Facing tropane alkaloid contamination in millet – Analytical and processing aspects

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Abstract

Thorn Apple or Jimson Weed (*Datura stramonium*) is a tropane alkaloid containing widespread pest plant growing in central Europe. This pest predominantly occurs in millet, buckwheat and maize cultivation. Therefore, sound cleaning of these crops is just as pivotal as reliable analytics. The cleaning of millet at laboratory scale with commercial mechanical cleaning devices, such as sieves, indented separators or table sorters without using colour sorters, reduces the dark coloured *Datura* seed content by up to 99%. Nevertheless, the few remaining *Datura* seeds (up to one nut per 30 kg) could lead to an exceedance of the European Union (EU) limit values for tropane alkaloids in baby food. However, plant material other than seeds or even invisible abrasion of *Datura* seeds can contaminate the millet during harvest, handling, cleaning processes or crop transportation. Contamination originating from adhesive dust can be removed by dehulling the kernels, and a reduction below the EU detection limit could be achieved.

Keywords: *Datura stramonium*, *Panicum miliaceum*, atropine, scopolamine, cereal cleaning

Introduction

The solanaceous herb *Datura stramonium* (Thorn Apple or Jimson Weed) is a widespread pest plant in central Europe (Steenkamp *et al.*, 2004). Karimmojeni *et al.* (2010) reported corresponding growth conditions for *D. stramonium* and maize. The possibility for populations within maize (*Zea mays*), buckwheat (*Fagopyrum esculentum*) and proso millet (*Panicum miliaceum*) cultures were attributed to the late emergence of these crops. Crops like wheat (*Triticum sp.*), rye (*Secale cereale*), oat (*Avena sativa*) and barley (*Hordeum vulgare*) are not affected since they form early closed populations before the germination of *Datura* seeds. This hindered the development of toxic plants such as *D. stramonium* within these crops (Hagood *et al.*, 1981; Kirkpatrick *et al.*, 1983). The primary risk for *D. stramonium* presence is the acute toxicity of high concentrations of tropane alkaloids (TA) distributed throughout the plant (Krenzelok, 2010; Miraldi *et al.*, 2001), which potentially contaminates the crop during harvest. At least 26 different alkaloids are detected in *D. stramonium* like the TA atropine (a racemic mixture of L- and D-hyoscyamine) and scopolamine in varying ratios (Berkov *et al.*, 2006; Krenzelok, 2010). At toxic doses, atropine and scopolamine affect the central nervous system (CNS) with restlessness, disorientation, hallucinations and delirium as major symptoms. At highly toxic doses, severe respiratory paralysis can also result in death (EFSA, 2013).

Intoxications because of food consumption have been described for buckwheat (Glaizal *et al.*, 2013; Perharič *et al.*, 2013) and millet contaminated with TA (Fretz *et al.*, 2007; Rwiza, 1991). Product contaminations are also known for maize products (e.g., popcorn) and food for infants and toddlers (EFSA, 2013; Mulder *et al.*, 2015, 2016). Further, contaminated maize silage becomes a serious challenge in animal feed regarding intoxications, decreased feed intake and anti-nutritive properties (Aboling *et al.*, 2019). Considering the degree of processing, Friedman and Levin (1989) reported very limited

degradation of TA during bread making. Hence, recoveries of 87% for breadcrumbs and 72% for bread crust were observed indicating, high stability of TA during thermal processing. These weak reduction findings imply that in addition to prevention, reliable analysis and a thorough removal of *Datura* seeds are highly recommended measures to prevent food contamination. The acute toxicity of TA combined with the frequent occurrence caused authorities to establish legal limit values. In the European Union (EU), the maximum content of 1 µg/kg atropine and scopolamine has been established for children and baby food (EU, 2016).

