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Are tropane alkaloids present in organic foods? Detection of scopolamine and atropine in organic buckwheat (*Fagopyron esculentum* L.) products by UHPLC-MS/MS.

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Abstract

A closer monitoring of tropane alkaloids (TA) in foods is now recommended by the European Commission, following a series of alerts related to the contamination of buckwheat with weeds of the genus Datura. A novel, accurate UHPLC-MS/MS method was developed and validated for the rapid detection of scopolamine and atropine in buckwheat foods. A suitable extraction protocol was set up to maximize recoveries and detection limits in different raw, processed and baked foods. The method offers good sensitivity, as LODs for scopolamine and atropine in buckwheat flour were obtained at 0.03 and 0.09 µg/kg respectively, with LOQs at 0.04 and 0.10 µg/kg. Precision (%RSD range) and accuracy (% recovery range) were 2.7-3.4% and 83-103%, respectively. The established method is suitable for routine determination of trace levels of TA and was applied to 26 different buckwheat-derived organic foods, detecting TA in 3 samples (13.9-83.9 µg/kg for atropine and 5.7-10.4 µg/kg for scopolamine). Only in one case the level of contamination was relevant in terms of food safety.

Keywords: food safety, buckwheat, tropane alkaloids, mass spectrometry, organic food.

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1. Introduction

Alkaloids are well-known for their remarkable toxicity and for a consolidated history of poisoning, due to incidental or deliberate food contamination (Koleva et al., 2012). In this regard, the once neglected presence of tropane alkaloids (TA) in foods has been recently reconsidered, leading to a specific recommendation by the European Commission (Adamse et al., 2014; Beuerle et al. 2013; EFSA 2015). Clear goals in this respect are the estimation of the actual level of human exposure by the western diet, the definition of foods most involved and the ascertainment of maximum levels for most common TA, namely atropine and scopolamine (Figure 1) (EFSA 2015). Such recommendations arise from the evidence that plants containing TA are intrinsic constituents of some food chains, in which distinct patterns were found for cereal based products, infant formulas and herbal teas. (Mulder et al., 2016). Cereal-based products, in particular, were mostly characterized by the presence of atropine and scopolamine, while ready-to-eat meals for children contained also tropine and pseudotropine. A wider range of TA including also convolvine was found in teas and infusion. These results highlight the need to include specific TA as targets in screenings, according to each food product group. Moreover, this evidence is the consequence not only of the presence of TA in specific developmental stages of certain crops (e.g. calystegines in *Solanum tuberosum* L.), but also of co-harvesting crops and specific weeds, drawing the attention for cereals and buckwheat-derived foods mostly on atropine and scopolamine (Griffin & Lin, 2000; Aehle & Drager, 2010). Plant families such as Cruciferae and Convolvulaceae may contain TA, but their agronomical relevance is lower and the knowledge regarding their toxicity is limited. According to EFSA opinion, TA can actually enter the food chains as a result of contamination especially due to seeds and fruits of some species of the Solanaceae family. In fact, in the recent past and on different continents, occurrence of TA in food was mainly associated to the cross-contamination of Solanaceae family, among them mainly the genera Hyoscyamus, Atropa and Datura (Kitano et al.,

2003; Chang et al., 1999; Van Raamsdonik et al., 2009; Fretz et al., 2007). Albeit whole Datura plants are known to contain TA (atropine and scopolamine, in particular), seeds are usually deemed to be the most frequent cause of contamination, due to their size and to the simultaneous maturation with crops like grains, cereals, legumes and pseudocereals sharing the same cultural cycle.

A simple seed contamination may be detected by visual inspection or reduced through sorting and cleaning by physical methods, but yet a minimal presence is relevant in flours and other processed materials, in which the presence of a single undetected Datura seed per million of crop seeds/fruits may pose relevant health risks. (Miraldi et al., 2001; Friedman & Levin, 1989; Lawrence et al., 1994). A variety of analytical methods have been set up in recent years, encompassing different approaches and matrices, thus allowing different degrees of sensitivity and workload (Aehle & Drager, 2010; Christen et al., 2013; Caligiani et al., 2011; Muder et al., 2015; Mulder et al., 2014; Shimshoni et al., 2015; Rancic & Spasic, 2009; Jakabová et al., 2012; Chow et al., 2012; Mol et al., 2011). Within this context, the recourse to liquid chromatography emerges as the leading approach for TA analysis and represents the method of choice by many laboratories, with numerous advantages following its combination with a MS detector (Christen et al. 2013). As a consequence of the high human sensitivity to these anticholinergic alkaloids, extremely low amounts of atropine derivatives may trigger severe poisoning symptoms and, therefore, analytical methods should be extremely sensitive and reliable. For such reason, the EU is fostering the availability of methods with LOQ for atropine and scopolamine "*preferably below 5 μg/kg and not higher than 10 μg/kg for agricultural commodities, ingredients, food supplements and herbal teas and lower than 2 μg/kg for finished foods and 1 μg/kg for cereal-based foods for infants and young children*" (European Commission, 2015). The literature at present only offers methods matching the upper limits of the requested sensitivity, with few exceptions (Mulder et al., 2015, Chen et al., 2017).

