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SHORT REPORT

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DETERMINATION OF TROPANE ALKALOIDS IN CORN PUFFS BY THE LC-MS/MS

ABSTRACT: The interest in tropane alkaloids (TA) as food contaminants is increasing. A sensitive and selective LC-MS/MS method was applied for the analysis of corn puff samples from the Serbian market. Only atropine was quantified in 22% of the samples. In case of scopolamine, although not quantified, it was detected in 22% of the samples. Whether the acute reference dose (ARfD) could be exceeded was checked on a case-by-case basis for the individual products under assessment. Due to their low body weight and relatively high snack consumption, preschool children were at the highest risk of TA exposure. Assuming that the average consumption is 50 g of corn puffs per day, the sample with the highest concentration of TAs (2.05 μg/kg, 1.58 μg/kg of atropine) could contribute with 32.0% to the ARfD, of which 24.7% owing to atropine. If the same amount of corn puffs is consumed by older age classes, corresponding exposure contributions to the ARfD would progressively decline, down to 8.4% for adult population. The study revealed no health risk from TAs exposure through the consumption of the corn puffs in Serbian population.

KEYWORDS: atropine, corn, food analysis, risk assessment, scopolamine

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INTRODUCTION

It is well known that certain plants, animals and microorganisms produce natural toxins which are not toxic to them, but can be toxic to humans when ingested through food. Those natural toxins have received significant attention nowadays as a potential health hazard to humans (Puvača et al., 2020). Bearing that in mind, the World Health Organization accentuates the importance of monitoring the most relevant natural toxins in food (Casado et al., 2020).

The tropane alkaloids (TAs) are a group of over 200 secondary metabolites, found in all parts of the tropane alkaloids producing plants (Mulder et al., 2016). Besides the *Solanaceae* family, tropane alkaloids are also found in the following families: *Convolvulaceae*, *Euphorbiaceae*, *Proteaceae*, *Brassicaceae* and *Erythroxylaceae* (Gutiérrez-Grijalva et al., 2020). The tropane alkaloids may be divided into: tropane alkaloids produced by the family *Solanaceae* (atropine (AT), scopolamine (SC), hyoscyamine) (Figure 1), coca alkaloids (cocaine) and a newly discovered group of tropane alkaloids – calystegines (Kohnen-Johannsen and Kayser, 2019). The toxic effects of the tropane alkaloids in humans are related to the inhibition of muscarinic acetylcholine receptors in the central and the autonomic nervous systems (EFSA, 2013).

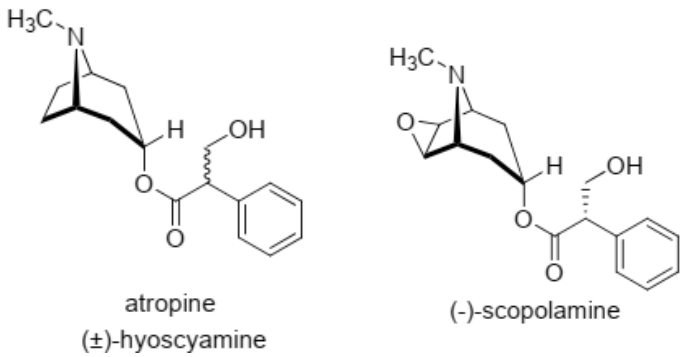

Figure 1. Chemical structure of atropine (racemic mixture of (±)-hyoscyamine) and scopolamine

The food contamination could be the consequence of the raw food material in which the TAs are naturally present. On the other hand, the contamination could occur through the co-harvesting plants, i.e. weeds containing tropane alkaloids, with the species of family *Solanaceae* being the most prominent, such as *Datura stramonium*. The parts of this plant have been found as the accidental impurities in the most important agricultural crops – maize, buckwheat, sunflower, soybean, millet and other (Gonçalves et al., 2020). In order to obtain more occurrence data on the presence of TAs in food, the EU Commission adopted Recommendation 2015/976/EU1 on the monitoring of the

presence of TAs in food (EC, 2015). However, the monitoring is limited due to the limited availability of reliable analytical methods combined with the appropriate sensitivity. The maximum level was established only in cereal-based foods for infants and young children, containing millet, sorghum, buckwheat, or their derived products (Regulation 2016/239), limiting atropine and scopolamine concentration to 1 μg/kg for each alkaloid (EC, 2016). The discussions are continuing to define the maximum levels on corn, buckwheat, millet and sorghum (grains and milling products). The herbal infusions are also under consideration.

