Elsevier Editorial System(tm) for Journal of Chromatography A

Manuscript Draft

Manuscript Number:

Title: Enantiomeric separation of (-) and (+)-hyoscyamine in Datura Stramonium and Brugmansia Arborea seeds and contaminated buckwheat by high performance liquid chromatography-tandem mass spectrometry

Article Type: Full length article

Keywords: atropine; (-)-hyoscyamine; Solanaceaes sedes; buckwheat; modified QuEChERS; chiral separation.

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Cover letter

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Almería, 31th July 2016

Dear Editor,

Please find enclosed the manuscript entitled "*Enantiomeric separation of (-) and (+)-hyoscyamine in Datura Stramonium and Brugmansia Arborea seeds and contaminated buckwheat by high performance liquid chromatography-tandem mass spectrometry* " by Marín-Sáez et al., for consideration of publication in Journal of Chromatography A, as an original research paper.

Datura Stramonium and *Brugmansia Arborea* belong to *Solanaceae* family plants which contain large amount of tropane alkaloids. These compounds have anticholinergic activity and products like buckwheat can be contaminated by these seeds, as it has been indicated by the European Food Safety Agency (EFSA). Atropine is the most important tropane alkaloid and it is the racemic mixture of (-)-hyoscyamine and (+)-hyoscyamine. Only the (-)-hyoscyamine is naturally formed and it is well known that (-)-hyoscyamine exhibit stronger anticholinergic effects than the (+)-hyoscyamine. For this reason the development of an extraction and chromatographic method that allow the determination of this enantiomer avoiding racemization is needed. For that, knowing the extraction conditions (pH, temperature, extraction time) that could affect this process is important.

Herein, we described a simple and fast analytical method for the determination of (-)-hyoscyamine and (+)-hyoscyamine in contaminated buckwheat and *Stramonium* and *Brugmansia* seeds using LC-MS/MS. During the extraction step, a modified QuEChERS extraction procedure was used. A chiral chromatographic method was developed for the separation of the two compounds. Therefore, the proposed method provided a fast and non-laborious method, which reduces the time employed to extraction and clean-up, ensuring the high selectivity required for complex analysis using mass spectrometry as detection technique. Good performance characteristics such as linearity, quantification limits and detection limits were obtained. Finally, the proposed method was applied to quantify (-) and (+) hyoscyamine and to evaluate the influence of pH, temperature and time on the racemization.

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Finally, we would like to indicate that this is the first application focused on the chiral separation of these type of compounds using a Chiralpak AY3 column and the evaluation of different conditions for the racemization in real samples. It 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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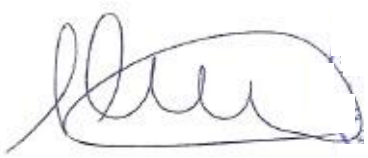

Yours Sincerely,

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Highlights

- Use of a modified QuEChERS procedure for the extraction of (-) and (+)-hyoscyamine
- Chiral chromatographic determination of both enantiomers and determination by LC-MS/MS
- Quantification in *Stramonium* and *Brugmansia* seeds and contaminated buckwheat
- Evaluation of the pH, temperature and time on the enantiomerization of atropine

Enantiomeric separation of (-) and (+)-hyoscyamine in *Datura Stramonium* and *Brugmansia Arborea* seeds and contaminated buckwheat by high performance liquid chromatography-tandem mass spectrometry

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Abstract

A new method has been developed for the enantioselective separation of (-) and (+) hyoscyamine in *Solanaceaes* seeds and contaminated buckwheat. A modified QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) extraction procedure using water and acetonitrile containing 1 % of acetic acid, and primary secondary amine (PSA) and graphitized carbon black (GCB) was applied as sorbents during clean-up stage. The extract was diluted with ethanol (50:50, *v/v*) prior to chromatographic analysis, and the separation was carried out applying a Chiralpak-AY3 column with ethanol (0.1 % diethanolamine) as mobile phase. After the optimization of the chiral separation, enantiomerization process of atropine was studied at different conditions such as temperature (30, 50 and 80 ºC) and pH (3, 5, 7 and 9). *Stramonium* and *Brugmansia* seeds were analyzed and the concentration of (-)-hyoscyamine was 1500 mg/kg and 320 mg/kg respectively. Contaminated buckwheat was also determined and (-)-hyoscyamine was detected at 170 µg/kg

Keywords: Atropine, (-)-hyoscyamine, *Solanaceaes* seeds, buckwheat, modified QuEChERS, chiral separation.

1. Introduction

Interest in natural toxins produced by fungi or plants has grown in recent years due to its toxicity and its impact on feeding and animal feed as well as on human food safety. Among these toxins, tropane alkaloids are compounds of great concern and they are the most commonly detected in *Stramonium* samples. Tropane alkaloids toxins are naturally produced by the *Solanaceae* family and in minor quantities by plants from other families as *Erythroxylaceae, Proteaceae, Euphorbiaceae, Rhizophoraceae, Convolvulaceae* and *Cruciferae* families [1]. Several crops such as buckwheat and related matrices can be contaminated by seeds belonging to these plants, which 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 [2]. Although animals can also be affected by these compounds, they are less susceptible than humans to poison by alkaloids, because the presence of an esterase enzyme, which efficiently biotransformed the atropine [3].