Following few episodes of mass intoxication from TA in buckwheat products in 2005 (in Slovenia) and in 2012 (in France), different surveys have been performed in European countries, namely Germany, Hungary, Slovenia, France and the Netherlands. The results highlighted different levels of contamination in a wide range of plant foods as in the case of flours, infant formulas, feed, silage products, botanicals or honey, both in terms of positive samples and of total TA (Perharič, 2005; Perharič et al., 2013; EFSA, 2008; Mulder et al., 2015; Mol et al., 2011; AFSSA, 2008). In particular, due to the concurrent fruit maturation and the similar seed size with *Datura* species, crops aimed at human consumption like linseed, soybean, millet,

pulses, sorghum and particularly buckwheat (*Fagopyrum esculentum* Moench) have been more often related to TA contamination and are, therefore, listed as targets for more careful scrutiny (EFSA, 2008; European Community, 2015). Due to the absence of gluten and to an encouraging nutritional profile, buckwheat is experiencing a renewed commercial interest and is more frequently sold as a flour. Moreover, it is increasingly used as a healthy ingredient in different processed foods including bread, pasta, snacks, cakes and specialty food for infant nutrition (Giménez-Bastida, & Zieliński, 2015). In most temperate climates, annual species known for atropine and scopolamine biosynthesis and accumulation like *D. stramonium, D. innoxia* and *D. ferox*, can easily thrive as weeds in buckwheat fields. Despite an adequate management, post-harvest handling and control, some seeds may go undetected to subsequent stages of the food chain making a certain degree of contamination by these TAs unavoidable (EFSA, 2008). Both *Datura* and *Fagopyrum* species in fact produce, and maturate almost simultaneously, a dehiscent fruit harboring seeds with similar size and weight. This may be particularly relevant for organic agriculture, in which a less strict weed management may allow an increased in-field presence of potentially dangerous plants alongside with crops (Bond & Grundy, 2001).

Therefore, in order to provide a new tool meeting EFSA requirements for the screening of tropane alkaloids in flour-based foods, we set up an accurate but simple UHPLC-MS/MS method, optimized for different food matrices. Being atropine and scopolamine the most prevalent TA in cereals and buckwheat flour, these compounds were chosen as targets. The fully validated protocol was then applied to a range of buckwheat flour samples and to different buckwheat containing foods, with the aim to provide a survey of the eventual presence of atropine and scopolamine in the organic food chain in Italy.

2. Materials and Methods

2.1 Chemicals and solvents.

Acetonitrile, Ethyl acetate, Methanol, formic acid, ≥ 99% HPLC grade, were purchased by Sigma Aldrich (Milan, Italy). Ultra-pure water, ≥ 99% HPLC grade, was produced in house by ultrafiltration using an Alpha-Q system (Millipore, Marlborough, MA, USA). Furthermore atropine, (≥ 95% (TLC), and scopolamine hydrobromide, (≥ 90% (TLC), obtained from Sigma Aldrich (Milan, Italy) were used for preparing standard solutions. For the

preparation of the extraction solvent with a final pH of 3.2, methanol and pure water with 0.2% acetonitrile and 0.2% formic acid were combined in a volume ratio 3:2 and prepared weekly.