For the determination of TA, chromatographic methods coupled with mass spectrometry are used in combination with other analytes (Berkov *et al*., 2006; Miraldi *et al*., 2001; Mulder *et al*., 2015). A good overview of the analysis of TA provided by Dräger (2002) is valid till today. TA is water-soluble; when been acidified, the extraction solvents are seen to contain traces of water. All plant material needs to be homogenised to tiny particle size for quantification of TA. Commonly, extraneous materials, for example, stones, dust, straw, chaff, stalks, particles of *D. stramonium* plants, etc., can be separated from millet grain during millet processing by using destoners and aspirators based on their density difference (Beta and Ndolo, 2019). The next stage involves the treatment of the cleaned millet through abrasion or friction mills for glumes (non-edible cellulosic tissue) removal, and decortication is performed with a centrifugal sheller or decorticators. Later, the millet is pulverised in steel or emery coated disc or stone mills (Gull *et al.*, 2016). To date, no data is available in the scientific literature about minimisation strategies of TA in millet contaminated with *D. stramonium* by applying typical cleaning techniques.

This study evaluated the effectiveness and applicability of common mechanical cereal cleaning techniques concerning the contamination level of *Datura* seeds within a millet batch. The effect of processing like transportation and hauling was estimated. Further, reliable and suitable analytics to monitor the results of the applied techniques were addressed. First, an overview of TA contents of *D. stramonium* plant and seeds are discussed. Second, experiments causing the abrasion on *Datura* seeds are estimated. Finally, millet containing *Datura* seeds was cleaned and dehulled using typical practical cleaning techniques, and each cleaning step was analysed.

Materials and Methods

Plant material

About 50 kg of uncleaned millet was purchased from Strobl Naturmühle (Linz-Ebelsberg, AT), *D. stramonium* plants were harvested outdoors of the Max Rubner-Institut (Detmold, DE), and *D. stramonium* seed material was collected from brownfield sites in Germany (BayWa agricultural company, Munich, DE). The millet lot was precleaned using a Labofix 90 laboratory grain cleaner (Schmidt-Seeger AG, Beilngries, DE) equipped with a 2.2 mm slot sieve. Additionally, two manual sieves with 2.0 mm and 1.6 mm pore size were applied. Subsamples were taken using a sample divider.

Chemicals and reagents

Liquid chromatography (LC)-mass spectroscopy (MS) grade methanol (CHROMASOLV®) and formic acid (MS grade with >98% purity) were purchased from Sigma-Aldrich Company (Steinheim, DE). Ultrapure water (Milli-Q Plus system, Millipore Bedford, MA) was used throughout the work. In all experiments, methanol/ultrapure water/formic acid, 600/400/4 (v/v/v), was freshly prepared and used as extraction solvent. Acetonitrile (ROTISOLV® high-performance liquid chromatography (HPLC) grade, Carl Roth Company, Karlsruhe, DE), atropine (purity 99%, Romer Labs Diagnostic GmbH, Tulln, Austria) and scopolamine hydrobromide trihydrate (purity ≥98%, HPLC grade, Sigma-Aldrich Company, Steinheim, DE) were used for preparing standard solutions. After every contaminated material use, the machinery and other equipment were disinfected with ethanol/formic acid, 1000/4 (v/v), ultrapure water and ethanol (Sigma-Aldrich). Matrix solid-phase dispersion material (MSPD; Strata®, Phenomenex, Aschaffenburg, DE) and n-hexane (ROTISOLV® HPLC grade) were utilised to degrease oily *D. stramonium* seeds during extraction.

Sample preparation

A ZM 200 Ultra Centrifugal Mill (Retsch, Haan, DE) with 14,000 rpm and 0.5 mm sieve pore size were applied to mill the samples. In addition, the samples were shaken by a laboratory shaker (GLF, universal model 3020, Burgwedel, DE), then centrifuged by a KR4i Large Capacity Centrifuge (JOUAN GmbH, Unterhaching, DE) and were mixed by a vortex mixer (Heidolph Instrument GmbH, Schwabach, DE). Before chromatographic analysis, samples were filtered using perfect flow® NYLON membrane filter, 0.2 µm pore size and 25 mm or 13 mm diameter (WICOM GmbH, Heppenheim, DE).