This family of compounds involves more than 200 compounds, although the compounds mostly studied are scopolamine, atropine and (-)-hyoscyamine [2]. Atropine is the racemic mixture of (-)-hyoscyamine and (+)-hyosciamine. However, only (-)-hyoscyamine has pharmacological activity and it is a more potent antimuscuranic agent than (+)-hyoscyamine or atropine [4]. This feature is very important for quality control in the pharmaceutical industry in order to achieve a better drug which should be enantiomerically pure, improving its efficiency [5].

However the current legislation established by European Union sets a limit for atropine of 1 µg/kg for the racemic mixture and the differentiation between both enantiomers are avoided. This could be due to there are not many studies where the enantioseparation of atropine was achieved [6]. Although information related to the racemization process is scarce, it is well-known that the extraction procedure can affect the ratio between both isomers, and therefore, the extraction conditions should be deeply studied in order to minimize this transformation process [2]. For this

reason the optimization of chiral analytical methods that allows the individual determination of each enantiomer is necessary [7]. In addition, it is also possible to find naturally racemization [8].

Up to our knowledge, currently there is not any extraction method in bibliography where the percentage of the (-)-hyoscyamine is 100%. This is because (-)-hyoscyamine could be degraded by two main routes: inversion of the natural (-)-enantiomer to the (+)-form till a racemic mixture is reached and hydrolysis to tropic acid and the corresponding tropane backbone, or dehydration to aposcopolamine/apoatropine [2]. Furthermore, it was observed that slight hydrolysis may occur at pH 3 [2], whereas the racemization occurs at pH higher than 3 and high temperatures [9], although this has not been studied in real samples. In order to get a reliable quantification of the enantiomers it is necessary the utilization of an extraction method which avoids racemization. The procedure should be performed either quickly or from moderately basified aqueous solution [10]. Therefore, the normal extraction procedures for tropane alkaloids are not valid [3,11], because they are long time consuming. Some extraction approaches used in the enantioseparation of atropine from plasma samples are based on liquid-liquid extraction with buffer phosphate solutions [12-14]. Acetonitrile and sonication was used to extract these compounds from drugs [15]. For the enantioseparation of the atropine enantiomers, few studies focused on the extraction of these compounds from plant material, like plants extract of the *Solanaceaes* family [16, 17] have been found but only one study has been developed in plant belonging to the species of *Stramonium* and *Brugmansia* or buckwheat, feed and similar matrices. It has been based on QuEChERS method followed by matrix solid phase dispersion (MSPD) [3], but the chiral separation was not achieved.

During the optimization of the extraction procedure, it is necessary to check if a racemization step is performed, and in order to evaluate it, enantiomeric separation should be carried out. Enantiomeric selectivity is usually achieved through the appropriate choice of a chiral stationary phase and mobile phase conditions. There are many studies related to the chiral separation of the racemic mixture of atropine. The enantioseparation can be carried out by capillary electrophoresis

(CE) [14,16-18], which have been focused on the enantiomeric impurity determination of active ingredients of chiral drugs, chiral resolutions of non-racemic mixtures and the determination of the stereochemical purity of the drugs [19]. In relation to the enantioseparation of atropine by liquid chromatography (LC), a α_1-acid glycoprotein-bonded stationary phase column (AGP column) is commonly used [12,15,20,21], but recently, other cheaper columns have been tested in order to achieve better chromatographic resolution, like Chirobiotic V column [13] or ChiraSpher NT column [4], but they are not checked with simple extraction method like QuEChERS.

The aim of this study was focused on the application of a simple extraction method using the QuEChERS methodology to extract the target compounds from *Daturae* and *Brugmansia* seed and buckwheat contaminated artificially. Moreover, a new chromatographic method using a Chiralpak AY3 has been developed for the enantiomeic separation of atropine, and finally the extraction conditions (pH, temperature and time) related to the racemization of the (-)-hyoscyamine in the (+)-hyoscyamine are studied.

2. Material and methods

2.1. Reagents and chemicals

Atropine reference standard was purchased from Sigma-Aldrich (St. Louis, MO, USA) and (-)-hyoscyamine reference standard was obtained from USP (Teddington, Middlesex, United Kingdom). Stock standard solutions of atropine and (-)-hyoscyamine (200 mg/L) were individually prepared by exact weighing of the solid substances and dissolved in 50 mL of ethanol (HPLC grade, J.T. Baker, Deventer, Holland), and they were stored at -20 °C in the dark. A mixture working standard solution was prepared at 5 mg/L with ethanol and stored in screw-capped glass tubes at -20 °C in the dark.

