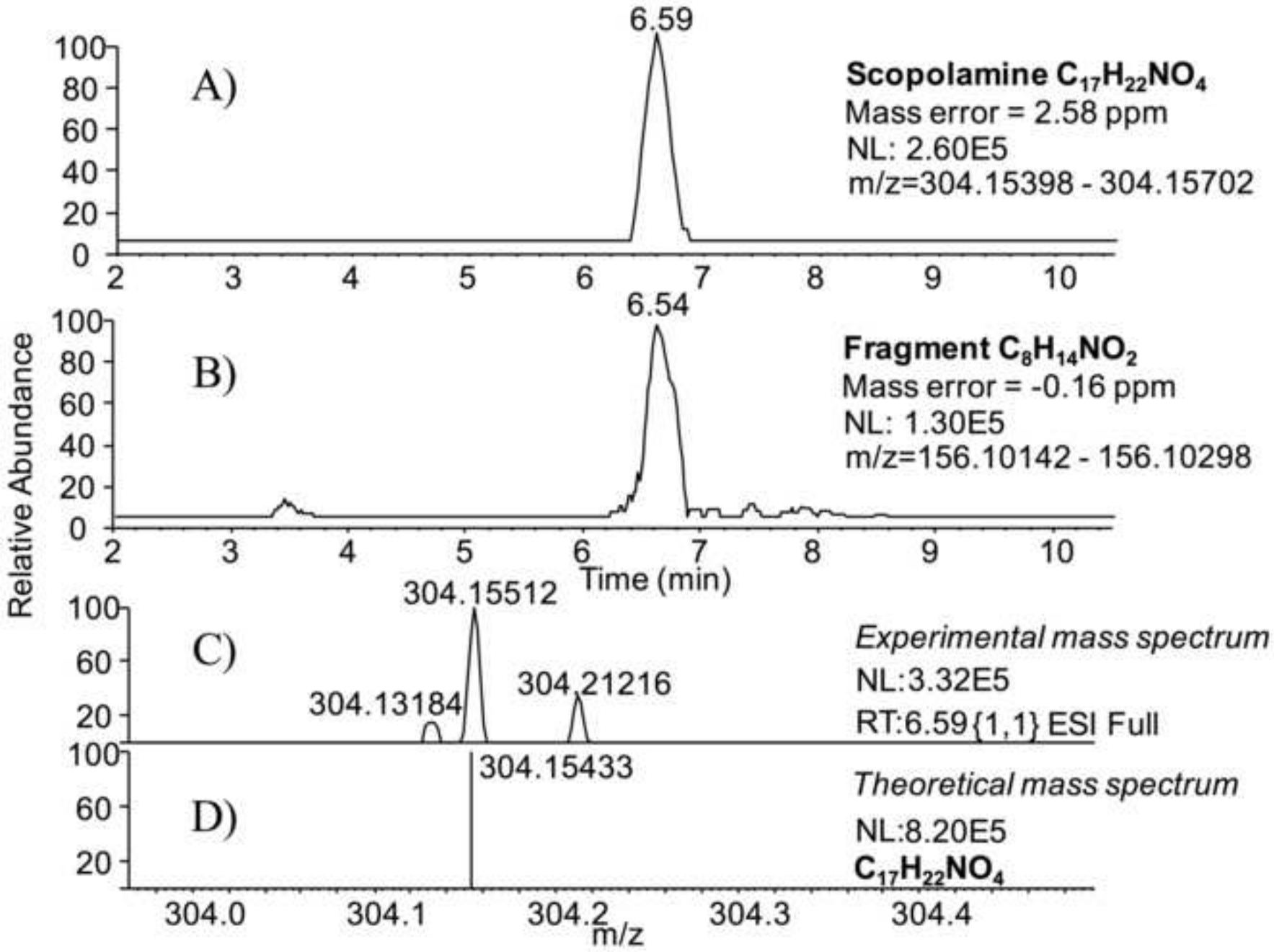

Tables

Table 1: Retention time windows (RTWs) and MS/MS parameters for the target compounds