Taking into account the growing interest in plant secondary metabolites, the aim of the present study was to investigate the presence of tropane alkaloids in corn puffs, popular extruded snacks made out of cornmeal, i.e. corn, by the liquid chromatography with tandem mass spectrometry (LC-MS/MS) and estimate the level of the exposure of Serbian population.

MATERIALS AND METHODS

Chemicals and reagents

Atropine and scopolamine reference standards were obtained from the Sigma-Aldrich. The standard solutions of atropine and scopolamine were prepared at 1 mg/mL in methanol, each. The working standard solution mixtures were prepared at the concentration of 10 μg/mL and 1 μg/mL in methanol and stored in the dark at -20 °C. Acetonitrile and methanol were purchased from J. T. Baker. Both organic solvents were HPLC Ultra Gradient HPLC grade. The formic acid was analytical grade (Fisher Scientific UK). The QuEChERS extraction (Cat. No. 5982-5650) and QuEChERS dispersive kit 15 mL (Cat. No. 5982-5156) were obtained from Agilent Technologies, USA.

Instrumentation

HPLC Agilent 1290 Infinity II chromatograph equipped with a quaternary pump, multisampler and column compartment thermostat was used for the detection of atropine and scopolamine. The HPLC system was coupled to an Agilent 6495 LC/TQ triple quadrupole mass spectrometer with AJS ESI (Jet Stream Technology Ion Source). The Zorbax Eclipse Plus C18 column Rapid Resolution HD (50x2.1mm, 1.8 μm particle size) was used for the chromatographic separation. The column temperature was held at 35 ºC and the injection volume for the LC system was 2 μL. The chromatographic separation of AT and SC was carried out with mobile phase consisting of water (A) and methanol (B), both containing formic acid (0.1%, v/v), in a gradient mode and flow rate of 0.25 mL/min. A gradient elution started at 5% of B and held 1 min. This

composition was increased to 40% B at 7 min, 90% B at 8 min and held for 2 min. The composition of the mobile phase returned to the initial conditions in one min and the system was equilibrated during two min. The total running time was 11 min. The ESI source was used with the following settings: drying gas (nitrogen) temperature 200 °C, drying gas flow rate 16 L/min, nebulizer pressure 30 psi, sheath gas temperature of 300 ºC, sheath gas flow 12 L/min and capillary voltage 3,000 V. The detection was performed using the dynamic multiple reactions monitoring mode (dMRM). The Agilent MassHunter software (version B.10.0 SR1 Agilent Tehnologies, 2006–2019) was used for the optimization and quantification.

Sample collection and preparation

Eighteen corn puffs samples were collected from the local shops and supermarkets in Novi Sad, Serbia. The sampling was performed in accordance with the EU directive 2002/63/EC. The samples were dry ground into powder prior to the analysis (particle size of less than 1 mm and sieved to obtain a homogenous sample particle size).

Atropine and scopolamine were extracted from ground corn puff samples using the QuEChERS method described on Figure 2.

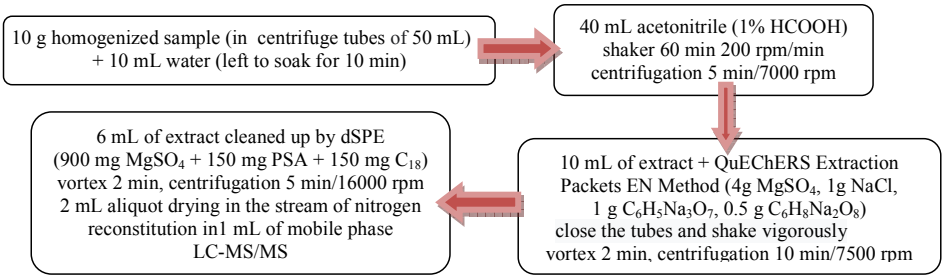

Figure 2. The steps of the atropine and scopolamine extraction

Acquisition parameters

Atropine and scopolamine were analyzed using ESI+ (electrospray positive ionization) by dynamic multiple reactions monitoring mode. The fragmentation of the protonated atropine and scopolamine ions yielded 3 product ions, respectively (Table 1). The most intense MRM transitions for atropine m/z 290.2 > 124.2 and scopolamine 304.2 > 138.2 were monitored for the quantification and the second most intense (other two) transitions were used for the confirmation (Vuković et al., 2018).