HPLC grade acetonitrile, isopropanol, acetic acid and acetone were obtained from J.T. Baker. Formic acid (Optima LC-MS), ammonium formate and diethanolamine (DEA) were obtained from

Fisher Scientific (Geel, Belgium). Methanol was obtained from Fluka (Steinheim, Suiza), while *n*-hexane was obtained from Scharlab (Barcelona, Spain). Triethylamine was purchased from Riedel-de Häen (Hannover, Germany). Graphitized black carbon (GBC) and primary secondary amine (PSA) were purchased from Scharlab (Barcelona, Spain). 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 (-) and (+)-hyoscyamine 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). An Astec Chirobiotic V column (250 x 4.6 mm, 5 µm particle size) from Supelco (Bellefonte, PE, USA) and Chiralpak AY3 column (150 x 4.6 mm, 3 µm particle size) from Daiciel (Barcelona, Spain), were employed for chiral separation. MassHunter (Agilent) was used for chromatographic data treatment.

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

2.3. Samples collection

Plant seed samples were collected from Huelva (Spain) and they are from *Solanaceaes* seeds, *Datura Stramonium* and *Brugmansia arborea* that contain the greatest concentrations of tropane alkaloids [2,22]. Buckwheat samples (*Fagopyrum esculentum*) were obtained from local supermarkets located in Almería (Spain). They were selected according to EFSA`s concern

regarding food control of tropane alkaloids [2] and previous studies [23]. Samples were ground into powder prior to analysis. Blank samples were used for the preparation of fortified samples during method validation.

The contamination of the buckwheat samples consisted in the addition of 1 g of *Stramonium* and *Brugmansia* seed to 1 kg of buckwheat, bearing in mind previous studies [11]. The mixture was homogenized before aliquots were collected to perform the extraction of the target compounds.

2.4. Sample preparation

The extraction of the three matrices (*Stramonium* seeds, *Brugmansia* seeds and contaminated buckwheat) was performed using an extraction procedure developed previously [23]. Briefly, this procedure was based on weighing 5 g of sample in a 15 mL polypropylene centrifuge tube, and mixed by vortex for 1 min with 10 mL of Milli-Q water. Then, 10 mL of acetonitrile solution containing 1% (*v/v*) of formic acid was added, and vortexed for 1 min. Then, 4 g of anhydrous sodium sulfate and 1 g of ammonium acetate were added to the mixture and this was homogenized by vortex for 1 min. The mixture was centrifuged at 5,000 rpm (4,136 ×g) for 5 min and one mL of the supernatant was transferred to a 15 mL centrifuge tube containing 25 mg of PSA and 25 mg of GBC. It was mixed by vortex for 1 min and then centrifuged at 5,000 rpm for 5 minutes. Finally, the supernatant was filtered with a 0.22 µm nylon syringe filter and diluted with ethanol (50:50, *v/v*) prior chromatographic analysis.

For *Stramonium* and *Brugmansia* seeds, only 0.1 g of samples was weighed due to the high concentration of tropane alkaloids [2,22].

For the evaluation of the extraction conditions and the ratio of the enantiomers in these conditions, different pHs (3, 5, 7 and 9) and temperatures (30, 50 and 80 ºC) were employed. The pH 3 buffer solution was prepared with monobasic sodium phosphate (1 M) and phosphoric acid (0.1 M) and the pH 5, 7 and 9 buffer solutions were prepared with monobasic potassium phosphate

153 (1 M)/dibasic sodium phosphate (0.1 M).

154

155 *2.5. LC-MS/MS analyses*

156 The chromatographic separation and determination of (-) and (+)-hyoscyamine were carried out
157 employing ethanol containing 0.1% DEA (*v/v*) as mobile phase, in isocratic mode for 10 minutes at
158 a flow rate of 0.4 mL/min. Injection volume was 5 µL and column temperature was kept at 25 ºC
159 (room temperature).

160 (-)-Hyoscyamine and (+)-hyoscyamine were ionized in positive ESI mode and detected using
161 selected reaction monitoring (SRM) mode. Source gas temperature and sheath gas temperature were
162 325 ºC and 400 ºC, respectively. Source gas flow and sheath gas flow were 5 L/min and 11 L/min,
163 respectively. Nebulizer was 45 psi. Capillary and nozzle voltage were 3500 V and 500 V,
164 respectively. MS/MS parameters are shown in Table 1.

165

166 **3. Results and discussion**

167 *3.1. Optimization of LC-QqQ-MS/MS*

168 The tandem mass spectrometry (MS/MS) parameters needed to characterize the compounds were
169 optimized in a previous study [23], where atropine, which was the mixture of (-) and (+)-
170 hyoscyamine was determined. Thus MS/MS transitions used to monitor both isomers are included
171 in Table 1.

172 Considering that for the enantioseparation of chiral compounds the stationary phase is a critical
173 factor, two columns, Chirobiotic V and Chiralpak AY3, were evaluated. First, the Chirobiotic V
174 column was tested, and different mobile phases found in bibliography for the separation of the
175 atropine were studied [3,13]. Initially, the conditions described by Jandrić et al. were checked [3].
176 In this study a mixture of water:acetonitrile (90:10 *v/v*) with 10 mM of ammonium formate as
177 aqueous phase (A) and methanol:acetonitrile (50:50 *v/v*) with 10 mM of ammonium formite as

organic phase (B), in isocratic mode, at a ratio of 20:80 (*v/v*) of A:B were used. The analysis time was 15 minutes and the flow rate was 1 mL/min. However, when these conditions were used a chiral separation was not achieved (Fig. 1a). In order to improve the separation, different percentages of A and B as 10:90, 30:70 and 40:60 (*v/v*) and different flow rates (0.25, 0.5 and 0.75 mL/min) were tested, but no separation was achieved.

