SCIENTIFIC REPORT

EFSA Journal

APPROVED: 18 December 2018

doi: 10.2903/j.efsa.2019.5574

Overview of available toxicity data for calystegines

European Food Safety Authority (EFSA),
Marco Binaglia, Katleen Baert, Marijke Schutte and Rositsa Serafimova

Abstract

Calystegines are polyhydroxylated nortropane alkaloids that have been found in various solanaceous foods, in particular in potatoes and aubergines. The biological activity and potential toxicity of calystegines are associated with their capacity to inhibit glycosidases and block carbohydrate metabolism inducing lysosomal storage toxicity. The present report summarises the retrieved information on the possible toxicity of calystegines. Only few *in vivo* short-term toxicological studies in rodents on individual calystegines or mixtures of calystegines were retrieved. Overall, these studies are insufficient to conclude on the possible chronic toxicity effects of calystegines in humans, in particular considering the short duration of the studies and potential lower sensitivity of rats and mice to glycosidase inhibitors, compared to other species such as goats and guinea pigs. Several studies and case reports were retrieved on the toxic effects induced in livestock or experimental animals following consumption or administration of plants containing calystegines. However, the concurrent presence of other alkaloids, in particular swainsonine, did not allow using these studies to draw conclusions on the toxicity of calystegines. Since no experimental data on genotoxicity of calystegines were retrieved, *in silico* predicting models were applied to identify possible alert for genotoxicity of five calystegines recently detected in food. In most of the cases, the outcome of the computational predictions indicated no alerts for genotoxicity; however, the low reliability of the results prevents a firm conclusion on the genotoxic potential of the substances. Overall, the available data do not allow drawing conclusions on the possible toxic effects of calystegines in humans or in livestock, and more data in relevant experimental models would be necessary to characterise the toxic profile of this group of substances.

Keywords: calystegine, nortropane, toxicity, plant toxins, Solanaceae, alkaloids

Requestor: European Commission

Question number: EFSA-Q-2018-00700

Correspondence: biocontam@efsa.europa.eu

Acknowledgements: The EFSA wishes to thank the following for the support provided to this scientific output: Andrew Worth and David Asturiol-Bofill (Joint Research Centre) for the support provided on the application of predictive *in silico* tools; Jan Alexander and Christiane Vleminckx for reviewing this scientific output.

Suggested citation: EFSA (European Food Safety Authority), Binaglia M, Baert K, Schutte M and Serafimova R, 2019. Scientific Report on the overview of available toxicity data for calystegines. EFSA Journal 2019;17(1):5574, 13 pp. https://doi.org/10.2903/j.efsa.2019.5574

ISSN: 1831-4732

The EFSA Journal is a publication of the European Food Safety Authority, an agency of the European Union.

Table of contents

1. Introduction

1.1. Background and Terms of Reference as provided by the requestor

1.1.1. Background

The European Food Safety Authority's (EFSA) Panel on Contaminants in the Food Chain (CONTAM) assessed in 2013 the risk for public and animal health related to the presence of tropane alkaloids in food and feed.

In the adopted opinion, it was acknowledged that only limited occurrence data was available, and a reliable exposure assessment could only be carried out for two tropane alkaloids atropine and scopolamine, covering only one food group (processed cereal based foods for infant an young children) and one age class (infants and young children. The CONTAM Panel therefore recommended the collection of more occurrence data for the various tropane alkaloids, and not only for atropine and scopolamine.

Taking into account the outcome of the EFSA opinion, the Commission established maximum levels for atropine and scopolamine in processed cereal based foods for infants and young children containing buckwheat, sorghum and millet. The Commission adopted also a Recommendation to obtain more occurrence data on the presence of tropane alkaloids in food.

In addition, EFSA outsourced a study on the occurrence of tropane alkaloids in food for human consumption from different geographic regions in Europe, to serve as supporting information to the CONTAM Panel for future exposure assessments for tropane alkaloids.

On request of the European Commission, EFSA assessed the human acute exposure to tropane alkaloids, taking into account the occurrence data available following the abovementioned Commission Recommendation and the study outsourced by EFSA.

High levels of calystegines (A3, A5, B1, B2, B3 and B4) were found in food from the Solanaceae family, such as potatoes, peppers, paprika and aubergines.

At its meeting in March 2018, the CONTAM Panel agreed that the information on toxicity of calystegines available in open literature is not sufficient to perform a comprehensive risk assessment for the presence of calystegines in the food chain.