Table 1. LC-ESI-MS/MS parameters for the analysis of AT and SC in MRM mode

TA	Molecular formula	Molecular weight (g/mol)	Retention time (min)	Precursor ion [M+H*] (m/z)	Product ion (m/z)	Fragmentation voltage (V)	Collision energy (V)
AT	$C_{17}H_{23}NO_3$	289.2	9.63	290.2	124.2*	150	24
					93.2	150	36
					77.1	150	68
SC	$C_{17}H_{21}NO_4$	303.2	8.42	304.2	156	150	12
					138.2*	150	24
					103.2	150	44

* Quantification product ion

RESULTS AND DISCUSSION

The previous studies (Vuković et al., 2018; Vuković et al., 2020) pointed out that the addition of the formic acid to the mobile phase resulted in more efficient ionization and gave the finer peak of the studied tropane alkaloids. The MRM chromatograms and mass spectra of atropine and scopolamine transitions are shown in Figure 3.

The limit of detection (LOD) was determined as the lowest concentration giving a response of three times the average baseline. The ratio signal/noise in the obtained chromatograms for the LOD was calculated by MassHunter Qualitative Software and was estimated to be 0.1 mg/kg for both tested compounds. The limit of quantification (LOQ) (1 mg/kg) was calculated as 3.3*LOD and was in accordance with the Commission Recommendation (EU) 2015/976 related to the LOQ: "preferably below 5 mg/kg and not higher than 10 mg/kg for agricultural commodities, ingredients, food supplements and herbal teas and lower than 2 mg/kg for finished foods and 1 mg/kg for cereal-based foods for infants and young children".

The quantification was carried out by "recovery calibration" method (a known amount of analyte is spiked into the sample before extraction begins) by adding the tropane alkaloids standards into each sample to five calibration levels of 1, 2, 5, 10 and 20 μg/kg. The obtained calibration curves (both atropine and scopolamine) were used for the calculation of the atropine and scopolamine concentrations in the samples. The calibration curves of the studied atropine and scopolamine in the range of 1–20 μg/kg are shown in Figure 4.