Another study, where the Chirobiotic V column was performed by Siluk et al [13], was also evaluated. In this case a mixture of methanol:acetic acid:triethylamine (100:0.05:0.04, *v/v/v*) (A) and a mixture of methanol:acetic acid:triethylamine (100:0.05:0.1, *v/v/v*) (B) were used as mobile phase. A gradient elution was performed, and the mobile phase composition was changed from 100 % A to 100 % B in 25 min, and after that, the mobile phase was returned to A in 5 min. As it can be observed in Fig. 1b, using these conditions the enantioseparation was not achieved. This procedure was also evaluated using formic acid instead of acetic acid and with different gradients, but no separation was obtained.

Bearing in mind these results, another stationary phase was tested. A Chiralpak column (Chiralpak AY3) was selected because it has been widespread used in chiral separations [24-26]. According to the manufacturer, optimum parameters for the separation of the enantiomers involve the use of normal phase conditions, using as mobile phase a mixture of hexane:ethanol:diethanolamine (80:20:0.1 *v/v/v*). If these conditions were applied, an extra step in the extraction procedure should be included because the extraction solvent was acetonitrile and this is immiscible with *n*-hexane. Thus, one mL of the extract was evaporated by nitrogen flow and the extract was redissolved with 0.5 mL of mobile phase, achieving the separation of (-) and (+)-hyoscyamine (Fig. 1c). The results shown that the separation was achieved but additional and time-consuming evaporation and reconstitution steps were necessary. In addition, the peak shape and sensitivity are not suitable for the detection of these compounds at low concentrations. Bearing in mind these results, the mobile phase was modified and ethanol, which was miscible with

203 acetonitrile, was employed as mobile phase. Thus a mixture of ethanol:DEA (100:0.1 *v/v*) was
204 tested as mobile phase, and a suitable separation of the components of interest (Fig. 1d) were
205 achieved, using isocratic mode. Moreover, the extraction solvent was diluted with ethanol (50:50
206 *v/v*). Thereby the peak shape and the sensitivity improved, as well as extraction time was minimized
207 because the evaporation step was avoided.
208
209 *3.2. Method evaluation*
210 The extraction procedure was based on a preliminary study [23] for contaminated buckwheat. For
211 *Solanaceaes* seeds this procedure is different because the concentration in tropane alkaloids is very
212 high (higher than 100 mg/kg) and several dilutions are necessary. For this reason, the same
213 extraction procedure but with a lower amount of sample and reagents than buckwheat was tested.
214 Thus, to 0.1 g of sample, 10 mL of water, 10 mL of acetonitrile containing 1% (*v/v*) of formic acid,
215 4 g of anhydrous sodium sulfate and 1 g of ammonium acetate were added, and after that, the
216 cleaning step indicated previously was used.
217 To inject the sample into the chromatrographic system, the dilution of the extract was evaluated in
218 order to obtain a better peak shape and decrease the matrix effect. The dilution was performed with
219 different solvents. If the mobile phase were hexane:ethanol:DEA (80:20:0.1 *v/v/v*), the dilution was
220 performed using *n*-hexane and when the mobile phase was ethanol:DEA (100:0.1 *v/v*), ethanol was
221 used to dilute the extract. Because the concentration of these compounds in the seeds are very high,
222 several dilutions, 1:50, 1:100, 1:200, 1:400 and 1:600 were performed from *Stramonium* and
223 *Brugmansia* seeds respectively, according to the concentration of atropine found in the bibliography
224 [2]. The best dilution was 1:100 for seeds. In addition, no significant racemization was observed
225 using this extraction and chromatographic procedure.
226 Despite the high concentration of the target compound in *Solanaceaes* seeds, limits of detection
227 (LOD) and quantification (LOQs) were determined in order to assure that in spite of the dilution,

the compounds could be detected and quantified. For that, low concentrations in solvent were injected because the concentration of the target compounds in the seeds are very high and no blank matrices are available. The lower limits of the method were defined as the lowest concentration for which peak area was three and ten times the signal-to-noise ratio respectively obtaining good peak shape (Fig. 2). The concentrations injected were from 0.1 to 5 µg/kg. The LOD for both compounds was 0.5 µg/kg and the LOQ was 1 µg/kg. The limit obtained are acceptable compared to the value set by European Union regulation [6], which establishes the LOQ for atropine (not for the isomers) in baby food prepared with buckwheat and products which containing millet, sorghum or its derivatives in 1 µg/kg. Setting a low limit of quantitation for enantiomeric separation is most useful than the quantification of the racemic mixture, since only one isomer has biological activity. In addition the limits obtained are similar [13] or even better than those obtained in previous studies, where the LODs and LOQs are 1.56 and 5.14 µg/kg respectively [15].

3.3. Analysis samples

Finally the developed method was applied to the analysis of *Stramonium* and *Brugmansia* seeds as well as buckwheat contaminated with *Datura* seeds.