Following discussions with Member States at meetings of the working group on Agricultural contaminants on 15 June 2018 and 13 July 2018, it was concluded that it would be appropriate to have an EFSA report on the available relevant toxicity data with an indication on possible risks for human health in relation with the abovementioned findings in order to be able to decide if and which further regulatory actions would be appropriate as regards the presence of calystegines in food to ensure a high level of human health protection.

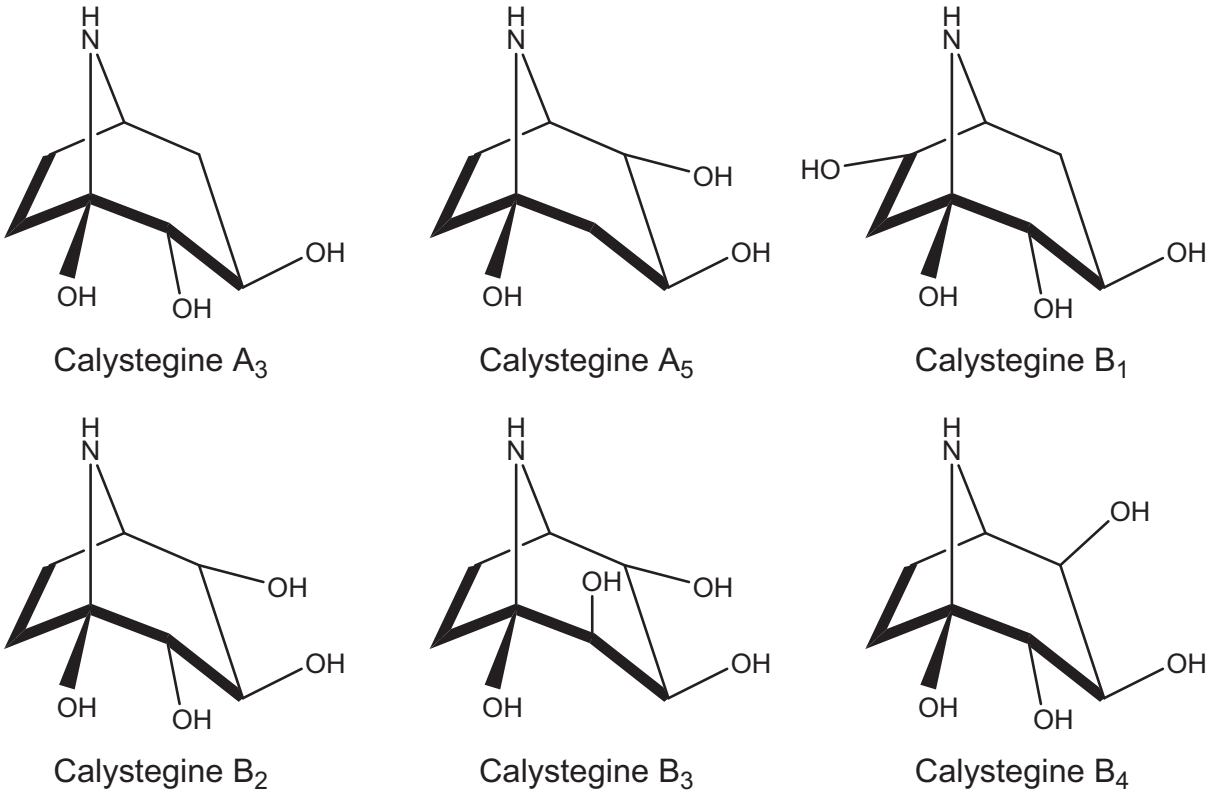
1.1.2. Terms of Reference

In accordance with Art. 31 of Regulation (EC) No 178/2002, the European Commission asks the European Food Safety Authority to provide a report on the toxicity of calystegines.

1.2. Additional information

Calystegines are plant alkaloids that are detected in several plant species including edible plants of the Solanaceae family (Andersson, 2002).

From a chemical point of view, calystegines are a group of alkaloids sharing a polyhydroxylated nortropane ring system (Andersson, 2002; Stegelmeier et al., 2013). The calystegines are divided into three groups differing on the increased degree of hydroxylation (groups A, B and C, containing three-four and five hydroxyl groups, respectively). Some examples of calystegine structures are reported in Figure 1.



Letters indicate the increasing degree of hydroxylation, whereas numbers are used to distinguish structural isomers and stereoisomers. A more comprehensive list of structures is reported in Andersson (2002).