Mixing process

The precleaned millet was mixed with *Datura* seeds (1 kg + 1 or 10 nuts), using a self-constructed laboratory mixer equipped with a glass bottle mimicking overhead

shaking for 15 min. Sampling was done every 5 min starting with a blank, and all devices were washed using aqueous ethanol/formic acid solution post the performance of each experiment to avoid carry-over.

Cleaning procedure

The *Datura* seeds (200) were weighted and mixed with 2 kg of millet for 5 minutes. Then the resultant mixture and was sieved through a 2.75 mm grain sieve, and the leftover material and the *Datura* seeds were weighed and counted, respectively. The screenings were transferred to ta Labofix 90-grain cleaner. All devices used were washed with aqueous ethanol/formic acid solution post each cleaning process to avoid sample contamination with TA.

Millet samples

The millet sample (2.5g) and 25 mL extraction solvent (methanol/water/98% formic acid 600/400/4 v/v/v) were vigorously shaken at 230 rpm for 30 minutes. Later the resultant mixture was centrifuged at 2687*g* for 10 minutes at 4°C and, the sample extracts were filtered into amber glass vials, vortexed (1–2 s, 2500 rpm) and analysed by LC-MS/MS for atropine and scopolamine levels. All millet samples were extracted as triplicates and measured with two injections each.

D. stramonium plant and seed samples

Seeds or other homogenised parts of the plants were crushed manually using a mortar and pestle. Samples (0.3 g homogenised seed or plant material powder) and 30 mL of extraction solvent (methanol/water/98% formic acid 600/400/4 v/v/v) were mixed and used for a single seed analysis. Extraction was performed using a laboratory shaker for 30 minutes. Later, the samples were centrifuged at 2687 g for 10 minutes at 4°C.

To 12 mL of seed extract, 0.6 g MSPD material was added, and the solution was vortexed for 30 seconds. The resultant sample was centrifuged at 1600*g* for 10 min at 4°C. After centrifugation, MSPD materials settled to the bottom of the centrifuge tubes and the extracts were stored separately for further analyses.

The extracts of *Datura* seeds were further diluted with the extraction solvent to reach a dilution of $1{:}10^8$ for analysis in the chromatographic system. All *Datura* samples except the single seeds were extracted as triplicates and measured with two injections each. Single seeds were extracted once and measured with two injections each.

HPLC-MS/MS

Chromatographic separation was carried out using a Shimadzu Prominence LC 20 system (Duisburg, DE). A Phenomenex reversed-phase column (150 × 2.0 mm) filled with Luna Phenyl Hexyl material (5 µm particle size) was used for the separation. The flow rate was 0.5 mL/min, and a volume of 10 µL was injected. A binary gradient consisting of water with 0.1% formic acid (solvent A) and methanol (solvent B) was applied. The best peak shape and separation was obtained with the following eluent gradient profile: start 10% B; 2 min: 10% B; 10 min: 80% B; 12 min: 80% B; 13 min: 10% B and 15 min:10% B. The temperature of the column was held at 40°C.

The mass spectrometer measurements were performed on a triple quadrupole/linear ion trap mass spectrometer, API 4000 QTrap (AB Sciex, Darmstadt, DE) It worked with electrospray ionization in the positive mode under the scheduled multiple reaction monitoring conditions. The curtain gas was set to 20 psi, collision activated dissociation gas to medium, GS1 to 55 psi and GS2 to 50 psi. The source temperature was 600°C, and ion spray voltage was 3500 V in positive mode. For each analyte, two mass transitions were recorded, applying individually optimised MS/MS conditions (Table 1).

Method optimisation and validation

Method optimisation and validation were performed using standard solutions or a spiked millet sample that

Table 1. Chromatographic and mass spectrometric parameters of the monitored ions.

Analyte	Retention time [min]	Precursor ion $(M+H)^+$	DP [V]	EP [V]	CE [V]	CXP [V]	Product ion
Atropine	6.2	290	93	10	38	8	124.1 (Q)
			89	10	75	14	93.1 (I)
Scopolamine	4.9	304.2	76	10	35	8	138.2 (Q)
			76	10	23	14	156.2 (I)

DP, declustering potential; EP, entrance potential; CE: collision energy; CXP: collision cell exit potential in volt; Q, transition used for quantification; I: transition used for identification.

was tested free of atropine and scopolamine for recovery experiments.