For the *Stramonium* and *Brugmansia* seeds, a standard addition calibration of diluted extract of the seeds (1:100) was prepared at concentrations ranging from 1 to 250 µg/kg, because the matrix effect despite the dilution performed, and the results are shown in Table 2. Finally for the contaminated samples of buckwheat the calibration curve was also performed by matrix-matched calibration.

The results show that the concentration of (-)-hyoscyamine is higher in *Stramonium* (1454 mg/kg) than in *Brugmansia* (327 mg/kg). They are similar to those obtained in previous studies where the concentration in *Stramonium* seeds was 1200-1600 mg/kg [11,27] and in *Brugmansia* plant was 200-600 mg/kg [22]. It is important to highlight that using the developed extraction procedure no

253 enantiomerization occurred, and in the raw material, only the the (-)-enantiomer was observed,
254 obtaining only 16 and 18 mg/kg for (+)-hyoscyamine for the *Stramonium* and *Brugmansia* seeds
255 respectively. Finally, the results in buckwheat contaminated with *Stramonium* and *Brugmansia*
256 seeds (1 g of seed in 1 kg of buckwheat) show that although the amount of seeds added to
257 buckwheat is small, the levels that EFSA states that can be found in contaminated feed and food are
258 exceeded (100 µg/kg) [2] and 170 µg/kg and 100 µg/kg were obtained for the samples contaminated
259 with *Stramonium* and *Brugmansia* seeds, respectively.
260
261 *3.4. Study of the pH and temperature conditions*
262 As it was mentioned above, the extraction procedure is the most important step for the chiral
263 analysis of atropine in order to avoid racemization [9,10]. It is important to evaluate those factors
264 that can affect the stereochemistry to provide information to the pharmaceutical industry to obtain
265 optically pure drugs. Moreover, it is important for the treatment during cooking where different pHs
266 and/or temperatures can be applied. For this reason, different conditions as pH and temperature
267 were evaluated in samples of *Solanaceaes* seeds.
268 First the effect of the pH during the extraction process on the racemization of atropine was
269 evaluated. To control the pH, different buffer solutions at 3, 5 (extraction pH), 7 and 9 pH were
270 prepared. The result obtained, expressed as a proportion of the (+) and (–) form was shown in Table
271 3 and it can be observed that the extraction pH does not affect the racemization during this
272 extraction procedure. This is important since the optimal pH of the mobile phase to get a good
273 separation should be slightly basic [15] as in this study, where the apparent pH of the mobile phase
274 is 8.7.
275 Then, the extraction time was also evaluated at the different pHs evaluated previously. In this
276 case, one hour was selected. The other conditions of the extraction procedure were described in
277 Section 2.4. It was observed that the proportion of (+)-hyoscyamine slightly increases (Table 3),

although the results are similar to those obtained using shorter extraction times.

Then the effect of the temperature was evaluated. In the same way that the extraction pH, different temperatures (30, 50 and 80 ºC) were evaluated during the extraction process at pH 5. The temperatures were applied to the mix of sample and water for thirty minutes, and after that, the extraction procedure was performed. Temperature did not affect significantly the racemization of (-)-hyoscyamine in (+)-hyoscyamine, obtaining a ratio 98.5(-):1.5(+) at 80 ºC (Table 3).

Finally, in order to verify the proposed racemization evolution with the pH and temperature described in bibliography [9] and to evaluate the time necessary to get the racemic mixture of 50:50 (±)-hyoscyamine, the combined influence of pH and temperature was evaluated. For this, the most unfavorable conditions were used. Thus, the samples were mixed with buffer solution at pH 9 and the extraction temperature was set at 80 ºC for 0.5, 1, 2 and 4 hours and 1, 2, 4 and 7 days. The result obtained were shown in the Fig. 3, and it can be noted that when basic pH and high temperatures are combined, racemization occurs and this racemization is higher when time is longer. Thus the enantiomerization is close to 50:50 ratio (enantiomerization 53:47 (-)-hyoscyamine:(+)-hyoscyamine) as described in bibliography [5]. It can also be noted that at extreme pH (pH 9) at long time (7 days) the compounds degrade and were not detected. This experiment was developed also with the normal extraction pH (pH 5) (Fig. 3) and it can be noted that at normal conditions (pH 5) but at high temperature (80 ºC), the racemization also occurs but more slowly. Moreover, it can be seen that the evolution of the racemization follows an exponential trend like other theoretical studies published previously [9] (Table 4).

4. Conclusions

This study has allowed the development of a simple method for the enantio-quantification of (±)-hyoscyamine in *Datura Stramonium* and *Brugmansia Arborea* seeds and contaminated buckwheat with low detection and quantification limits, with a stationary phase previously not used for this

purpose. The method was applied to evaluate the racemization of (-)-hyoscyamine in (+)-hyoscyamine in enantiomerically pure *Solanaceaes* samples. For this, different pH and temperature conditions were evaluated, obtaining that the racemization only occurs when high temperatures are applied during long periods of time. This effect is more pronounced at basic pH (pH 9). The developed method allows an analysis without racemization and it can be applied in real samples of *Solanaceaes* samples and buckwheat and other contaminated matrices proposed by EFSA [2].