Figure 1: Chemical structure of six calystegines

Despite sharing a similar ring system, calystegines being nortropanes, as they lack the nitrogen associated methyl group, do not share the bioactivity of tropane alkaloids. However, some calystegines were detected, together with other alkaloids, in poisonous plants associated with livestock conditions known as plant-induced storage diseases. As other polyhydroxylated cyclic alkaloids, some calystegines are known glycosidase inhibitors. Inhibition of various glycosidases can induce cellular toxicity via the accumulation of partially catabolised saccharides in the lysosomes (Stegelmeier et al., 2013).

The presence of calystegines in edible plants from the Solanaceae family has been known since they were identified in the 1990s, in particular in sweet peppers, aubergines, potatoes and tomatoes (Andersson, 2002). Asano et al. (1997) analysed several edible fruits and vegetable for the presence of calystegines A3, B1, B2 and C1. Higher total levels were found in aubergines (up to 80 mg/kg fresh weight), sweet peppers (53 mg/kg fresh weight) and sweet potatoes (up to 44 mg/kg fresh weight), whereas relatively lower levels were found in tomatoes and potatoes. B2 was the individual calystegine quantified at higher levels in most of the samples (up to 73 mg/kg fresh weight in aubergines). Levels of calystegines A3 and B2 in different potato cultivars were reported by Friedman et al. (2003) to vary from 5.4 to 68.1 mg/kg (fresh weight), with B2 present at higher levels in most of the samples.

Recently, Mulder et al. (2016) analysed the levels of six calystegines (A3, A5, B1, B2, B3, and B4) in 308 potato product samples (297 fresh potato samples and 11 processed potato products), 90 samples of aubergines and 6 samples of bell peppers collected in various European Union Member States (EU MSs). Calystegines were detected in all the potato product samples and in about 93% of the aubergine and bell pepper samples. Fresh potatoes showed higher levels (mean concentration of 164.0 mg total calystegines/kg, maximum level of 507.3 mg total calystegines/kg), followed by processed potato products (mean and maximum levels of 95.6 and 207.7 mg total calystegines/kg) and aubergines (mean and maximum levels of 21.1 and 181.5 mg total calystegines/kg). At individual level, A3, B2 and B4 were the calystegines most commonly detected in potato products, with A3 also as the one with higher mean and maximum levels (around 100 and 400 mg/kg, respectively). Aubergines and bell peppers showed a different pattern, with A3, B1 and B2 as the most commonly

detected substances and with B2 as the one measured at higher mean and maximum levels (around 15 and 125 mg/kg, respectively). Additional analysis of two samples of sweet potatoes was also performed and in those samples B1 and B2 were identified as the main calystegines.

2. Data and methodologies

2.1. Literature search

A literature search was performed in four databases using the strategy summarised in Table 1.

Table 1: Overview of the performed literature search strategy and outcome

Search database	String	References found
PubMed	(toxin* OR toxic*) AND calystegin*	21
Embase	tox* AND calystegin*	28
Web of Science	(tox* AND calystegin*)[topic]	29
Scopus	(tox* AND calystegin*)[ti,ab, keywords]	27

Overall, excluding duplications, 42 references were found. Six articles were excluded by the screening of titles and abstracts, based on the lack of relevance. Full-text screening was performed on the remaining 36 articles; these could be divided into four broad categories:

1) *in vivo* toxicity reports of plants containing calystegine alkaloids (19 articles);
2) *in vivo* toxicity studies of individual calystegine alkaloids (3 articles);
3) chemical analysis/occurrence of calystegine alkaloids (8 articles); and
4) *in vitro*/*in vivo* studies of biological activity/mode of action (6 articles).

Additional papers were identified via the bibliography cited in the retrieved references. This technique is known as snowballing.

