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LC-MS/MS DETERMINATION OF TROPANE ALKALOIDS IN MAIZE CROP *

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Summary: The undemanding LC-MS/MS method was developed for the synchronized analysis of atropine and scopolamine in maize crop. The dSPE was carried out with 1% acetic acid in acetonitrile/water and a mixture of magnesium sulphate, sodium chloride and sodium citrate. The analytes were separated on a Zorbax XDB C18 column using methanol/water as the mobile phase in gradient mode. The detection was done using a tandem masss spectrometry (MS/MS) in the positive ESI. The tropane alkaloids exhibited excellent linearity in the range of 2-20 µg/kg with the LOQ of 5 µg/kg for maize. The extraction recoveries of atropine and scopolamine were 65.7 and 85.5% with the intraday RSDr 10.25 and 4.29%, respectively. The validated method was appled to real maze samples. One sample contained 18.8 µg/kg of atropine and 6.3 µg/kg of scopolamine.

Key words: tropan alkaloids, Datura spp., maize, LC-MS/MS.

INTRODUCTION

The family *Solanaceae* (Nightshade) is well-known for producing the toxins named tropane alkaloids (TA) (Jandric et al., 2011) and the most comonly investigated are (-)-hyoscyamine and (-)-scopolamine. Atropine is the racemic mixture of (-)-hyoscyamine and (+)-hyoscyamine of which only the (-)-hyoscyamine enantiomer is found to have anticholinergic activity (EU 2015/976).

Datura is a frequently present weed in the cultivated fields, and ruderal habitats. Its seeds cannot be easily removed from the cultivated grains such as maize, sorghum, barley and wheat by sorting and cleaning. Beside *Datura stramonium* many other speaces including Atropa belladonna and Solanum nigrum are significant producers of TA which makes the plants potentially poisonous, especially their seeds (Jandric et al., 2011).

Both humans and animals are affected by the TA (scopolamine and atropine) found in family *Solanaceae.* Because of the fact that biotransformation of atropine and scopolamine is more efficient in animals, due to an esterase enzyme they are less suceptible to the toxic effect of alkaloids.

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The accidental contamination and the use of plants from the *Solanaceae* family for the pharmaceutical purposes are the main sources of human exposure to atropine and scopolamine. The contamination can be chronic due to continuous ingestion of small amounts of contaminated food and the acute intoxication (a huge amount in a single dose) (Cirlini et al., 2018).

Chen et al. (2017) indicate that The European Food Safety Authority (EFSA) state that the acute reference dose (ARfD) has been set at 0.016 µg/kg body weight, taking into account boht atropine and scopolamine.

There are no established maximum residue levels in food or feed for TA. Practically all detected values above the Limit of Quantifications (LOQs) indicate that the samples are not for consumption. Namely the LOQ for atropine and scopolamine must not exceed 5 µg/kg and must not be higher than 10 µg/kg for agricultural products including teas, components and food supplements. Also it should be lower than 2 µg/kg for the finished foods and 1 µg/kg for cereal-based foods for babies and small children (EU 2015/976).

Within this low established LOQs values for atropine and scopolamine, the only analytical method for their determination, which could be used for sauch detections, in traces, is the liquid chromatography with tandem mass spectrometry (LC-MS/MS).

Due to the above mentiond, the LC-MS/MS was applied for the analyses of atropine and scopolamine in eleven maize samples.

MATERIAL AND METHODS

Chemicals and apparatus. 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 prepared in methanol (HPLC grade, Sigma), and they were stored at -20 C in the freezer. A mixture working standard solution was prepared at 5 mg/L with methanol and stored in the dark at -20 C, too. HPLCgrade methanol and acetonitrile (100% purity) were obtained from J.T. Baker Chemicals (Netherlands). Formic acid was purchased from Fisher Scientific UK (Loughborough, UK). The Agilent Bond Elut EN Buffered Extraction kit (p/n 5982-5650) and Bond Elut QuEChERS EN Dispersive SPE kits for Fruits and Vegetables with fats and waxes (p/n 5982-5158) were purchased from Agilent (Agilent Technologies Inc.CA, USA). An Agilent series 1200 HPLC system (Agilent Technologies) equipped with a G1312B binary pump, a G1367D autosampler, a G1379B degasser, a G1316B column compartment temostat, The HPLC system was coupled to an Agilent triple quadrupole mass spectrometer (6410 B) coupled to an electrospray ionization source (ESI+). A Zorbax XDB C18 column (50x4.6 mm, 1.8 µm particle size) from Agilent (San Jose, CA, USA) was employed for separation. The chromatographic determination of atropine and scopolomine was carried out employing a binary mobile phase with methanol (A) and an aqueous solution of formic acid (0.1%, v/v) (B). A gradient elution started at 90% of B and held 4 min at flow rate of 0.4 mL/min. This composition was reduced to 5% B in 10 min, and held for 5 min. The composition of the mobile phase returned to the initial conditions in 2 min and the stationary phase was equilibrated during 2 min. The total running time was 17 min. The injection volume was 5 µL and column temperature was kept at 25 ºC. The ESI source values were as follows: drying gas (nitrogen) temperature 350 °C, drying gas flow rate 10 L/min, nebulizer pressure 40 psi and capillary voltage 3500 V. The detection was performed using the multiple reactions monitoring mode (MRM). The Agilent MassHunter software (version B.06.00 Agilent Tehnologies, 2012) was used for optimization and quantification.