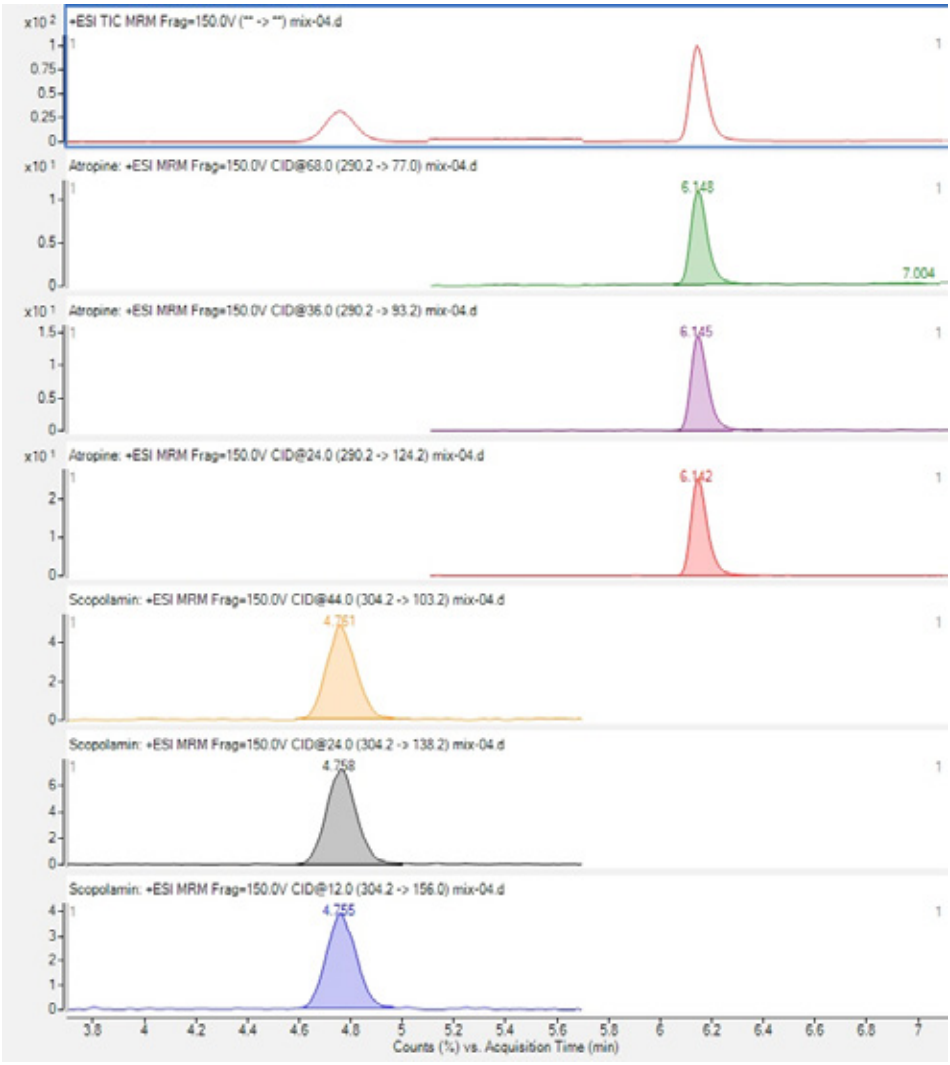

Figure 3. MRM chromatograms and mass spectra of AT and SC

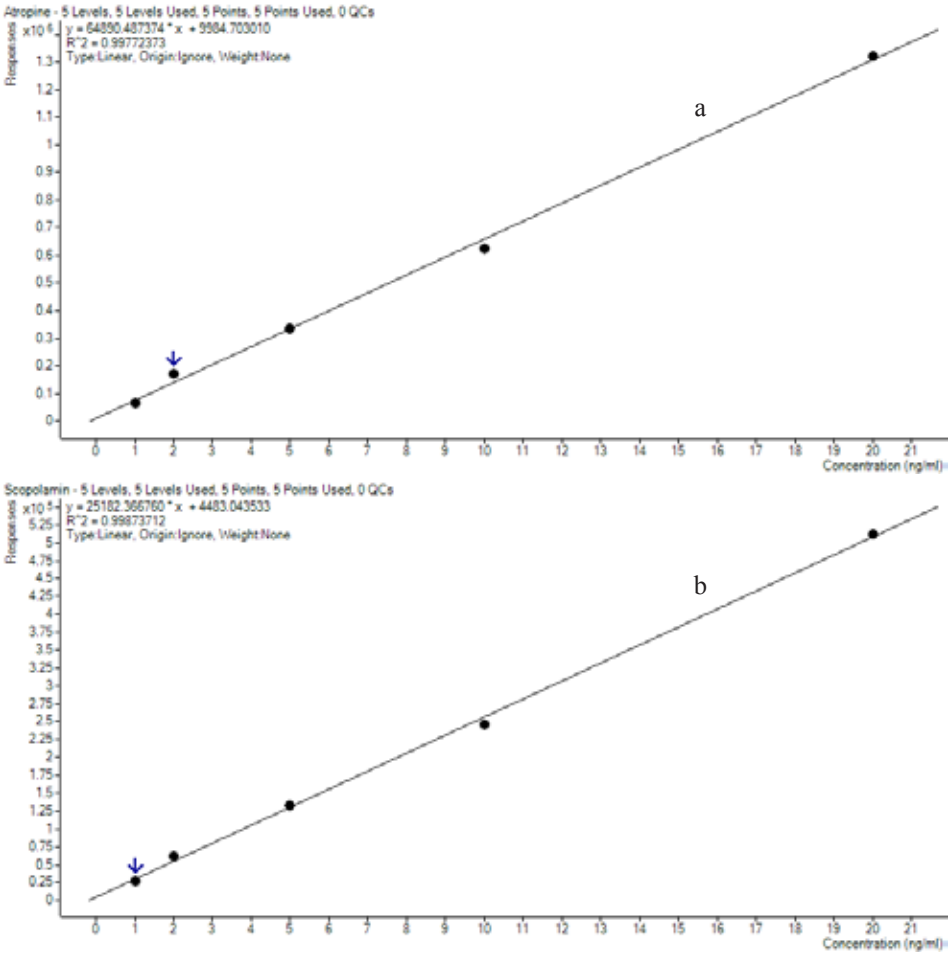

Figure 4. Calibration curves of atropine (a) and scopolamine (b) in matrix

Determination of tropane alkaloids in corn puffs

A total of 18 corn puffs samples were analysed for the presence of atropine and scopolamine. As shown in Table 2, only atropine results were quantified (22%, all the remaining 78% left-censored results were reported as below the LOD). With regard to scopolamine, 22% of the results were reported as numerical values, but below the LOQ.

