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Development of a DNA-based Assay to Detect and Quantify Tropane Alkaloids Producing Thornapple Contaminations in Processed Food

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Abstract: Contaminates such as pesticides, toxic molecules of natural origin, genetically modified organisms and others can occur in processed food, especially if the main ingredient grows in open fields exposed to the environment. In particular, some health threatening toxic compounds are natural ingredients of plants that grow wild next to vegetables intended for consumption and can therefore enter the crop yield and stay there undetected. The tropane alkaloids-containing nightshade thornapple *Datura stramonium*, often grows in close vicinity to *millet* (*Panicum miliaceum*) a widely cultivated cereal, representing an important nutrient source in different countries of Asia and Africa. Discriminating thornapple from *millet* during harvest is not easy and consequently, millet-containing food products are often contaminated with tropane alkaloids from thornapple. In this work, two DNA specific hydrolysis probe qPCR methods were developed for *Datura stramonium* and *Panicum miliaceum* in order to detect thornapple contamination in *millet*-containing food products. The specificity and sensitivity of the developed assay system allows for its application in screenings during food product testing.

Keywords: *Datura stramonium* · *Millet* food preparation · Real-time PCR · Specific hydrolysis probe · Tropane alkaloids

1. Introduction

The cultivation of plants for food is subject to different types of risk such as meteorological disturbances, environmental pollution or parasites. Moreover, plants of different type but growing under similar conditions might either compete for space and resources or grow in perfect symbiosis, making it very difficult to distinguish the farmed from the contaminating plants during harvest.

If the crop is to be used for food processing, it is essential that the raw material is free of any kind of contaminants of both chemical (*i.e.* environmental pollutants) and natural origin (*i.e.* parasites or other plants) especially if these represent a threat to human or animal health.

Millet is an annual grass belonging to the family of *Poaceae*; it is cultivated all over the world, especially in Asia where in certain regions it can represent more than 20% of the annual crop, and in Africa where the annual production reached 16 Mio t in 2012. *Millet* is also a basic ingredient for various processed food products like baby food, cereal-based products or fodder. In Europe, the most cultivated millet species is the *Proso millet* (*Panicum miliaceum* (Fig. 1A right panel)) which tolerates different weather conditions and numerous soil compositions. Due to its versatility and non-demanding farming properties, it often grows alongside other plants that are not intended for food processing.

In the case of the nightshades such as thornapple (*Datura stramonium* (Fig. 1A left panel)) and deadly nightshades (*e.g. A. belladonna*), contamination of the processed food might result in a health threat. In fact, both thornapples and deadly nightshades contain tropane alkaloids such as atropine and scopolamine. Tropane alkaloids produce an anticholinergic and antimuscarinic effect[1] which allows atropine to be used as a drug in cardiology and anaesthesiology or as an antidote in organophosphate intoxication.[2] However, side effects upon unintentional ingestion may be severe. Symptoms can range from dry mouth, tachycardia, disturbed speech, restlessness and fatigue on a peripheral level to hallucinations, delirium and coma on the central nervous system level.[2–4] A toxic minimal dose for humans has been observed at doses as low as 50 µg/kg body weight but can be much higher depending on various factors such as route of uptake and general health.

The tropane alkaloid-producing plant *Datura stramonium* grows under similar conditions as millet and tends to reach approximately the same height, making a selective harvest very difficult. Furthermore, the shape and colour of the seeds are very similar and are not easily discriminated during food processing. These reasons are at the origin of the contamination that has been found in some *millet*-processed food and that can lead to unintentional intake of tropane alkaloids.

Processed food (*e.g. Millet*) that is at risk of containing traces of tropane alkaloids of nightshade origin, is subjected to routine analysis, consisting mostly of high performance chromatography coupled with mass spectrometry detection methods.[5–8] These tests, however, are very expensive with respect to 'sample to result time' and equipment costs. So far, screening analysis on a DNA level has not been developed, although the advantages of a DNA-based over a LC/MS-based assay are evident on different levels (*i.e.* for economical and material resources reasons). In fact, due to the amplification of the signal obtained through a PCR-based amplification method, a few mg (less than 100 mg) of starting material are sufficient to extract sufficient DNA to test, possibly enabling an improvement on the limit of detection level.

In this report, we describe the development of a *Datura stramonium* and a *Millet*-DNA-based TaqMan assay system as an alternative to existing methods for screening analysis and detection of traces of native atropine-producing plants. Both systems were tested on raw materials (*i.e.* seeds) as well as three processed *Millet* infant food preparations, which have been previously found positive to both atropine ranging from 1.4 µg/kg to 36.5 µg/kg total food weight and scopolamine (12.5 µg/kg total food weight) during LC MS-MS routine analysis performed at the Cantonal Laboratory Basel-Stadt, Switzerland.