Validation parameters. The proposed method was validated by determining the limits of quantification (LOQs), and the limit od detection (LODs). The linearity was estimated from standard addition calibration curves constructed using three concentration levels ranging from 2- to 10-fold increase in the LOQs of each analized sample, and the correlation coefficients (R^2) were above 0.99. The recoveries (at three concentration levels of 1-, 2-, and 3-fold increase in the LOQ). The LOD was estimated from the chromatogram of the lowest level of calibration using the Agilent MassHunter software (Agilent Technologies, B.06.00) for those concentrations that provide a signal to noise ratio of 3:1. The LOQ was defined as the reference value 5 µg/kg in consideration of Commission Recommendation (EU) 2015/976. The recovery studies were performed on two spiking levels (5 and 10 µg/kg) in three replicates. The method precision is expressed as the repeatability (RSD%) based on recovery experiments.

Sample collection. All analyses samples were unprocessed maize from local producer, the Republic of Serbia. The sampling was performed in accordance with the general principles and methods of the European Commission (EC) directive 2002/63/EC for establishing MLs in food commodities. All the samples were placed in polythene bags, labelled, and transported to the laboratory for processing. Samples were ground into powder prior to analysis. The blank samples were used for the preparation of fortified samples during the optimization of sample extraction procedure and method validation.

Sample preparation. All samples were ground, homogenized, and weighed. A 5 g of homogenized sample was placed into a 50-mL centrifuge tube. The spiking samples for calibration were fortified with appropriate working

spiking solution to yield a 2; 5 and 10 µg/kg concentracion in the samples. An 10 ml of water awas added to each tube and vortexed for 1 min and equilibrated for 10 min. Next, 10 mL aliquot of acetonitrile containing acetic acid (1%, v/v) was added and shaken by vortex for 1 min. An Agilent Bond Elut QuEChERS EN extraction salt packet, containing 4g anhydrus MgSO4, 1 g NaCl, 1 g three sodium citrate and 0.5g disodium hidrogen citrate sesquihydrate, was added directly to each tube. The tubes were sealed tightly and shaken vigorously for 20 second by hand and 15 min/250 rpm by orbital shakers. The sample tubes were centrifuged at 4000 rpm for 5 min. The supernatants were then transferred to 15-mL QuEChERS d-SPE kits consisting of 150 mg of C18 sorbent and 900 mg of $MgSO_4$, vortexed for 5 min, and centrifuged at 4000 rpm for 10 min. The obtained mixtures were transferred and dried under nitrogen gas at 45 ºC until the volume was <0.3 mL. The residues were reconstituted in the mixture methanol/water up to 2 mL, vortexed, centrifuged at 7000 rpm (Eppendorf Centrifuge 5430) and filtered through Whatman Mini-UniPrep™ syringeless filters prior to LC-MS/MS analysis.

RESULTS

The optimization of the LC-MS/MS parameters was performed by the direct infusion of standard solutions of atropine and scopolamine in the concentration of 1 mg/L, prepared in methanol:water (50:50, v/v) at a flow rate of 0.1 mL/min, using a restriction capillary instead of a chromatography column and massHunter Optimizer software (Agilent). Both compounds were analyzed using ESI+ and the protonated molecule [M + H]+ was the most intense parent ion for both compounds. The fragmentation of the protonated molecular ion at m/z 290.2 of atropine yielded 4 product ions at m/z 142.2, 93.2, 77.1, and 67.1. The most abundant product ion 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. The fragmentation of the protonated molecular ion of scopolamine yielded 4 product ions at m/z 156.1, 138.2, 103.2, and 77.1. The most abundant product ions at m/z 138.0 were formed by loss of tropic acid ($C_9H_{10}O_3$,166 Da) from protonated molecular ion at m/z 304.2. The most intense transitions for atropine m/z 290.2 > 124.2 and scopolamine 304.2 > 138.2 were selected for quantification, while the other three transitions were used for the confirmation (Table 1).