2.2. *In silico* predictions

In view of the lack of genotoxicity data, *in silico* prediction was performed on the six calystegines recently detected in food by Mulder et al. (2016), i.e. calystegines A3, A5, B1, B2, B3 and B4. *In silico* predictions were performed via the application of two free available (VEGA and Toxtree) and four commercial software tools (TIMES, DEREK Nexus, ACD Percepta and ADMET Predictor). All of the systems predict *in vitro* mutagenicity (Ames), four of them predict *in vitro* chromosomal aberrations and DEREK Nexus, TIMES and Toxtree provide also predictions for some *in vivo* genotoxicity endpoints (see Table 2). In addition, the Danish (Q)SAR database was checked for predictions available for the substances of interest. Danish (Q)SAR database includes predictions from more than 200 (Q)SARs, from free and commercial platforms, for more than 600,000 substances. The database could be searched for available predictions for a particular substance or for predictions based on similar substances, with the threshold of similarity defined by the user. The predictions for the following genotoxicity endpoints were found in the Danish (Q)SAR database: *in vitro* mutagenicity (Ames), *in vitro* chromosome aberrations in Chinese hamster ovary (CHO) and lung (CHL) cells, *in vitro* mutations in thymidine kinase locus in mouse lymphoma cells, *in vitro* mutations in hprt locus in CHO cells, *in vitro* unscheduled DNA synthesis (UDS) in rat hepatocytes, *in vitro* Syrian hamster embryo (SHE) cell transformation, *in vivo* sex-linked recessive lethal (SLRL) test in *Drosophila melanogaster*, *in vivo* micronucleus test in mouse erythrocytes, *in vivo* dominant lethal mutations in rodents, sister chromatid exchange in mouse bone marrow cells, *in vivo* comet assay in mouse.

Table 2: Information on the applied software tools

Software	Endpoint/model	Algorithm	Availability	Web link
Toxtree v3.1.0	*In vitro* mutagenicity (Ames) carcinogenicity and mutagenicity *in vivo* micronucleus in rodents	Knowledge based	Freely available	http://toxtree.sourceforge.net/
Vega v.1.1.4	*In vitro* mutagenicity (Ames)	Statistical	Freely available	https://www.vegahub.eu/

Software	Endpoint/model	Algorithm	Availability	Web link
TIMES 2.27.15	*In vitro* mutagenicity (Ames) *In vitro* chromosome aberrations *In vivo* clastogenicity *In vivo* micronucleus	Hybrid	Commercial	http://oasis-lmc.org/
ADMET Predictor v9.0	*In vitro* mutagenicity (Ames) chromosome aberrations	Statistical	Commercial	https://www.simulations-plus.com/software/admetpredictor/
ACD Percepta Release 2017.2.1 Build 2977	*In vitro* mutagenicity (Ames)	Statistical	Commercial	https://www.acdlabs.com/
Derek Nexus: 6.0.1, Nexus: 2.2.2	*In vitro* chromosome damage *In vitro* non-specific genotoxicity *In vitro* photo-induced chromosome damage *In vitro* photo-induced non-specific genotoxicity *In vitro* photomutagenicity *In vivo* chromosome damage *In vivo* mutagenicity *In vivo* non-specific genotoxicity *In vivo* photo-induced non-specific genotoxicity	Knowledge based	Commercial	https://www.lhasalimited.org/

3. Assessment

3.1. Biological activity and toxicological mode of action

The biological activity of calystegines is related to the affinity of some of them to bind glycosidases resulting in the inhibition of carbohydrate metabolism. As such, they are part of a wider family of natural polyhydroxylated cyclic alkaloids acting as glycosidase inhibitors. Among them, 1-deoxynojirimycin, castanospermine and swainsonine were studied in particular for their pharmacological applications (Stegelmeier et al., 2013). The affinity and potency of various calystegines were studied *in vitro* on enzymes isolated from different species, including bovine, rat and human liver. A comprehensive overview of the *in vitro* affinities of different calystegines is given by Andersson (2002) and Stegelmeier et al. (2013). Differently from swainsonine, a potent inhibitor of α-mannosidase and mannosidase II (see e.g. Molyneux et al., 1994; Ikeda et al., 2003), and castanospermine, α-glucosidase inhibitor (see e.g. Saul et al., 1984), calystegines, including those most commonly detected in edible solanaceous plants (A3 and B2) are mainly associated with inhibition of α- and β-galactosidases, β-glucosidase and β-xylosidase (Asano et al., 1997; Ikeda et al., 2003). *In vitro* data indicate that the inhibitory potency of calystegines increases proportionally with the degree of hydroxylation (i.e. calystegines C > B > A).