2.2 Plant and food material

To obtain an actual TA profile from a supposed in field contaminant, a sample of dried *Datura stramonium* seeds was obtained by the Botanical Garden of the University of Parma from plants grown in 2014 and stored at -20°C until analysis. Twenty-five commercial samples, namely 12 flours, 6 pasta and 8 bakery products, belonging to different brands and containing different amounts of buckwheat were purchased during 2015 from different groceries and supermarkets in Northern Italy. The sample size for flour, pasta, and bakery products was about 1 kg, 0.5 kg, and 0.25 – 0.5 Kg, respectively. For each sample, information on the formulation and on the percentage of buckwheat were recorded, when available, together with the actual weight of package (Table 1). For sample preparation, a subsample (20 g) was obtained from the whole content of the package, after grinding (0.2 mm mesh) and mixing. Datura seeds were finely ground and carefully mixed, before storage. All ground materials were subsequently kept at -20° C and in the dark until analysis.

2.3 Preparation of standard solution for atropine and scopolamine

A standard stock solution at the concentration of 5.0 mg/L was prepared, by dissolving 0.50 mg of atropin-/scopolamin powder in 100 mL methanol, in a volumetric flask. standard working solutions at 5.0 µg/L was then prepared by proper dilution of the stock solution. Both standard solutions were stored at 4° C in the dark and used for 3 months. A six-point calibration curve was obtained for both atropine and scopolamine (Figure X), by injecting in triplicate solution at the following concentrations: 0.05, 0.10, 0.25, 0.50, 0.75, 1.00 µg/L prepared in a blank extract, obtained from TA-free buckwheat flour and pasta samples, corresponding to a contamination range of 2.5 – 50 µg/kg.

2.4 Sample preparation

For sample extraction, 5.00 g of each ground sample was mixed with 25.0 mL of extraction-solvent (dilution factor: 1:5, v/v), then placed in a shaker for 90 minutes at room temperature. After that, the samples were centrifuged (15 min, 4000 rpm). A volume of 5.00 mL of the clear solution was collected and diluted 1:10 with the extraction solvent before analysis (total dilution factor: 1:50 v/v).

2.4 Instrumental analysis

The chromatographic analysis was performed by UHPLC-MS/MS. The set up consisted from an UHPLC Ultimate 3000 separative module (Dionex, Sunnyvale, CA, USA), coupled with a TSQ Vantage triple quadrupole (Thermo Fisher, Waltham, MA, USA). The separation of tropane alkaloids was achieved by using a RP-C18 Kinetex column (2.6µ, 100A; 100x2.10 mm) from Phenomenex (Torrance, CA, USA) operated at a column temperature of 40° C.

The mobile phase consisted of bi-distilled water (Eluent A) and methanol (eluent B). Both eluents were acidified with 0.2% formic acid. The flow rate was set at 0.35 mL/min, column oven temperature was set at 40ºC, sample temperature was 20ºC and the injection volume was 4µL. The gradient was set as follows: the first step was a linear gradient from 3% B to 20% B in 6 min, followed by a second step to 80% B in 6 min. The eluent B was decreased to the initial conditions in 4 min, and the reconditioning step at 3% eluent B was kept for 4 min. The total run time was 20 min. Compounds were monitored by ESI ionization in positive mode (spray voltage = 3200 V), with the capillary temperature at 270 °C. The vaporizer temperature was kept at 200 °C, the sheath gas flow was set at 50 units and the auxiliary gas flow at 5 units. Detection was obtained in multiple reaction monitoring (MRM) modality. The following transitions were monitored: 290 →93 (CE = 33 eV) and 290 → 124 (CE = 25 eV) for atropine; 304 →103 (CE = 35 eV), 304 →138 (CE = 19 eV) and 304 →156 (CE = 16 eV) for scopolamine..

2.5 Method performance parameters

Method performance was evaluated by assessing several parameters, i.e. linearity, specificity, limit of detection, limit of quantification, accuracy as recovery, matrix effect, and precision (RSD%). Specificity was evaluated by analysing six blank samples and verifying the absence of interferences in