It is important to notice full co-occurrence, as well as the ratio of atropine to scopolamine in the range from 3.4 to 3.9, coherent with a potential contamination with *Datura stramonium*. It would be valuable to know whether and how much of tropane alkaloids was lost during the extrusion – the food processing technique used in the corn puffs production.

Table 2. Summary of the presence of atropine and scopolamine in corn puffs

Parameter	Atropine	Scopolamine	Sum of atropine and scopolamine
N	18	18	18
N pos (%)	4 (22.2)	4 (22.2)	4 (22.2)
min c (µg/kg)	1.03	0.29	1.32
max c (µg/kg)	1.58	0.47	2.05

N – number of the samples; pos – positive samples; c – concentration.
LOD = 0.1 µg/kg and LOQ = 1.0 µg/kg for atropine and scopolamine.

The surveys performed in the European countries reported different levels of contamination, both in terms of the fraction of positive samples and of total TA content, in a broad range of foods (flours, infant formulas, botanicals or honey). It was confirmed that atropine and scopolamine were the most frequently found TAs in cereal-based foods (Mulder et al., 2016; EFSA, 2018). Over the last five years, Rapid Alert System for Food and Feed (RASFF, https://ec.europa.eu/food/safety/rasff_en) reported 24 notifications related to TAs in food, out of which 14 related to cereals and bakery products (made from corn, millet, buckwheat and soy), nine to tea and herbal preparations and one to infant food. The highest measured concentrations were 180 and 36 mg/kg of atropine and scopolamine in baking mix, respectively, as well as 213 and 44.7 mg/kg in herbal infusion. It is interesting to note that the country of origin of two products was Serbia (corn grits and peppermint). A peppermint sample from Serbia contained even 200.5 and 488.7 mg/kg of atropine and scopolamine, respectively.

The most recent research considering TAs has been published by Vuković et al. (2021). The study included 71 food product samples, such as corn puffs, popcorn, corn and corn grits. The TAs detections above the LOQ (2 µg/kg, which is in accordance with the Commission Recommendation (EU) 2015/976), appeared in 29.57% of the analyzed samples. According to the literature data, these concentrations can have serious negative effects on human and animal health. The highest mean concentrations of atropine and scopolamine were detected in corn grits samples, followed by popcorn and corn.

Health risk assessment

The acute reference dose (ARfD), i.e. the amount of substance, expressed on a body weight basis, that could be ingested via food over a day without a risk for the consumer health, was established by the European Food Safety Authority (EFSA) at 16 ng/kg bw per day for the sum of atropine and scopolamine (EFSA, 2013). However, a tolerable daily intake (TDI) for chronic exposure was not established, since the TAs "are not bio accumulative, or genotoxic, and do not exhibit chronic toxicity" (EFSA, 2013). Whether the ARfD

could be exceeded was estimated on a case-by-case basis for the individual products under assessment, based on the measured atropine and scopolamine concentrations and the estimated corn puffs consumption. The acute exposure to atropine and scopolamine through the consumption of corn puffs, estimated on a per day basis across age classes, is presented in Tab. 3, as well as the exposure to the sum of atropine and scopolamine, calculated as the sum of both alkaloids in the same sample.

Table 3. Exposure assessment to atropine and scopolamine through consumption of corn puffs, across age classes.

	Exposure (ng/kg bw)		% of group ARfD		> ARfD
Age class	Min	Max	Min	Max	%
			Atropine		
Preschool children	2.6	4.0	16.1	24.7	0
Children 7–10 y.	1.6	2.4	9.8	15.0	0
Adolescents 11–14 y.	1.0	1.5	6.2	9.5	0
Adults 15+ y.	0.7	1.0	4.2	6.5	0
			Scopolamine		
Preschool children	0.7	1.2	4.53	7.3	0
Children 7–10 y.	0.4	0.7	2.75	4.5	0
Adolescents 11–14 y.	0.3	0.5	1.74	2.8	0
Adults 15+ y.	0.2	0.3	1.19	1.9	0
			Sum of atropine and scopolamine		
Preschool children	3.3	5.1	20.6	32.0	0
Children 7–10 y.	2.0	3.1	12.5	19.4	0
Adolescents 11–14 y.	1.3	2.0	7.9	12.3	0
Adults 15+ y.	0.9	1.3	5.4	8.4	0