Acknowledgments

In appreciation of the Ministry of Economy and Competitiveness (MINECO) and the European Regional Development Fund (ERDF) (project ref. CTQ2015-69899-R) for financial support.

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402 **Figure captions**

403 **Fig. 1.** Enantioseparation of atropine (solvent standard at 200 µg/L) with different column and
404 chromatography conditions: a) Chirobiotic V ((A) water:acetonitrile (90:10 *v/v*) and (B)
405 methanol:acetonitrile (50:50 *v/v*) both with 10 mM of ammonium formate); b) Chirobiotic V
406 (methanol:acetic acid:triethylamine (A) 100:0.05:0.04, *v/v/v* and (B) 100:0.05:0.1, *v/v/v*); c)
407 Chiralpak AY3 (hexane:ethanol:diethanolamine (80:20:0.1 *v/v/v*)); d) Chiralpak AY3 (ethanol:DEA
408 (100:0.1 *v/v*)).

409 **Fig. 2.** Peak shape for the limit of detection (LOD) for (±) hyoscyamine (0.5 µg/kg).

410 **Fig. 3.** pH and time influence in the enantiomerization of (-)-hyoscyamine in *Stramonium* seeds at
411 80 ºC.

Figure

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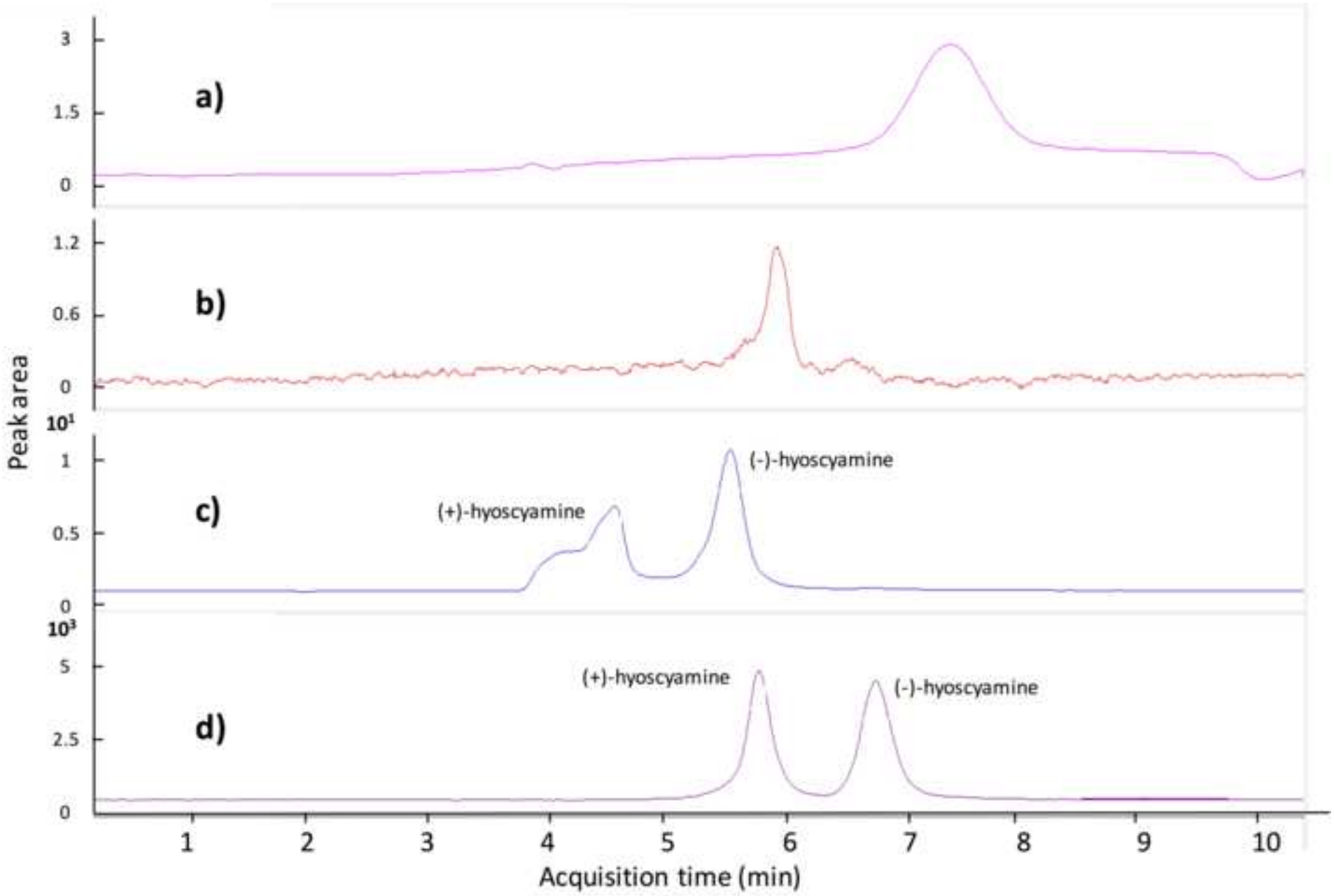