Compound	RTW (min)	Precursor ion		Fragments		
		Exact mass	Mass error (ppm)	Exact mass	Molecular formula	Mass error (ppm)
Anisodamine	6.58-6.96	306.1699	2.66	140.1070	$C_8H_{14}NO$	-0.93
				91.0542	C_7H_7	4.54
				122.0964	$C_8H_{12}N$	-4.64
Scopolamine	6.75-6.98	304.1543	3.83	138.0913	$C_8H_{12}NO$	4.84
				121.0648	C_8H_9O	4.61
				156.1019	$C_8H_{14}NO_2$	1.88
Atropine	6.69-6.90	290.1751	3.21	103.0542	C_8H_7	4.98
				124.1121	$C_8H_{14}N$	4.46
				93.0699	C_7H_9	3.36
Littorine	6.60-6.81	290.1751	4.58	124.1121	$C_8H_{14}N$	3.42
				142.1226	$C_8H_{16}NO$	3.09
				93.0699	C_7H_9	3.79
Aposcopolamine	6.28-6.61	286.1438	-1.30	103.0542	C_8H_7	4.20
				138.0913	$C_8H_{12}NO$	-2.90
Homatropine	6.68-6.88	276.1594	4.27	124.1121	$C_8H_{14}N$	4.95
				142.1226	$C_8H_{16}NO$	2.47
				93.0699	C_7H_9	2.72
Apoatropine	6.26-6.60	272.1645	-1.86	124.1121	$C_8H_{14}N$	2.85
				93.0699	C_7H_9	3.15
				103.0542	C_8H_7	4.59
Cuscohygrine	11.31-11.49	225.1961	-1.95	84.0808	$C_5H_{10}N$	4.09
Scopoline	7.84-7.98	156.1019	-0.03	138.0913	$C_8H_{12}NO$	0.79
				96.0808	$C_6H_{10}N$	0.04
Tropine/ Physoperuvine	7.83-8.10	142.1226	2.53	98.0964	$C_6H_{12}N$	4.73
				96.0808	$C_6H_{10}N$	3.27
				93.0699	C_7H_9	4.65
Pseudotropine	7.85-8.13	142.1226	-0.99	96.0808	$C_6H_{10}N$	4.83
				124.1121	$C_8H_{14}N$	2.85
				93.0699	C_7H_7	1.43
Tropinone	7.56-7.95	140.1070	2.92	98.0964	$C_6H_{12}N$	-0.87
				57.0573	C_3H_7N	3.66
Tropane	7.72-7.86	126.1277	3.76	95.0855	C_7H_{11}	3.08
				70.0651	C_4H_8N	0.06

Table 2: Recoveries obtained for the different extraction procedures evaluated from spiked samples at 100 µg/kg

Compounds	Recoveries[a,b]			
	Extraction 1	Extraction 2	Extraction 3	Extraction 4
Anisodamine	79 (10)	62 (12)	69 (3)	95 (3)
Scopolamine	72 (13)	53 (11)	70 (4)	94 (4)
Atropine	73 (7)	51 (10)	62 (5)	91 (2)
Littorine	70 (2)	52 (5)	63 (3)	103 (1)
Aposcopolamine	87 (1)	61 (6)	48 (3)	95 (4)
Homatropine	66 (11)	50 (15)	62 (2)	93 (2)
Apoatropine	93 (2)	63 (3)	55 (1)	99 (1)
Scopoline	19 (4)	30 (6)	23 (1)	93 (6)
Cuscohygrine	17 (11)	20 (18)	39 (11)	93 (14)
Tropine/Physoperuvine	24 (3)	29 (3)	38 (6)	91 (2)
Pseudotropine	23 (13)	25 (10)	26 (5)	89 (6)
Tropinone	40 (3)	48 (5)	57 (3)	91 (4)
Tropane	19 (14)	25 (11)	58 (15)	95 (4)