Sample extracts in the concentration range of 0.2 to 5.0 ng/mL were calculated against a solvent calibration curve for both analytes (Table 2). The regression coefficients for atropine and scopolamine were observed as 0.9997 and 0.9994, respectively. Further experiments with an internal standard calibration or a matrix calibration curve (data not shown) revealed no significant differences concerning the calculated results.

Recoveries were determined by spiking 2.5 g of a blank millet sample at low, middle and high concentration levels in five replications. The limit of detection (LOD) and limit of quantification (LOQ) were approximately estimated with three-and 10 times the signal-to-noise ratio (S/N) of the chromatograms, respectively.

Results and Discussion

D. stramonium plant parts and seeds

A whole *D. stramonium* plant was harvested from the outdoors of the institute, and all parts were analysed individually. The TA contents are shown in Table 3, and were found to vary from 80 ± 4 mg/kg atropine and 16 ± 1 mg/kg scopolamine for the stem material and up to 2590 ± 6 mg/kg atropine and 442 ± 6 mg/kg scopolamine for unripe seeds.

It was remarkable to see the transmission of the TA from the seeds to the capsule during ripening, as concentrations in nuts and capsules show opposing trends for unripe and ripe material. A strongly varying TA-content could be measured within a single *D. stramonium* plant seeds with 46 mg/kg atropine and 11 mg/kg scopolamine to 3064 mg/kg atropine and 352 mg/kg scopolamine (Table 4).

These findings contrast the study of Miraldi *et al.* (2001), who analysed *D. stramonium* plants grown in Tuscany, Italy. Their study outcomes indicated that the stem material showed the highest TA levels in young plants, and reported seed concentrations were up to 387 mg/kg in adult plant seeds, approximately 87% less than the highest TA content observed in this study. However, the analysed and compared plants were grown under different environmental and nutritious conditions. The high nitrogen requirements of *D. stramonium*, the varied growth conditions in Italy and Germany and grades of maturation could lead to different TA synthesis and translocation processes within the characterised plants as reported for

Table 2. Performance characteristics and statistical parameters of the studied techniques: linear range, R^2, LOD, LOQ, repeatability, intermediate precision and recovery.

Analyte	Linear range [ng/mL]	R2	LOD [ng/mL]	LOQ [ng/mL]	Repeatability [%] n = 5	Intermediate precision [%] n = 5	Recovery [%] n = 5
Atropine	0.05–5.0	0.9999	0.003	0.01	1.2[c]	3.6[c]	85[a] 101[b] 108[c]
Scopolamine	0.05–5.0	0.9999	0.008	0.025	1.1[c]	3.5[c]	119[a] 92[b] 97[c]

[a]0.05 ng/mL; [b]1.0 ng/mL; [c]2.5 ng/mL.
R^2, coefficient of determination; LOD, limit of detection; LOQ, limit of quantification.

Table 3. Tropane alkaloid contents in dry mass of *D. stramonium* plant material.

Material	Atropine [mg/kg dm]	Atropine, SD [mg/kg dm]	Scopolamine [mg/kg dm]	Scopolamine, SD [mg/kg dm]
Stem	80	4	16	1
Leaf	434	7	91	1
Capsule (unripe)	225	7	86	2
Capsule (ripe)	625	28	327	15
Seeds (unripe)	2590	6	442	6
Seeds (ripe)	1827	45	566	9

SD, standard deviation.

Table 4. Tropane alkaloid contents of a single *D. stramonium* plant seeds.

Seed	Weight [mg]	Atropine [mg/kg]	Scopolamine [mg/kg]
Seed 1	**9.928**	2718	340
Seed 2	7.523	3064	207
Seed 3	7.743	2494	195
Seed 4	7.520	1489	352
Seed 5	8.895	2282	161
Seed 6	6.524	46	12
Seed 7	7.757	545	58
Seed 8	7.143	1429	103
Seed 9	7.136	102	11
Seed 10	7.545	1125	89
Average	7.771	1694 ± 1012	188 ± 110

the glycoalkaloid synthesis within the solanaceous herb *Solanum nigrum* (Edmonds *et al.*, 1997).