the atropine/scopolamine retention time range (t_R ± 2.5%). Linearity was verified in the 0.05–10 µg/L range. In particular, different solutions were obtained by diluting the standard stock solution in three different not contaminated sample extracts (buckwheat flour and pasta) at 8 concentration levels. Quantification was performed on the basis of calibration curves constructed by linear regression analysis of the area versus the concentration of the injected analytes. Moreover, for sample concentrations outside the calibration and linearity range, a proper dilution with extraction solvent must be done in order to interpolate the results. Matrix effect was defined in the 0.05 – 10 µg/L range using three different not contaminated sample extracts (buckwheat flour, pasta and cake) spiked with increasing concentration of atropine and scopolamine, by the quotient of the slope of the matrix-matched regression curve and the slope of standard regression curve. A value > 100% indicates signal enhancement, whereas a value < 100% shows signal suppression (Sulyok et al., 2007). Limit of detection (LOD) and the limit of quantification (LOQ) were derived from the signal-to-noise ratio (3/1 and 10/1, respectively). Repeatability, expressed as relative standard deviation (RSD%), was evaluated at three different spiking levels evenly distributed along the linearity range (0.05, 0.5 and 10 µg/L). Each concentration level was injected 6 times over 3 days (n = 18). The RDS% values corresponding to intra- e inter-day precision (day 2 and day 3, respectively) below 15%, were considered as acceptable (Shah et al. 2000). Accuracy, in terms of trueness, was assessed by the analysis of spiked samples at six different concentration levels. Calculated and nominal concentrations were then statistically compared. Recovery was evaluated by analyzing blank samples spiked at 6 concentration levels in the range 0.05 – 10 µg/L, and by statistically comparing the calculated and the nominal values.

2.6 Statistical analysis.

Calibration data were statistically evaluated by an ANOVA test ($\alpha = 0.05$), performed with the Statistical Analysis Tool of Microsoft Excel 2003, Microsoft Office Professional Edition 2003 (Microsoft Co., Santa Rosa, CA, USA).

3. Results and Discussion

The presence of tropane alkaloids deriving from incidental contamination of seed crops by plants of the *Datura* genus has been reported in different

countries. Authorities are actively promoting a more careful screening of those food chains most likely to be exposed to TA contamination, but a widespread monitoring is precluded by the limited availability of reliable methods combining sensitivity and speed. As a consequence, maximum levels of exposure to TA in foods are not yet available, so as a more precise knowledge of their actual occurrence in western diet. Thus, we focused our effort on a flexible protocol and on optimizing results in terms of sensitivity and recovery on different food matrices. Tropane alkaloids are small-sized, polar analytes that are easily resolved by high performance liquid chromatography-mass spectrometry, whose quantification may take particular advantage from their sensitive detection in positive ion electrospray ionization (ESI+). Encouraged by the recent success obtained by Adamse and Van Egmond in 2010, and more recently by Mulder et al. in 2015, we focused our attention on a similar strategy (Adamse and Van Egmond, 2011, Mulder et al., 2015).

3.1 Method development

Different approaches were undertaken to maximize extraction yields while minimizing processing steps and reducing the time needed for sample preparation. Different combinations of solvents were tested using a spiking concentration of 10 µ/Kg (Table 3) and the best combination in terms of recovery was obtained using CH_3OH: H_2O (3:2 v/v) acidified with 0.2% HCOOH + ACN 0.2% solution, which was therefore chosen as the elective extraction solvent. As the literature frequently reported on the degradation of tropane alkaloid reference compounds (Kirchoff et al., 2004), we tested their stability under laboratory conditions. Both analytes showed excellent stability at room temperature for 48 hours, revealing an revealing an RSD% of 2.4 % for scopolamin and 10.5 % for atropine at the lowest concentration of the calibration curve (0.05 µg/L for both TA) . Frozen TAs stock solutions were stable over 3 months of observation; in contrast, refrigerated TAs stock solutions (+ 4°C) showed a sharp degradation after 90 days.

3.1.1.Method validation. Optimized UHPLC-MS/MS method was subjected to validation in terms of precision, accuracy, linearity, detection and quantitation limits, following recommendations of the International Conference on Harmonisation (ICH, 2005). Full validation data for the method are reported in Table 2. Limits of detection (LOD) and of quantification (LOQ) were determined in blank extract, and were found to be 0.03 µg/Kg and 0.1 µg/Kg for scopolamine, and 0.09 µg/Kg and 0.30 µg/Kg for atropine, respectively. Linearity, determined in blank matrix over the range 0.05 µg/L to 10 µg/L (8 concentration levels, each injected in triplicate), was fully satisfactory, with a $r^2 > 0.99$ for both scopolamine and atropine in all the

considered matrices. Matrix effect was calculated by matrix-matched experiments over the calibration range, using blank samples spiked at the required levels. Signal suppression or enhancement (SSE%) was calculated according to Sulyok et al. (2007), by comparing the slope of the regression curve obtained for standard solution and the slope of the regression curve obtained in the matrix. The SSE% values obtained for scopolamine and atropine provided different results according to each matrix, pointing out the necessity of a matrix-matched calibration for each category of commercial products (flour, bakery, pasta).