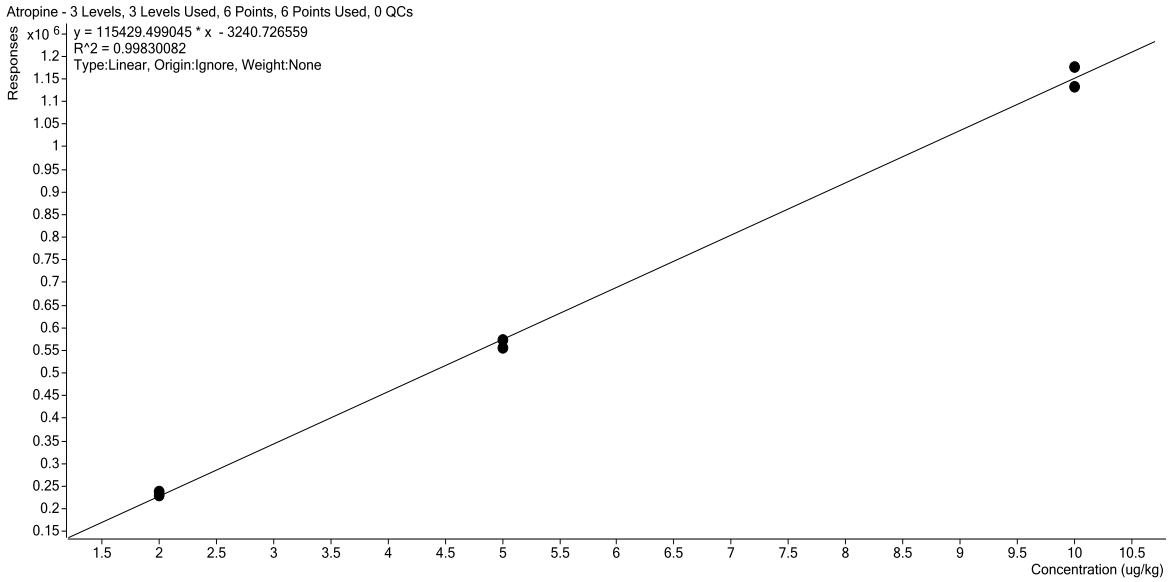
Table 1. MS/MS conditions

Analite	Rt (min)	Transitions (*m/z*)	CE (V)	Frag. (V)	Analite	Rt (min)	Transitions (*m/z*)	CE (V)	Frag. (V)
Atropin	9.69	290.2 -> 124.2	24	96	Scopolamine	8.85	304.2 -> 156.1	12	92
		-> 93.2	36	96			-> 138.2	24	92
		-> 77.1	68	96			-> 203.2	44	92
		-> 67.1	56	96			-> 77.1	70	92

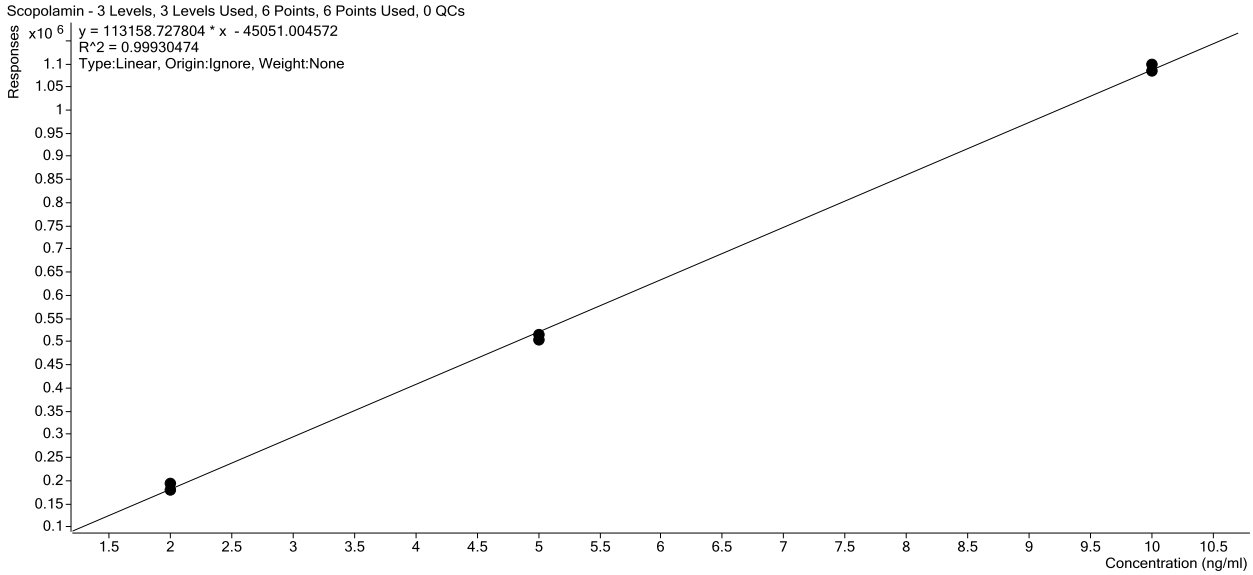
The best recovery was obtained by the use of the mixture of acetonitrile/acetic acid (99/1, v/v), because atropine and scopolamine are very polar compounds and the partition behavior was pH-dependent (Zheng W et al. 2018, Miyazaki et al, 1993).