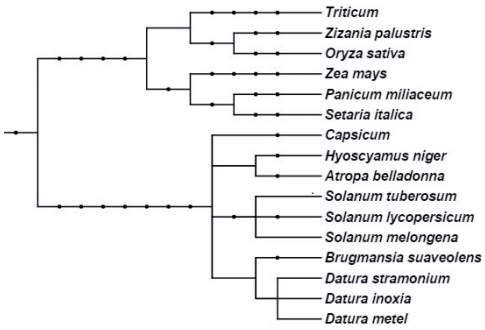
2. Results and Discussion

2.1 *Datura stramonium* DNA Sequence Alignment

The nucleotide database NCBI was searched for DNA sequences of *Datura stramonium* of >300 bp length and these sequences were compared to sequences of *Poaceae*, the family name for grasses including the nutritionally important

A

B



C

Datura stramonium	c a c t t g c a c t t c a t c c t g a a a a t c a
Acnistus arborescens	t g a . . . . . c . . . . c c . . . t .
Brugmansia arborea	t . . . . . a . . . . . c . . . . t
Capsicum lycianthoides	t g a . . . . . c . t . c . . . . c . . . . .
Eriolarynx fasciculata	t g a . . . . . c . g . . c . . . . t .
Iochroma nitidum	t g a . . . . . c . . . . c . . . . t .
Nicotiana paniculata	t t a . . . . . t . a t . . . c c . . . a .
Saracha quitensis	t g a . . . . . c . . . . c . . . . t .
Solanum aviculare	t g a . . . . . c . t . c . . . . c . . . . .
Solanum valdiviense	t g a . . . . . t . c . . . . c . . . . .

Fig. 1. (A) *Datura stramonium* (left panel) and *Panicum miliaceum* (*millet*, right panel). Picture sources: *https://www.magicgardenseeds.de/Wissenswertes/Wei%C3%9Fer-Stechapfel-%28Datura-stramonium%29-A.DAT03-* and *https://antropocene.it/en/2017/08/22/panicum-miliaceum/* (B) Taxonomic genetic tree of *Solanaceae* (bottom clade) and *Poaceae* (top clade). Sequence data originate from NCBI. Taxonomic tree was generated using ITOL (Interactive Tree of Life). (C) Alignment of *Datura*-related species of the region of binding of the developed hydrolysis probe. Mismatches over the probe are indicated, whereas consensus to *Datura* is shown as dots.

millet, rice, wheat, maize and others. As *Datura stramonium* contamination is preferentially found in food based on plants belonging to the *Poaceae*, DNA sequences that showed >50% identity to known *Poaceae* sequences were discarded in order to avoid any false positives when performing the DNA based assay (Fig. 1B). The remaining sequences were compared among different *Solanaceae* in order to be able to choose one that allows for discriminating between *Datura* species and the other nightshades. As a result, we chose the sequence encoding the granule-bound starch synthase I (GBSS1) gene (Fig. 1C). Its database sequence was confirmed by sequencing the *Datura GBSS1* gene from seeds purchased from a local shop. (Fig. 1). A similar approach was followed to find the suitable target gene *GOT2* (encoding the mitochondrial aspartate aminotransferase) for qPCR studies.

2.2 *PCR Assay System – Sensitivity*

Based on the database and sequencing data, a specific hydrolysis probe assay was developed for both *Datura stramonium* and *Panicum miliaceum*.

The efficiencies of both developed systems were assessed on a serial dilution of DNA ranging from 50 to 0.195 ng per reaction (Fig. 2). The *Millet* system showed an efficiency of 0.96 with an $R^2 = 0.990$ whereas the *Datura* system showed an efficiency of 0.98 with an $R^2 = 0.993$. Mean of Ct values and standard deviations of the measured dilutions is summarized in Table 1 in the Supplementary Data. Based on the genomic size of the two plants[9] and the measured amount of DNA, the copy number per reaction calculates to 11111, 2778, 694, 174, 43, 11 and 3 copies of the diploid *Datura* genome and to 24038, 6010, 1502, 376, 94, 23 and 6 copies of the diploid *Millet* genome, respectively. The *Datura* assay showed a limit of detection of roughly 11 diploid cells, whereas down to approximately 23 diploid cells can be detected with *Millet* assay.