Precleaned millet

The raw millet (50 kg) was purchased from a cereal mill to obtain homogeneous millet material and was cleaned. The cleaned millet was divided into five fractions, and TA content was analysed, including the cleaned millet sample material for further studies. The coarse material, containing mostly seeds like soybean, lupine or cereal kernels from the indented separator section of the Labofix 90, showed TA contents below LOD. The fraction obtained from the aspiration fraction of the Labofix 90 contained 12.81 ± 0.52 µg/kg of atropine and 4.58 ± 0.2 µg/kg of scopolamine. The highest amounts of TA were found within the manual sieve fraction below 1.6 mm (144.77 ± 14.38 µg/kg of atropine and 53.20 ± 5.45 µg/kg of scopolamine). The cleaned millet sample was also contaminated by 1.75 ± 1.30 µg/kg of atropine, indicating pre-existing contamination with *D. stramonium* or other Solanaceae species. The scopolamine content was analysed below LOD in the cleaned millet. A thorough cleaning of cereals is highly recommended even if no apparent contamination with toxic material is visible, especially for small scale laboratory experiments. As high amounts of TA along with the fatty characteristic of the seeds make for a challenging homogenisation of artificially contaminated samples.

Artificial contamination of millet with *D. stramonium* seeds

The cleaned millet was artificially contaminated with *D. stramonium* plant seeds and vigorously mixed to mimic the potential abrasion of *Datura* seeds during transportation, relocation and processing (Figure 1A). Mixing 1 kg millet with one *D. stramonium* plant seeds led to a continuous increase in atropine content from 0.79 ± 0.09 µg/kg to 1.96 ± 1.76 µg/kg post mixing for 15 minutes. Ten *D. stramonium* seeds were added to the millet sample to increase the simulated contamination level (Figure 1B). The initial atropine content in the precleaned sample was 2.15 ± 0.05 µg/kg and upon adding 10 seeds and mixing for 5 minutes, dropped to 0.65 ± 0.02 µg/kg. The atropine content increased continuously to 1.06 ± 0.13 µg/kg with continuous mixing of 15 minutes. The scopolamine content also dropped after adding the *Datura* seeds and increased continuously during mixing time.

Here, it is presumed that shortly after adding the *Datura* seeds, the friction among the millet kernels could brush the pre-existing TA contamination off. Aqueous *Datura* plant liquids can adhere to cereal surfaces during harvest causing invisible contamination after drying. Further mixing, the *Datura* seeds could abrade fatty, highly adhesive seed material onto the millet surface. These assumptions indicate that millet containing *Datura* seeds should be moved as little as possible.

Addressing the drop of TA contents shortly after the start of the mixing process in *Datura* seed-free millet by