Recovery for TAs calculated by matrix-matched calibration experiments was in the range 78 – 103% for all considered categories. Recovery rates decreased at low spiking concentration, leading to a lower accuracy for concentrations close to the LOQ. This effect could be a consequence of the already discussed influence of matrix interferences. Considerably the recovery was constant during the whole validation period. Intra- and interday repeatability were calculated in terms of RSD % at three different concentration levels (0.05, 0.5 and 10 μg/L for both TAs); the results are showed in Table 2.

3.1.2.Advances provided by the method. Figures 2 and 3 depict respectively a typical chromatogram of a buckwheat flour spiked with atropine and scopolamine (Fig. 2) and with Datura seeds (Fig. 3). The sensitivity of the presented method is largely compliant with limits suggested by EFSA and better than those recently provided by means of methods based on Orbitrap-LC, GC-MS or LC-MS/MS and developed for food matrices (Caligiani et al., 2011; Mulder et al., 2015; Mol et al., 2011; Chen et al., 2017). Given their small molecular size and polarity, for the routine analysis of TA in food both GC and HPLC methods are available, with sensitivities ranging between 0.3 and 5 μg/Kg for LOD, and between 1 and 6 μg/Kg for LOQ. The most sensitive, multi-analyte HPLC-MS/MS method published was focused on grain-based products for infants and children, with LOD in the range of 0.3 μg/Kg and recoveries exceeding 88% for both atropine and scopolamine, but being the target of this method the detection of both ergot and tropane alkaloids in a largest array of matrices, no precise tailoring on buckwheat was performed and the source of the contaminated commercial samples was not determined (Mulder et al., 2015). As described in Table 2, our protocol provides a net improvement to the reported methods in the literature in terms of sensitivity, with LOD for scopolamine and atropine in the range of 0.03 μg/Kg and 0.09 μg/Kg respectively, while LOQ were obtained at 0.10

189 and 0.30 μg/Kg.

190

191 **3.2 Screening of different commercial organic samples**

192 A further goal of our work was to provide a first estimate of the presence on TA in organic buckwheat products from the Italian market. A previous
193 survey conducted in our laboratory did not detect TA in conventional buckwheat foods purchased in Italy in 2011, but the inter-year variability
194 observed in other countries and the improved sensitivity of the present method prompted us to a more careful inspection (Mulder et al., 2015; Caligiani
195 et al., 2011). Organic foods were chosen as, on a theoretical basis, organic agriculture may be more prone to TA contamination due to a different weed
196 management in crop fields that, if not properly conducted, may increase the risk of co-harvesting *Datura* and *Fagopyrum* seeds. The results of our
197 survey involved 26 samples, 3 of which were positive for TA (Table 3). The most contaminated sample was a buckwheat flour, in which 83.9 μg/Kg of
198 atropine and 10.4 μg/Kg of scopolamine were detected. Minor amounts of atropine were found in a pasta sample (21.3 μg/Kg), and in one bakery
199 snack (13.9 μg/Kg). Scopolamine was found in buckwheat flour and pasta, together with high levels of atropine (10.4 μg/Kg and 5.7 μg/Kg,
200 respectively).

201 The atropine:scopolamine ratio of 20:80 in positive samples and the comparison of the UHPLC-MS/MS profile of pure jimsonweed seeds (Figure X)
202 are coherent with a potential *Datura stramonium* contamination (Steenkamp et al., 2004).

203 Despite the limited numbers, our data are in agreement, both in terms of overall incidence and of quantitative amounts, with those emerging from
204 recent surveys conducted elsewhere in Europe, in which TA were present in approximately 20% of the samples with the highest occurrence level at 100
205 μg/Kg for atropine and 47 μg/Kg for scopolamine (EFSA, 2008 ; Mulder et al., 2015). The contamination level recorded in sample BF2 was close to
206 the limit set by French authorities in 2008, defining an intervention threshold at 100 μg/kg buckwheat flour for the sum of atropine and scopolamine
207 (Afssa, 2008). On the contrary, values recorded for Italian samples are remarkably lower than those detected in Slovenia in 2013 by Peharic *et al*. and
208 in 2007 and 2008 by AFSSA in France. In particular, on these occasions TA contamination concerned 40% of the samples and total TA exceeded 7400