Plenty of matrix compounds from maize can be present in the raw extracts during the liquid extraction (the mixture of water and acetonitrile) of the atropine and scopolamine. The co-extracted substances could interfere with the atropine and scopolamine and lead to significant matrix effects. We investigated a number of approaches with PSA and C18, as sorbents when dispersive solid phase extractions (d-SPE), MycoSep (Romer), MycoSpeen (Romer), Bond Elut Mycotoxin (Agilent) were used to remove the co-extractant substances (Jadrić et al, 2011; Jakabová et al, 2012). In these stududies only PSA and C18 sorbent showed good results, while in the other stududies, investigated compound were missing or the recovery was very poor.

The accuracy was investigated through the recovery trials and spiked blank samples at two levels (5 and 10 µg/kg) were evaluated. Each fortified concentration was repeated three times and the results are shown in Table 2. The average recoveries of scopolamine and atropine were 85.5 and 65.7%, respectively. The recoveries obtained in this work could be acceptable according to the European Commission SANTE/11813/2017 which indicated that the recoveries should be in the range of 70–120%. The precision, expressed as a relative standard deviation (RSD), was evaluated in terms of repeatability and the obtained values were lower than 17%. The matrix –matched calibration curves were linear over the calibration range from 2 to 20 µg/kg (Image 1-2).