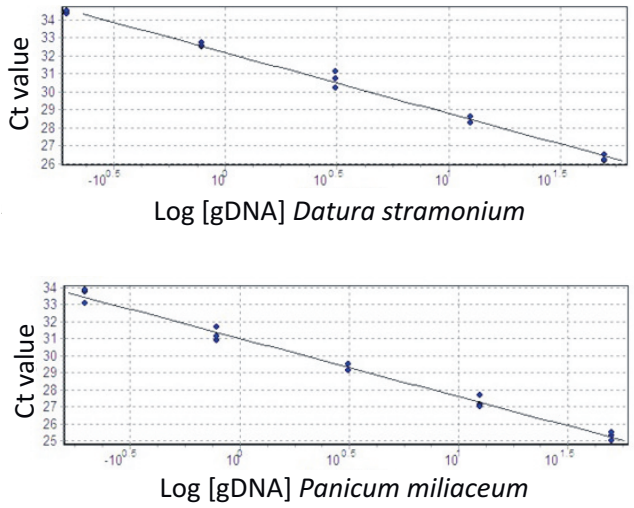


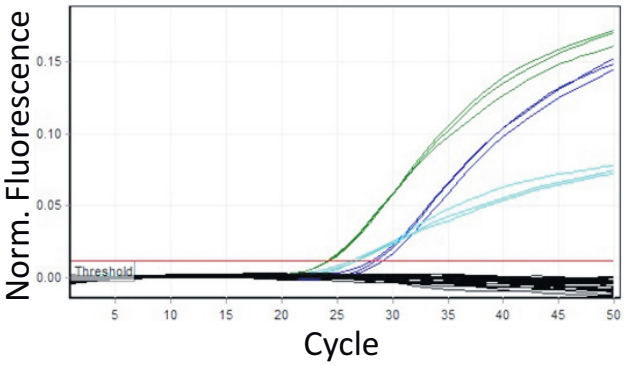
Fig. 2. Standard curves of *Datura stramonium* (upper panel) and *Panicum miliaceum* (lower panel) consisting of a serial dilution containing respectively 50, 12.5, 3.125, 0.781, and 0.195 ng per reaction. Reactions were performed in technical triplicates. Datura reaction efficiency E = 0.98 (R^2 = 0.998); *Panicum miliaceum* reaction efficiency: E = 0.96 (R^2 = 0.990).

2.3 *PCR Assay System – Specificity*

To assess the specificity of the developed *Datura* assay, reactions containing 50 ng of DNA each of different related belonging either to the *Solanaceae* or to the *Poaceae* family were performed. Of all species tested, only the *Datura sp. D. stramonium, D. metel* and *D. inoxia* showed a well detectable PCR signal (Fig. 3) with Ct values of 26.36, 30.69 and 30.14, respectively. As the Taqman probe sequence was designed for *D. stramonium*, it is not 100% complementary to the other two *Datura* species which explains the higher Ct values obtained with *D. inoxia* and *D. metel*.

2.4 *Contaminated Millet Samples*

Total DNA of the three lyophilized *Millet*-based food samples 99A, 26A and 70A obtained from the Cantonal Laboratory Basel-Stadt was isolated in order to detect the *Datura* contamination using the developed qPCR assay system. These samples were previously selected because they showed an unusually high content of tropane alkaloids during LC/MS analyses. The target DNA amplification was performed using 50 ng gDNA per reaction under the conditions described above for both the *Millet* and the *Datura* systems. As shown in Fig. 4 right panel, the Ct values of both the *Millet* seeds (positive control) and contaminated sample 99A were identical (Ct mean values 25.26 and 25.27, respectively) indicating that the main plant ingredient in the sample was *Millet*. As expected from the presence of tropane alkaloids, we were able to detect *Datura* genomic DNA



	Species	Mean Ct	SD
	Datura stramonium	26.36	0.13
	Datura metal	30.69	0.41
	Datura inoxia	30.14	0.21
	Solanum melongena	n.d.	n.a.
	Brugmansia arborea	n.d.	n.a.
	Panicum miliaceum	n.d.	n.a.
	Oryza sativa	n.d.	n.a.
	Hyoscyamus niger	n.d.	n.a.
	Atropa belladonna	n.d.	n.a.
	Solanum tuberosum	n.d.	n.a.
	Capsicum	n.d.	n.a.
	Solanum lycopersicum	n.d.	n.a.
	H_2O	n.d.	n.a.