μg/kg in one french sample and 38000 μg/kg in one sample from Hungary (Peharic et al., 2013; AFSSA 2008). Nevertheless, a 94.3 μg/kg TA content cannot be considered negligeable from a food safety standpoint and reinforces the need for a punctual monitoring of the buckwheat market and the enforcement of precise management of Datura weeds in buckwheat fields, both organically or conventionally grown. In fact, assuming mean thousand seed weight values for buckwheat and Datura seeds (http://data.kew.org/sid/weight.html) and a standardised alkaloid content in the latter, the addition of one Datura seed per million buckwheat seeds would result into 7.4 mg/kg of atropine and 1.8 mg/kg of scopolamine (Friedman & Levin, 1989), a value that translates to approximately 10 seeds per 200 kg in our most contaminated flour sample. As this ratio may be easily achievable by co-harvesting a single *Datura stramonium* plant in a buckwheat field, a careful scrutiny at field level must be encouraged to prevent the contamination at its source. For instance, a full-grown Datura stramonium plant may produce approximately between 1000 and 2000 seeds, whose presence in a buckwheat crop may lead to a critical contamination of approximately 20-40 tons of flour. Furthermore, TA are fairly stable during drying and heat treatment and may be present at 72–100% after baking bread from wheat flour contaminated with *Datura stramonium* (Friedman and Levin, 1989). As a consequence, a daily portion of bread (160 g) prepared with the most contaminated flour detected in this survey would result in an intake of approximately 17 μg of TA. This amount is sufficient to exceed the NOAEL for atropine and the Acute Reference Dose (ArfD) recently established by CONTAM panel for atropine and scopolamine, according to the same elegant rationale suggested elsewhere (Mulder et al., 2015). A similar approach, however, cannot be applied to the contaminated pasta sample, for which the likely dilution and degradation of TA in boiling water would reduce considerably the actual intake.

4. Conclusions

There are actually two major sources of human exposure to TA: accidental contamination of foodstuffs and the deliberate use of Datura plants in herbal preparations. The former falls in turn into two categories, one involving continuous low-level contamination due to reiterated ingestion and one involving occasional, acute intoxication due to the ingestion of massively contaminated foods. Both require the availability of sensitive and easy-to-perform analytical methods. The validated UHPLC-MS/MS ESI+ method presented herein proved to be both reliable and serviceable for screening

traces of tropane alkaloids in buckwheat not only as a raw material, but also as an ingredient in processed and baked foods. It can be thus applied as a screening tool for a large number of matrices along the buckwheat supply chain, to monitor the potential presence of these harmful contaminants. It must be noted that the low percentage of contaminated samples in the limited screening hereby provided is undoubtedly a favourable event, but the severity of potential intoxications and in particular the high level reported for one organic buckwheat flour sample suggest the adoption of precaution and underlines the focus put by EFSA on this topic. In particular, organic buckwheat samples in Italy may present a degree of atropine and scopolamine contamination comparable to the one recently described for various cereal-based products in Europe (Mulder et al., 2016). The availability of a method capable of spotting trace levels of tropane alkaloids also in baked products is relevant as these substances are known to survive bread-baking conditions. Given the limited number of producers, we cannot exclude that the positive samples detected in our survey may have been originated by a single in-field contamination, leading to suggest the pertinence of a closer control at the very beginning of the food chain.

5.Conflict of interests. Having read and understood the journal policy on declaration of interests, the authors declare no competing interests.

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329 **Table 1: Commercial, organic food samples and their ingredients**

330

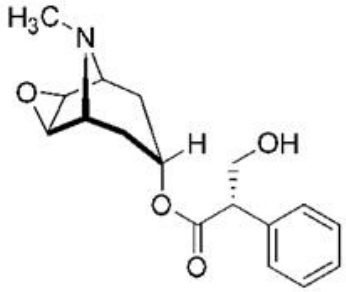
	Sample	Description
Buckwheat flour	BF1	100% buckwheat
	BF2	100% buckwheat
	BF3	100% buckwheat
	BF4	100% buckwheat
	BF5	100% buckwheat
	BF6	100% buckwheat
	BF7	100% buckwheat
	BF8	100% buckwheat
	BF9	100% buckwheat
	BF10	10% buckwheat, 90% maize
	BF11	100% buckwheat
	BF12	100% buckwheat
Buckwheat pasta	BP1	30% buckwheat flour, 70% durum wheat
	BP2	25% buckwheat flour, 75% durum wheat
	BP3	100% buckwheat
	BP4	100% buckwheat
	BP5	100% buckwheat
	BP6	100% buckwheat
Buckwheat bakery	BB1	Crackers: 100% buckwheat
	BB2	Crackers: 100% buckwheat
	BB3	Crackers: 40% buckwheat, 40% rice
	BB4	Plumcakes: 20% buckwheat
	BB5	Crackers: 100% buckwheat
	BB6	Crackers: 100% buckwheat
	BB7	Crackers: 100% buckwheat
	BB8	Crackers: 100% buckwheat