Picture 1. Atropine calibration courve

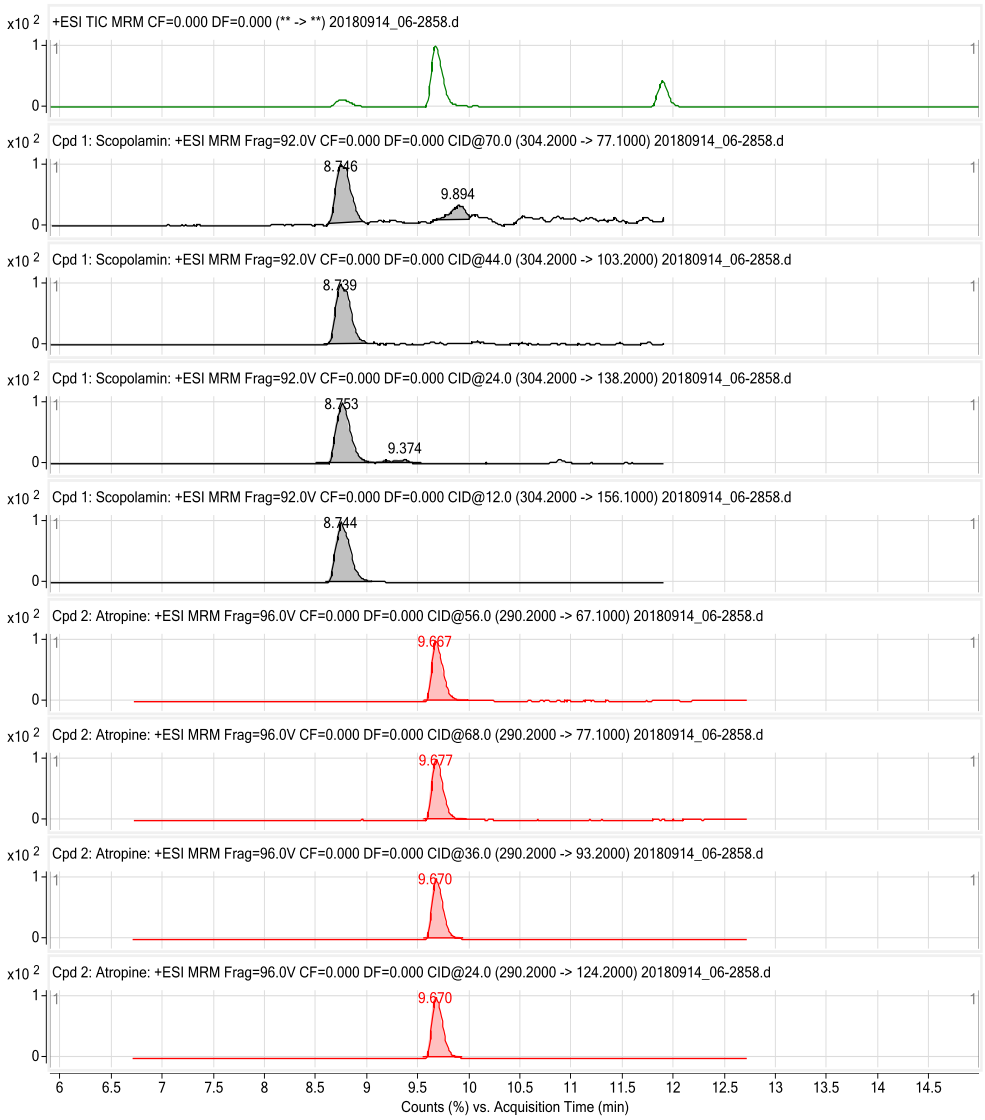


Picture 2. Scopolamine calibration courve

The limits of detection (LODs) were defined as the lowest concentration for which area values were three times the ratio of signal-to-noise and calculated by MassHunter software. LODs for atropine and scopolamine were 5 µg/kg, respectively, while LOQs of atropine and scopolamine were set at 5 µg/kg, for both of them. These limits were checked in the terms of recovery and repeatability.

Chromatographyc separation of atropine and scopolamine shown in image 3.

Image 3. LC-MS/MS chromatograms of maize sample (18.8 µg/kg of atropine and 6.3 µg/kg of scopolamine)



DISCUSSION

There are no established maximum residue levels in food or feed for scopolamin and atropine. All the detections above the LOQs (for atropine and scopolamine must not exceed 5 µg/kg and must not be higher than 10 µg/kg for agricultural products) indicate that the samples are not for consumption. Namely, the LOQ fot the components and food supplements should be lower than 2 µg/kg for the finished foods and 1 µg/kg for cereal-based foods for babies and small children (EU 2015/976).

Of all the analysed maize samples (11), only one was with detections of both investigated TA: 18.8 µg/kg of atropine and 6.3 µg/kg of scopolamine.

CONCLUSION

This study proposes an undemanding LC-MS/MS method for the determination of scopolamine and atropine in maize using a modified QuEChERS. The modified QuEChERS technique is based on acetonitrile extraction and clean-up stage with the mixture of PSA and C18. The validation showed great linearity, accuracy, precision and LOQs. The analyses of 11 maize samples showed the presence of scopolamine and atropine in only one sample in the concentration of 18.8 µg/kg of atropine and 6.3 µg/kg in case of scopolamine.

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LC-MS/MS ODREĐIVANJE TROPANSKIH ALKALOIDA U KUKURUZU

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Izvod: *Brza i jednostavna LC-MS/MS metoda je razvijena za istovremeno određivanje atropina i skopolamina u kukuruzu. Tehnika dSPE se izvodi 1% sirćetnom kiselinom u smeši acetonitrila i vode i prisustvu soli magnezijum sulfata, natrijum hlorida i natrijum citrata. Jedinjenja se razdvajaju na hromatografskoj koloni, Zorbax XDB C18 koristeći metanol/vodu (sa 0,1% mravljom kiselinom) kao mobilu fazu pri protoku 0,4 mL/min u gradijentnom režimu rada. Detekcija se vrši pomoću masenog spektrometra sa trostrukim kvadrupolom sa +ESI. Tropanski alkaloidi pokazuju odličnu linearnost u opsegu od 2-20 µg/kg sa granicom kvantifikacije za kukuruz od 5 µg/kg. Prinos ekstrakcije za atropin i skopolamin je viši od 80% sa ponovljivošću manjom od 17%. Razvijena metoda je primenjena na 11 realnih uzoraka neobrađenog kukuruza, u jednom uzorku je nađena koncentracija atrpina i skopolamina iznad referentne vrednosti (18,8 µg/kg atropina i 6,3 µg/kg skopolamina).*

Ključne reči: tropanski alkaloidi, *Datura* spp., kukuruz, LC-MS/MS.

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