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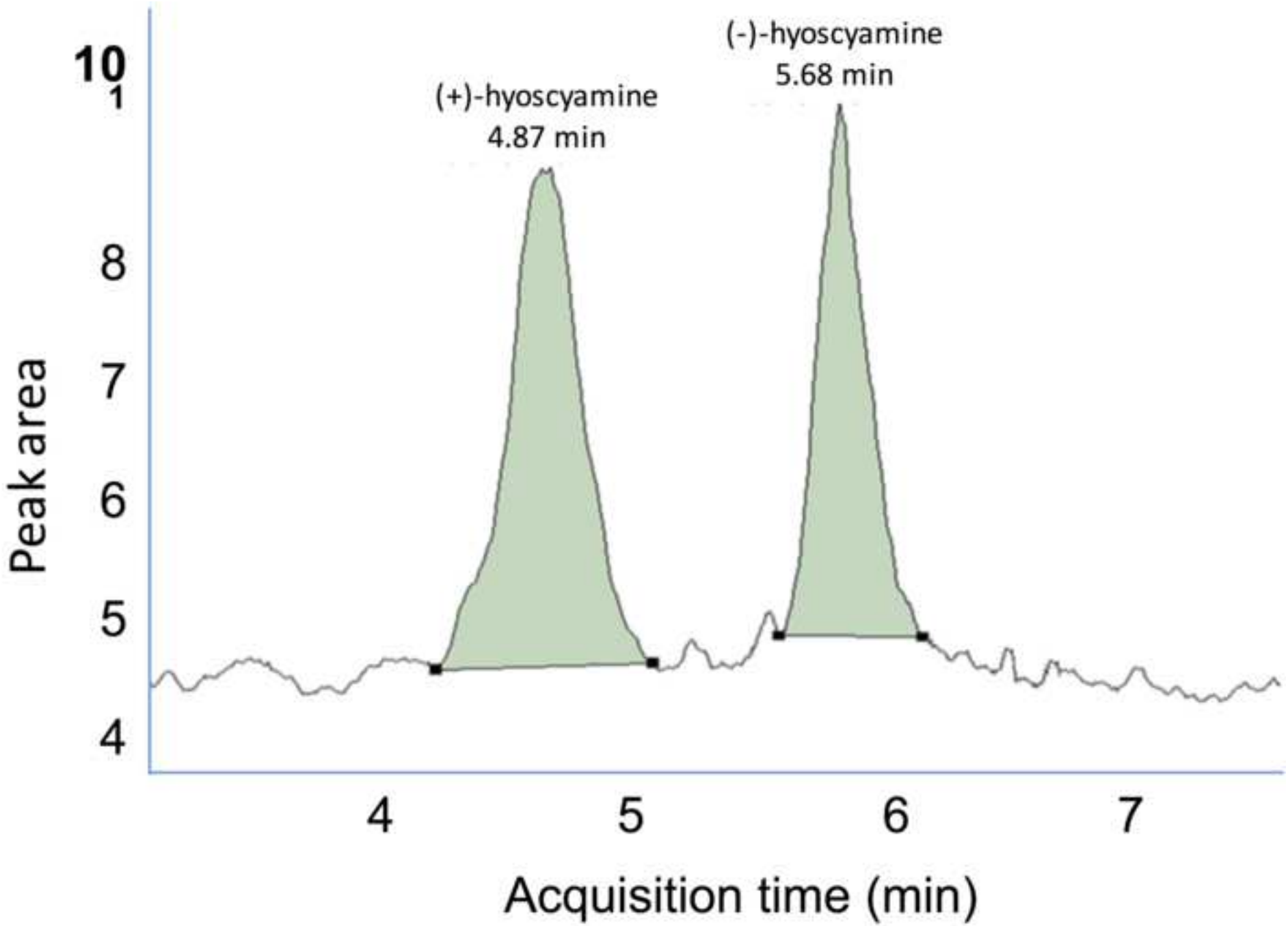