Table 2: Quality parameters of the method, calculated from blank samples spiked with alkaloid standards

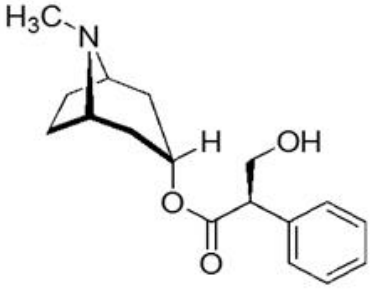
	Scopolamine	Atropine
Linearity range (µg/Kg)	0.05 - 10	0.05 - 10
RR% Flour	78	83
SSE% Flour	119	126
RR% Pasta	99	103
SSE% Pasta	133	141
RR% Bakery products	102	88
SSE% Bakery products	75	77
Slope	135928 ± 2982	154054 ± 2810
Intercept	5810 ± 262	18119 ± 1220
LOD (µg/Kg)	0.03	0.09
LOQ (µg/Kg)	0.10	0.30
Intraday Repeatability at 0.05 µg/Kg (RSD%)	3.4	10.7
Intraday Repeatability at 0.5 0.05 µg/Kg (RSD%)	5.2	1.8
Intraday Repeatability at 10 0.05 µg/Kg (RSD%)	2.3	2.8
Interday Repeatability at 0.05 µg/Kg (RSD%)	2.4	10.5
Interday Repeatability at 0.5 µg/Kg (RSD%)	9.5	6.9
Interday Repeatability at 10 µg/Kg (RSD%)	1.8	7.1

Table 3: Optimization of the extraction method: recovery rates of blank samples spiked with alkaloid standards

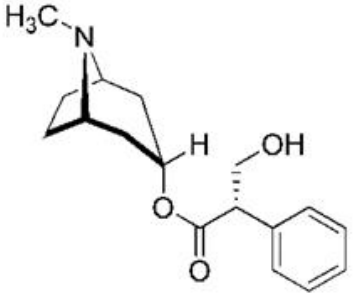
	Scopolamin		Atropine	
Extraction Solvent	**Recovery rate (%) Flour**	**Recovery rate (%) Pasta**	**Recovery rate (%) Flour**	**Recovery rate (%) Pasta**
CH_3OH: H_20 (3:2)	45	82	61	86
CH_3OH: H_20 (3:2) + 0.2 % HCOOH	75	92	110	54
CH_3CN: H_20 (3:2)	69	81	87	92
CH_3CN: H_20 (3:2) + 0.2 % HCOOH	88	89	111	79
CH_3OH: H_20 (3:2) + 0.2 % HCOOH + 0.2% CH_3CN	98	102	105	104