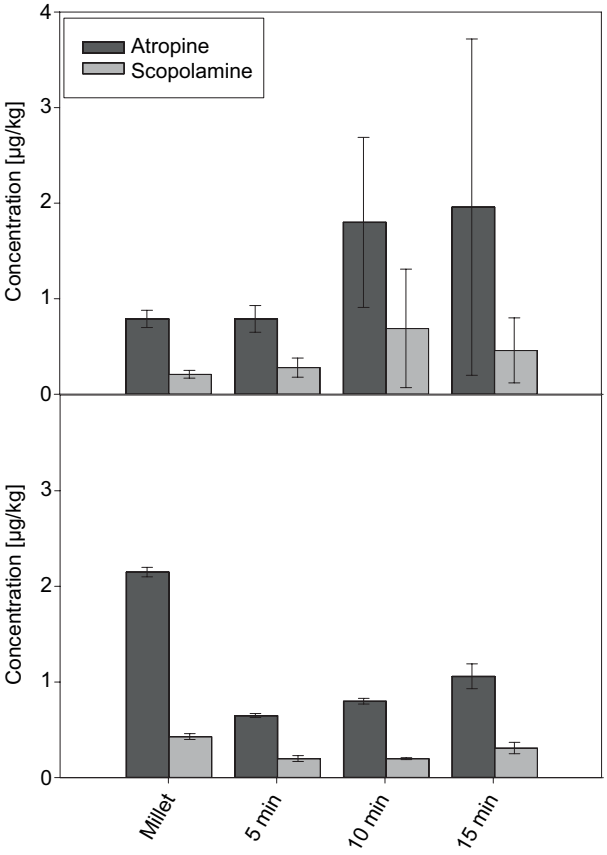

Figure 1. Tropane alkaloid content levels in the pre-cleaned millet; After mixing with one (A) and 10 *Datura* seeds (B).

typical milling techniques, like scouring and brushing, could decrease TA contents in millet lots before dehulling. Considering the number of applied *Datura* seeds, the total amounts of atropine and scopolamine within the two experiments in this study are notable. The difference in the initial TA contents of the millet material underlined the inhomogeneity caused by punctually high TA concentrations, despite using a sample divider.

Cleaning of the contaminated millet

The effectiveness of common cereal cleaning techniques was evaluated by contaminating 2 kg millet with 200 *D. stramonium* plant seeds (1.675 g). After distributing the seeds homogeneously throughout the millet sample, the cleaning was performed (Figure 2A). By sieving, 136 seeds were removed, and 1967.8 g millet and 64 seeds mixture were passed to the next stage. In the next cleaning step using a Labofix 90 device, a cylindrical slot sieve with a pore size of 1.75 × 20 mm removed 61 seeds. The resultant contained 1608.8 g millet and three seeds which were passed through the indented separator section. Finally, the remaining three seeds were removed by hand to obtain a *Datura seed*-free material for the dehulling experiment. The 200 recovered seeds showed a loss of 114.11 mg by weight. The TA contents of the *Datura* seedless millet were evaluated post every cleaning step. Figure 1A shows the initial TA content dropped after mixing and stayed low at sieving. After Labofix 90, the TA content increased erratically. By dehulling the *Datura* seedless millet, the TA content could be decreased below LOD. The removed husks showed high TA contents of 5.93 ± 1.30 µg/kg of atropine and 2.55 ± 0.01 µg/kg of scopolamine.

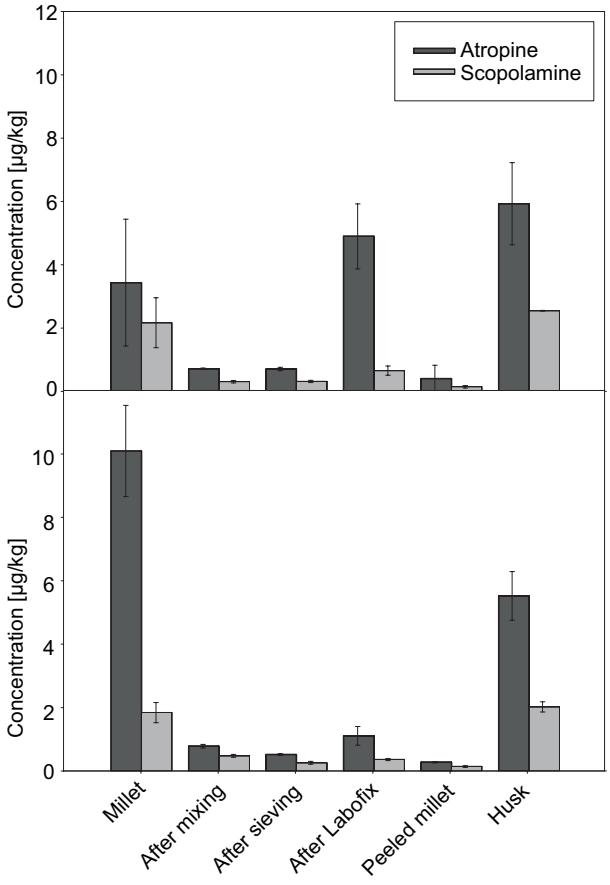

Figure 2. Tropane alkaloid contents of millet during cleaning procedures; experiment (A) and replicate (B).