Figure

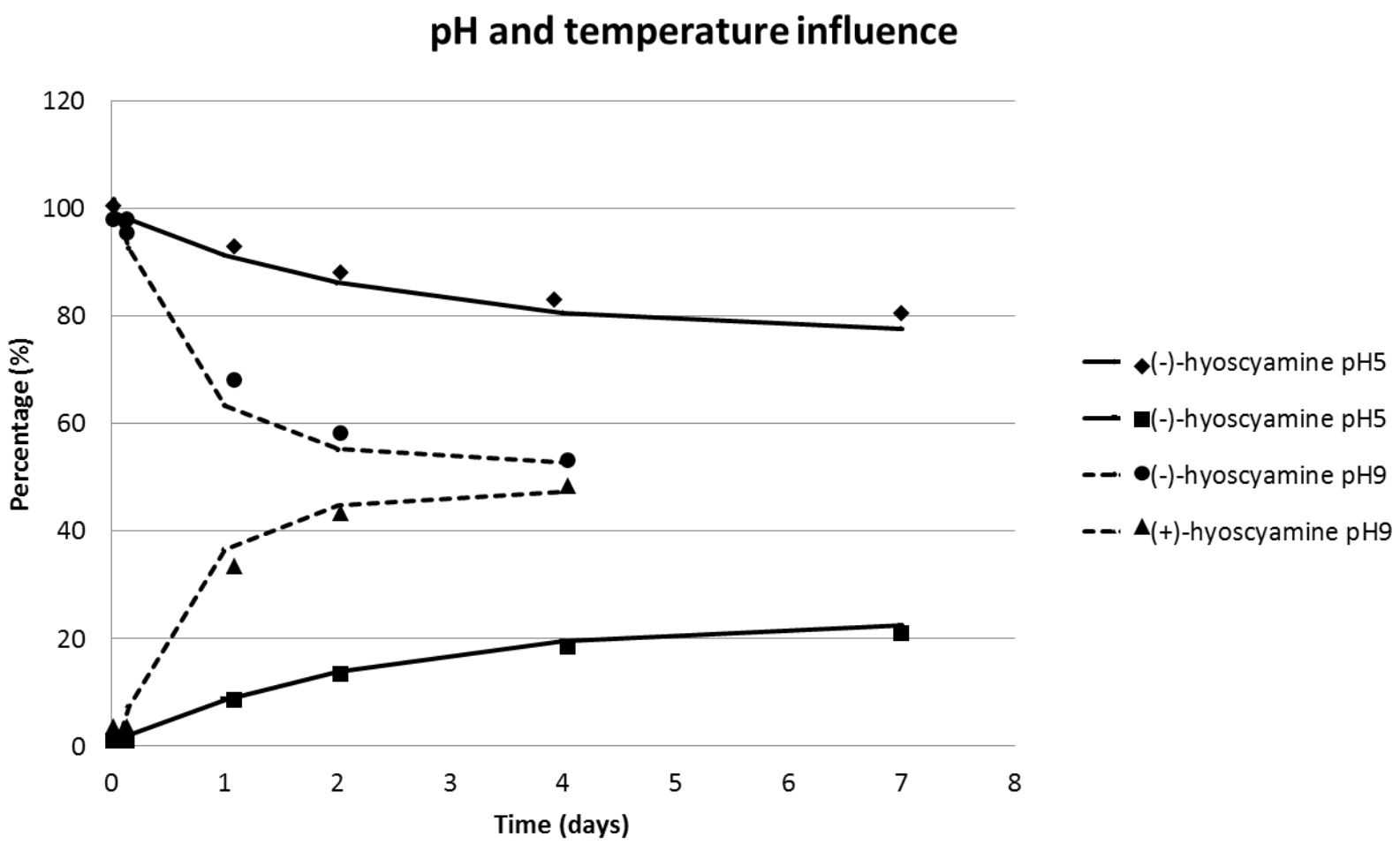
pH and temperature influence
120
100
80
60
40
20
0
Percentage (%)
0
1
2
3
4
5
6
7
8
Time (days)
(-)-hyoscyamine pH5
(-)-hyoscyamine pH5
(-)-hyoscyamine pH9
(+)-hyoscyamine pH9

Table 1. Retention time windows (RTWs) and MS/MS parameters of (+,-) hyoscyamine

Compound	RTW (min)	Precursor ion (*m/z*)	Product ion (*m/z*)[c]	Ion ratio (%)
Hyoscyamine	4.27-5.11[a] 5.42-5.99[b]	290.2 (70)[d]	**124.0** (20)[e]	100
			93.0 (28)	42
			91.0 (48)	21
			77.0 (50)	26

[a] Retention time window (RTW) for (+)-hyoscyamine

[b] RTW for (-)-hyoscyamine

[c] Transition in bold was used for quantification.

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

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

Table 2. Concentrations (mg/kg) of (+,-) hyoscyamine in the tested samples

Sample	(-)-hyoscyamine	(+)-hyoscyamine
Datura seeds	1454.30 (7)[a]	16.21 (11)
Brugmansia seeds	326.91 (9)	17.88 (9)
Buckwheat contaminated with *Stramonium*	0.17 (15)	0.01 (12)
Buckwheat contaminated with *Brugmansia*	0.10 (6)	0.01 (3)

[a] Relative standard deviation is given in brackets (n = 4).

Table 3. Study of the enantiomerization when different pH and temperature conditions were applied.

	Compound	pH[a]				Temperature (ºC)[c]		
		3	5	7	9	30	50	80
Racemization (%)	(-)-hyoscyamine	99.40 (98.90)[b]	99.12 (98.81)	98.93 (98.03)	98.42 (97.86)	99.00	98.91	98.42
	(+)-hyoscyamine	0.60 (1.04)	0.88 (1.18)	1.07 (1.96)	1.58 (2.13)	1.00	1.09	1.57

[a] Proportions obtained when 10 minutes was used as extraction time, when temperature was set at 80 ºC.

[b] Proportions obtained after one hour in brackets.

Table 4. Adjustment of the evolution of racemization with pH and time

Conditions	(-)-hyoscyamine		(+)-hyoscyamine	
	R^2	Equation	R^2	Equation
pH=5	0.997	$Y=76.47 + 22.89*e^{(-x/2.31)}$	0.997	$Y=23.52 - 22.89*e^{(-x/2.31)}$
pH=9	0.986	$Y=53.14 + 50.02*e^{(-x/0.63)}$	0.986	$Y=46.85 - 50.02*e^{(-x/0.63)}$