(-)- scopolamine



(-) – Hyoscyamine



(+) – Hyoscyamine

atropine

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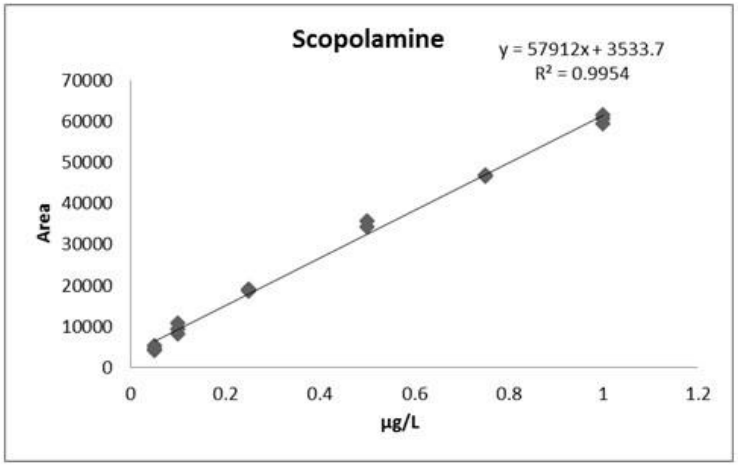
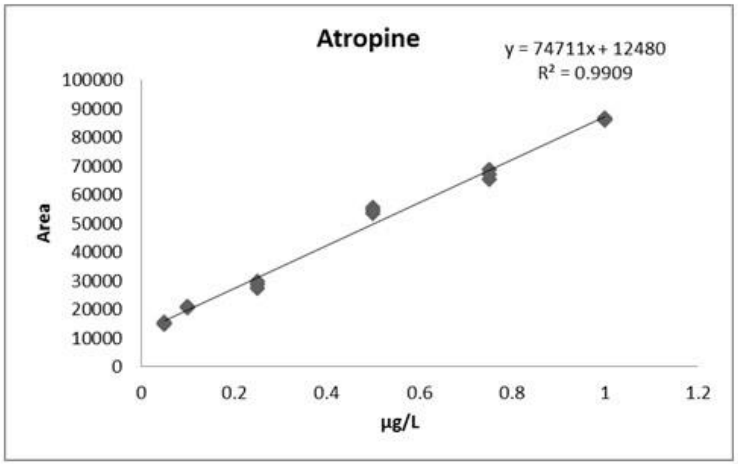
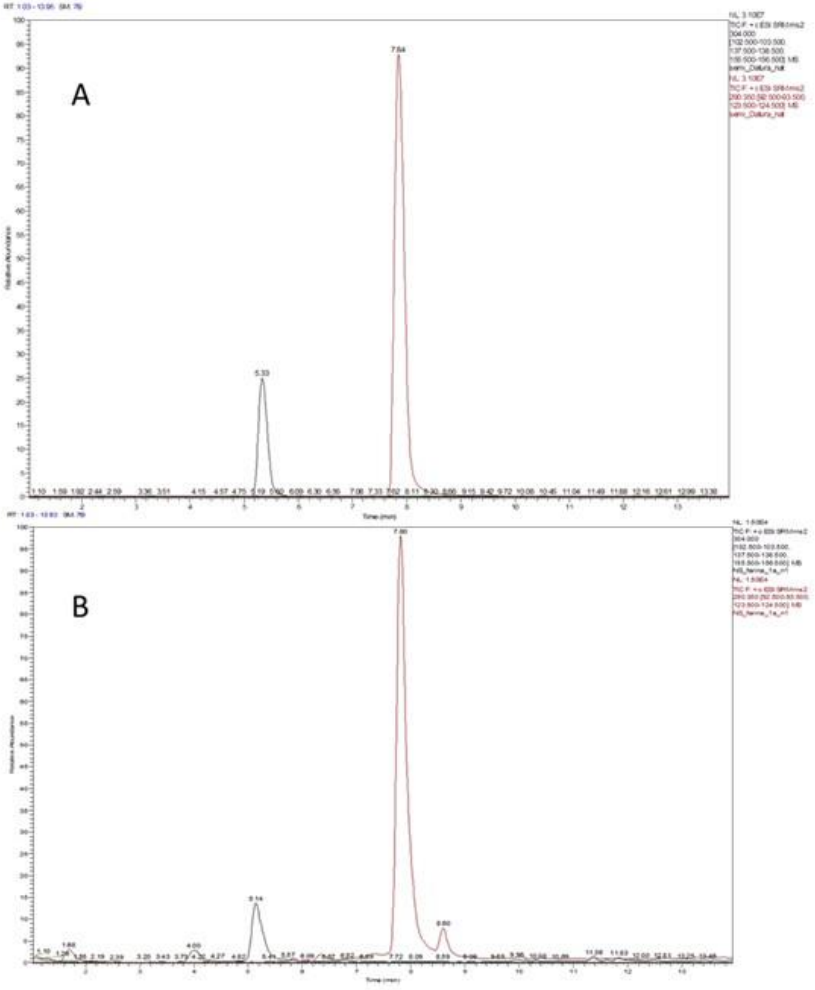




Calibration parameters	scopolamine	atropine
b	3533.7	12480
S_b	572.7	1004.5
a	57912	74711
S_a	1014.9	1790.9
$S_{y/x}$	1487.4	2626.7
df	15	16
r^2	0.9954	0.99089
F	3255.7	1740.3
SSreg	7202704342	1.2E+10
SSresid	33184841.6	1.1E+08



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