[a] Extraction 1= QuEChERS extraction; Extraction 2 = Basified QuEChERS; Extraction 3 = Methanol 0.5% acetic acid extraction; Extraction 4= Methanol:water 0.5% acetic acid (2:1 *v/v*)

[b] Repeatability is given in brackets (n=5)

Table 3: Linearity, matrix effect and lower limits for the target tropane alkaloids in cereal samples

Compounds	Linearity (R^2)	Matrix effect with SPE purification[a]				Limits with SPE purification (µg/kg)			Limits without SPE purification (µg/kg)		
		Buckwheat	Millet	Soy	Linseed	LOD	LOI	LOQ	LOD	LOI	LOQ
Anisodamine	0.997	0.332	0.635	0.431	0.543	0.5	0.5	1	1	2	2
Scopolamine	0.993	0.198	0.303	0.352	0.334	1	1	2	2	3	5
Atropine	0.997	0.315	0.375	0.351	0.338	0.5	1	1	1	2	2
Littorine	0.996	0.289	0.391	0.361	0.345	0.5	1	2	5	5	10
Aposcopolamine	0.996	0.174	0.203	0.271	0.131	1	1	2	2	2	5
Homatropine	0.997	0.218	0.328	0.330	0.283	0.1	0.5	0.5	2	5	5
Apoatropine	0.997	0.212	0.465	0.384	0.213	1	1	2	2	2	5
Scopoline	0.996	0.029	0.091	0.054	0.013	1	1	2	10	10	25
Cuscohygrine	0.995	0.592	0.412	0.396	0.521	2	3	3	5	5	10
Tropine/Physoperuvine	0.996	0.031	0.062	0.005	0.012	0.5	0.5	1	10	10	25
Pseudotropine	0.996	0.042	0.078	0.011	0.030	0.5	0.5	1	10	10	25
Tropinone	0.996	0.044	0.067	0.002	0.013	0.5	1	2	10	10	25
Tropane	0.995	0.024	0.032	0.005	0.013	0.1	0.5	0.5	1	1	2

[a] Calculated as the quotient between the matrix and solvent slopes

Table 4: Validation parameters of the optimized method

Compounds	Matrix	Recoveries[a]			Interday precision (n=5)		
		1 µg/kg	5 µg/kg	50 µg/kg	1 µg/kg	5 µg/kg	50 µg/kg
Anisodamine	Buckwheat	63 (10)	84 (7)	91 (2)	11	6	1
	Millet	72 (8)	90 (5)	93 (4)	-	5	-
	Soy	66 (11)	84 (9)	81 (3)	-	8	-
	Linseed	67 (12)	71 (8)	75 (3)	-	9	-
Scopolamine	Buckwheat	-	94 (5)	86 (4)	-	6	8
	Millet	63 (9)	91 (3)	90 (3)	-	5	-
	Soy	-	91 (8)	84 (6)	-	8	-
	Linseed	-	72 (8)	83 (5)	-	6	-
Atropine	Buckwheat	65 (10)	91 (7)	91 (4)	13	10	5
	Millet	76 (5)	93 (6)	92 (4)	-	7	-
	Soy	63 (11)	90 (13)	86 (6)	-	11	-
	Linseed	63 (13)	79 (9)	83 (3)	-	10	-
Littorine	Buckwheat	-	75 (7)	80 (1)	-	10	2
	Millet	74 (11)	87 (6)	88 (1)	-	4	-
	Soy	-	81 (11)	79 (8)	-	9	-
	Linseed	-	65 (8)	84 (3)	-	6	-
Aposcopolamine	Buckwheat	-	74 (6)	75 (6)	-	15	7
	Millet	70 (15)	92 (7)	97 (6)	-	11	-
	Soy	-	60 (7)	78 (8)	-	9	-
	Linseed	-	75 (10)	83 (9)	-	10	-
Homatropine	Buckwheat	66 (8)	86 (6)	93 (1)	13	12	2
	Millet	81 (8)	94 (5)	89 (3)	-	8	-
	Soy	70 (9)	84 (10)	86 (2)	-	10	-
	Linseed	68 (11)	72 (5)	81 (6)	-	12	-
Apoatropine	Buckwheat	-	60 (5)	68 (2)	-	6	3
	Millet	68 (12)	73 (6)	82 (3)	-	10	-
	Soy	-	60 (8)	69 (5)	-	7	-