ARfD – group acute reference dose for the sum of atropine and scopolamine (16 ng/kg bw per day) (EFSA, 2013). y – years of age. Consumed amount of corn puffs: 50 g (one pack). Body weight: preschool children 20 kg, children (7–10 y.) 33 kg, adolescents (11–14 y.) 52 kg, adults (15+ y.) 76 kg.

The moderate differences were observed between the minimum and maximum exposure estimates for the age class. Due to their low body weight and relatively high snack consumption, preschool children were at the highest risk to the TA exposure. For a preschool child of around 20 kg bw, the ARfD would correspond to an intake of 320 ng TAs per day. Assuming the consumption of 50 g of corn puffs (one pack) per day, the sample with the highest TA concentration (2.05 µg/kg of total TAs, 1.58 mg/kg of atropine) could contribute with 102.5 ng of TAs (32.0% of the ARfD, of which 79.0 ng (24.7%) as atropine contribution). The ARfD would be exceeded when the product contained more than 6.4 µg TAs per kg. If the same amount of corn puffs is consumed by

older age classes, the corresponding exposure contribution to the ARfD would progressively decline with the increase of age and average body weight, down to 8.4% (6.5% owing to atropine) for the adult population. As shown in Table 3, the fraction of the products under assessment that would cause exceeding 10% of the ARfD was 22% for the preschool children and the children, 6% for the adolescents, while for the adults that level was not reached by any of the products. The study revealed no health risk from tropane alkaloids exposure through corn puffs for Serbian population.

The snacks are especially popular with the children for which the highest mean acute exposure for the sum of atropine and scopolamine has been observed in the assessment conducted by the EFSA (children 0.97–18.91 ng/kg bw/day, toddlers 1.82–18.65 ng/kg bw/day and other children 1.13–18.13 ng/kg bw/day) (EFSA, 2018). From a toxicological point of view, if ARfD is exceeded, adverse health effects can no longer be ruled out.

CONCLUSION

The study results do not indicate a serious health concern related to the exposure to the tropane alkaloids through the consumption of corn puffs commercialized on the Serbian market. The younger age groups (preschool children and children) are acutely exposed to the higher levels of the tropane alkaloids compared to the older age groups.

The study included only one type of food with a limited number of samples. Bearing in mind that the consumption of snacks is increasing, especially among the children and adolescents, obtained insight into the actual contamination levels is considered important. Furthermore, the study results highlight the importance of monitoring the tropane alkaloids in other food categories that could potentially be contaminated with the tropane alkaloids.

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ОДРЕЂИВАЊЕ ТРОПАНСКИХ АЛКАЛОИДА У КУКУРУЗНОМ ФЛИПСУ ПРИМЕНОМ LC-MS/MS

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РЕЗИМЕ: Интересовање за тропанске алкалоиде као контаминанте хране је у порасту. За анализу кукурузног флипса са тржишта Србије примењена је осетљива и селективна LC-MS/MS метода. Атропин је квантификован у 22% узорака. У случају скополамина, који није квантификован, детектовано је у 22% узорака. Да ли може доћи до прекорачења акутне референтне дозе проверено је за сваки појединачни производ који је уврштен у процену. Мала телесна маса и релативно велике конзумиране количине снек-производа истичу предшколску децу као групу у највећем ризику од изложености тропанским алкалоидима. Под претпоставком о конзумирању 50 грама кукурузног флипса дневно, узорак с највећом концентрацијом тропанских алкалоида (2,05 μg/kg, 1,58 mg/kg атропина) може допринети са 32,0% од акутне референтне дозе, од чега 24,7% одговара атропину. Уколико исту количину кукурузног флипса конзумирају старије узрасне групе, допринос следствене изложености тропанским алкалоидима би прогресивно опадао до 8,4% за одраслу популацију. Студија није указала на здравствени ризик услед изложености тропанским алкалоидима путем конзумирања кукурузног флипса за популацију у Србији.

КЉУЧНЕ РЕЧИ: анализа хране, атропин, кукуруз, процена ризика, скополамин