Fig. 3. Specificity test. 50 ng of DNA of different origin of related plants were assessed in technical triplicates. Non template reactions were performed with H_2O instead of DNA. Reaction threshold was set at 0.0248 and mean Ct and standard deviation were assessed (n.d.= not detected, n.a.= not applicable).

(Ct value of 39.39) in this sample. Considering the amount of total DNA of 50 ng used for the analysis, it can be concluded that the *Millet* preparation 99A contained a contamination of *Datura stramonium* of approximately 0.02%. In contrast to sample 99A, relatively high Ct values of samples 26A and 70A were measured using the *Millet* assay (Ct mean values 33.15 and 33.46, respectively). A possible explanation for this shift is the fact that these samples were prepared for LC/MS analyses and that this preparation may lead to a partial destruction of the genomic DNA to a form that is not PCR amplifiable anymore. This in turn would mean that during possible application of our test in routine analyses, part of the sample taken would have to be prepared for DNA analyses. Preferably, this would include simple freezing of the sample until analysis.

3. Conclusion

Two DNA assays based on specific hydrolysis probes aimed at the detection and quantification of *Datura stramonium* and *Panicum miliaceum* were developed and reaction efficiency and limit of detection were assessed. The system aimed at *Datura* was additionally tested for its specificity with related species and both systems were used to determine the contamination found in *Millet* food preparations that were previously found positive for tropane alkaloids. A contamination of *Datura stramonium* of 0.01 ng gDNA/50ng total gDNA was found in the sample 99A. This sample showed a concentration of 36.5 µg/kg atropine and 12.5 µg/kg scopolamine in a previous LC/MS based analysis. In contrast, the contaminated samples 26A and 70A, which contained 3.1 µg/kg and 1.4 µg/kg atropine, respectively, showed only very little or no *Datura* DNA contamination (in sample 26A a signal appeared in only one out of three replicates, whereas the sample 70A did not show any signal). It is possible that in those samples, the process of drying and milling the food sample destroyed the genomic DNA. For screening reasons with our DNA based assay system, this would be inacceptable. To circumvent this, screening of preprocessed food for the presence of *Datura stramonium* genomic DNA would be necessary. Due to the low cost and high throughput systems, many more tests can be performed which in turn would lead to a diminution of the probability of samples remaining undetected. Since the amount of *Datura* genomic DNA may not necessarily fully correlate with the amount of tropane alkaloids, our DNA based assay system is not intended to replace the direct measurements of tropane alkaloids. Rather, we propose that the relatively few positive samples expected during our newly developed high throughput screening procedure can subsequently be tested using the conventional LC/MS based assay for confirmation and quantitation of the tropane alkaloids.

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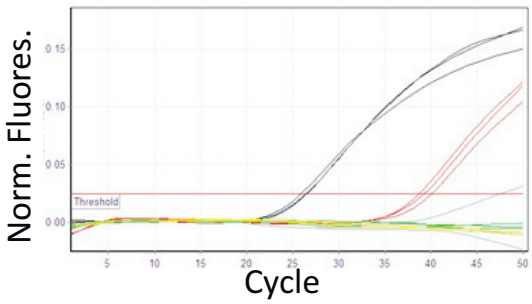

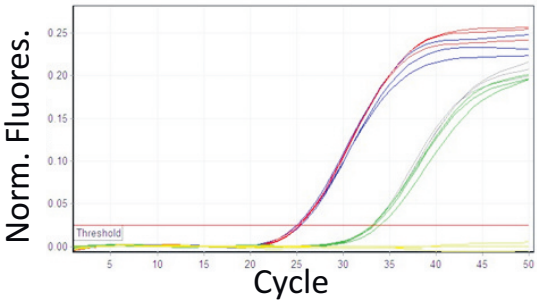

		Mean Ct	SD
	Datura	26.36	0.33
	26A	47.65	n.a.
	70A	n.d.	n.a.
	99A	39.39	0.67
	H_2O	n.d.	n.a.

		Mean Ct	SD
	Millet	25.26	0.24
	26A	33.15	0.07
	70A	33.46	0.49
	99A	25.27	0.10
	H_2O	n.d.	n.a.

Fig. 4. Real time PCR of contaminated samples. Left panel: *Datura* assay system, right panel: *Millet* assay system. Each reaction contained 50 ng of DNA, and DNA from *Millet* and *Datura* seeds were used as positive control. Non template reactions were performed with H_2O instead of DNA. Reaction threshold was set at 0.0248 and mean Ct and standard deviation were determined and are shown below. All reactions were performed in technical triplicates. n.d.= not detected, n.a.= not applicable.

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