	Linseed	-	61 (8)	69 (3)	-	11	-
	Buckwheat	-	67 (14)	81 (8)	17	11	13
Scopoline	Millet	64 (19)	74 (5)	89 (3)	-	9	-
	Soy	-	69 (16)	90 (8)	-	17	-
	Linseed	-	83 (14)	91 (9)	-	7	-
	Buckwheat	-	66 (9)	76 (8)	-	14	4
Cuscohygrine	Millet	-	71 (10)	89 (5)	-	14	-
	Soy	-	61 (13)	82 (11)	-	14	-
	Linseed	-	62 (12)	79 (8)	-	15	-
	Buckwheat	67 (13)	84 (11)	80 (2)	16	14	6
Tropine/	Millet	72 (8)	90 (8)	85 (5)	-	10	-
physoperuvine	Soy	34 (15)	95 (12)	109 (4)	-	15	-
	Linseed	63 (12)	81 (11)	82 (3)	-	16	-
	Buckwheat	74 (19)	84 (16)	87 (9)	15	16	5
Pseudotropine	Millet	66 (12)	97 (9)	91 (6)	-	17	-
	Soy	53 (13)	95 (15)	101 (11)	-	14	-
	Linseed	73 (11)	79 (12)	85 (9)	-	11	-
	Buckwheat	-	79 (19)	88 (3)	-	20	7
Tropinone	Millet	-	70 (11)	92 (5)	-	16	-
	Soy	-	62 (20)	97 (6)	-	20	-
	Linseed	-	74 (13)	88 (5)	-	13	-
	Buckwheat	63 (20)	64 (16)	88 (9)	15	17	9
Tropane	Millet	63 (18)	69 (15)	81 (4)	-	13	-
	Soy	47 (17)	60 (16)	69 (11)	-	18	-
	Linseed	63 (16)	68 (17)	72 (10)	-	13	-

[a] Intraday precision is given in brackets (n=5)

Table 5: Concentrations (µg/kg) of detected compounds in the contaminated samples

Compounds	Concentration (µg/kg)[a]				
	Brugmansia contamination		*Stramonium* contamination		Millet flour positive sample
	1 g/kg[b]	0.5 g/kg	1 g/kg	0.1 g/kg	
Anisodamine	114.6 (13)	45.6 (10)	22.9 (6)	2.6 (8)	5.4 (4)
Scopolamine	693.5 (12)	277.2 (6)	89.7 (4)	6.4 (11)	23.4 (6)
Atropine	291.9 (7)	142.4 (8)	1846.9 (1)	168.5 (6)	12.7 (6)
Littorine	16.4 (9)	4.5 (12)	139.0 (3)	13.2 (13)	2.8 (19)
Homatropine	337.4 (8)	142.7 (10)	7.2 (4)	-	-
Apoatropine	-	-	3.6 (4)	-	-
Scopoline	42.8 (11)	20.7 (16)	17.3 (6)	-	-
Tropine/physoperuvine	18.1 (6)	6.6 (10)	17.1 (5)	2.5 (12)	-
Pseudotropine	5.5 (14)	2.4 (12)	3.4 (8)	-	-
Tropinone	-	-	9.7 (7)	-	6.0 (1)

[a] Repeatability is given in brackets (n=3)

[b] 1 g/kg = 1g seeds per 1 kg of buckwheat