In the replicates (Figure 2B), the 200 *Datura* seeds weighed 1.819 g. During the initial cleaning step, 163 seeds were sieved, and the resultant mixture contained 1965.4 g millet and 37 seeds. These were passed through a Labofix 90 device which removed 33 seeds. The mixture obtained contained 1635.5 g millet and four seeds after passing the indented separator section without removing any seeds. A *Datura*-free material for the dehulling experiment was obtained by manually removing the leftover seeds. The 200 recovered *Datura* seeds showed a loss of 120.22 mg by weight. Figure 1B shows that the high initial TA content dropped after mixing and stayed low during sieving. Again, after Labofix 90, the TA content increased. By dehulling the completely cleaned millet, the TA content could be decreased below LOD. The removed husks showed high TA contents of 5.53 ± 0.77 µg/kg of atropine and 2.03 ± 0.16 µg/kg of scopolamine.

Repeatedly a rise in the TA contents in millet increased after the Labofix 90 treatment. The obtained result is counterintuitive, as using a Labofix treatment, needed to reduce the contamination and not enhance the TA content. Here, supposedly the indented separator section (indented cylinder) of the Labofix 90 device caused vigorous mixing and abrasion of millet and seed material since the *Datura* seeds showed significant weight losses during the experiments. Graeber *et al.* (2016) presented a similar phenomenon for the separation of ergots (*Claviceps purpurea*) out of rye using an industrial indented separator. The ergot alkaloid content increased after this cleaning step. However, in the addressed publication, the shown ergot alkaloid data was interpreted as a measuring artefact. We assume that *Datura* seeds and ergots have high-fat contents that make the abraded material strongly adhere to the cereal surface (Franzmann *et al.*, 2010; Friedman and Levin, 1989).

In general, thorough removal of *Datura* seeds is highly indicated considering the high TA amounts. Even one remaining *Datura* seed per 30 kg of millet could lead to an exceedance of the EU limit values for baby food, presuming a homogeneous distribution of seed material in the prepared foodstuff (Commission Regulation (EU), 2016/239). Assuming an extremely limited spatial

distribution of such material, single packages of the corresponding lot could pose serious health risks. The experiments with practical cleaning procedures demonstrated the presence of minimal seeds in the millet material, which could be eliminated by currently available colour sorters in the last cleaning step. Here the mechanical cleaning techniques are pivotal since the performance and throughput of colour sorters increase with decreasing concentrations of extraneous material.

Conclusions

This study showed the several processing aspects that need to be considered to produce safe and less TA-contamination millet products, especially for children's and baby food. The high concentrations of TA distributed throughout the entire *Datura* plant indicate different contamination pathways along the processing chain from harvest to decortication. Contamination of millet with damaged *Datura* plant material inside the harvester, for example, can lead to invisible TA-contamination or abrasion of the *Datura* seed material in the cleaning process. Second, the high concentration of TA within the *Datura* plant could lead to inhomogeneities with highly punctual contaminations, which would pose a critical challenge for a statistically representative sampling on the one hand and food safety on the other. Therefore, in many products made from millet that are considered safe, some isolated packs may contain harmful TA levels. Third, the millet contaminated with *Datura* seeds needs to be processed gently to avoid the abrasion of the seed material with complete disposal of nuts at each processing step. Fourth, while the behaviour of *Datura* seeds within the decortication process still needs to be evaluated, complete seed removal before dehulling is necessary. The high energy input required for decortication could otherwise lead to further abrasion of the *Datura* seeds, which could adhere to the millet surface.

The study outcomes indicated that a safe millet product is only possible when the *Datura* seeds are eliminated without traces before milling, which cannot be guaranteed in all cases. Ideally, using agronomic techniques before harvest to ensure food safety will eradicate the total occurrence of *D. stramonium* in millet crops. If any visible *Datura* plant material is detected, it must be abolished before further processing, including the adhesive abrasion of the plant material on the millet surface.

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