The toxic potential of calystegines is also related to their ability to inhibit glycosidases (see e.g. Molyneux et al., 1995). The insufficient activity of lysosomal glycosidases may cause the accumulation of partially catabolised saccharides and lead to lysosomal storage toxicity. As an example, the effects induced in livestock and experimental animals by exposure to swainsonine are similar to those observed in the genetic lysosomal storage disorder α-mannosidosis (Huxtable et al., 1982; Armién et al., 2007). Congenital α-mannosidosis is known to occur in humans, cattle, cats, mice and guinea pigs (Cholich et al., 2013). In addition to the different target enzymes, in general, calystegines show lower inhibition potency than swainsonine, this possibly explaining main role of swainsonine in livestock intoxications from *Ipomoea carnea* (see Section 3.3). α- and β-galactosidases deficiencies are also related to known human genetic diseases (Fabry's disease and Gauchner's disease, respectively); therefore, the inhibitory activity of calystegines may represent a relevant mode of action in particular in susceptible subjects with lower expression of these enzymes. Albeit *in vitro* data indicate that swainsonine and calystegines exhibit their glycosidase inhibition potential in different species, *in vivo* data on exposure to plants/plant extracts containing these alkaloids suggest a marked species-sensitivity, with rat and mouse showing in general a lower response than cattle, goat, horse and guinea pig. For example, neurological effects are among the main signs of intoxication following exposure to *I. carnea* or other *Ipomoea* spp. in livestock and guinea pigs (De Balogh et al., 1999; Barbosa et al., 2006; Armién et al., 2007; Cholich et al., 2009, 2013); however, no signs of

neurotoxicity were observed in rats orally exposed to *I. carnea* alkaloids, (Hueza et al., 2005), and only relatively high parental doses of swainsonine induced neurotoxicity in mice (Stegelmeier et al., 2008). This apparent species specificity is documented in particular for swainsonine; however, its relevance for calystegines is uncertain. Moreover, the causes and the human relevance of the species-specificity are unknown.

3.2. *In vivo* studies on individual calystegines or mixtures of thereof

Only sparse information was retrieved on *in vivo* toxicity of isolated calystegine alkaloids. In particular, the three studies summarised below were conducted in rats and mice, possibly species of lower sensitivity to lysosomal storage toxicity.

Hueza et al. (2005) exposed via gavage for 14 consecutive days female Wistar rats (6/group) to either an aqueous extract of *I. carnea* (15 g/kg body weight (bw)), or to the following doses of the single alkaloids isolated from the extraction: 1.8 mg/kg bw swainsonine, 3.0 mg/kg bw calystegine B1, 2.4 mg/kg bw B2, 1.2 mg/kg bw B3 or 1.8 mg/kg bw C1. Two control groups of six animals each were administered the vehicle (tap water) via the same administration route. The doses of individual alkaloids were equivalent to the respective concentrations contained in the aqueous fraction of 15 g/kg bw. No mortality or clinical signs of toxicity were observed in any groups. No gross lesions were observed during gross pathology examinations. Histopathological evaluation of the group treated with the aqueous extract showed the presence of marked cytoplasmatic vacuolation in several tissues including thyroid follicular epithelium, liver (hepatocytes and Kupffer cells), pancreatic acinar cells and renal tubule epithelium. Rats exposed to swainsonine showed similar effects, but with lower severity and only in the thyroid and kidney. No histopathological changes were observed in the groups exposed to calystegines. No effects were observed in the central nervous system in any treatment groups.

Stegelmeier et al. (2008) studied the comparative toxicity of different alkaloids with glycosidase inhibiting potential in male Swiss Webster mice (3/group). The individual alkaloids extracted and purified from ground plant materials were: swainsonine, castanospermine and calystegines A3, B2 and C1. Substances were administered for 28 consecutive days via osmotic minipumps. Each alkaloid was administered in three doses as outlined in Table 3, and a concurrent vehicle control group was included. No mortality, clinical signs of toxicity or significant gross lesions were observed in any treatment groups. Histopathological examinations revealed increased numbers of eosinophilic granulated cells in the liver sinusoids, accompanied by swollen hepatocytes with minimal cytoplasmic vacuolation in mice exposed to calystegine A3 at 140 mg/kg bw per day. No histopathological changes were observed in the groups exposed to the other calystegines. Swainsonine induced cellular vacuolation in kidney and thyroid at $\geq$ 1.56 mg/kg bw per day, and extensive vacuolation was observed at the highest tested dose in several tissues including the central nervous system. At the highest tested dose, castanospermine induced mild vacuolation in the kidney and thyroid, and minimal hepatocyte vacuolation.

Table 3: Projected and actual doses of various alkaloids delivered to Swiss Webster mice for 28-days via osmotic minipumps (Stegelmeier et al., 2008)

Alkaloid	Projected dose (mg/kg bw per day)	Mean delivered dose ± SD (mg/kg bw per day)
Calystegine A3	1.0	1.2 ± 0.2
	10.0	13.5 ± 2.1
	100.0	140.2 ± 7.7
Calystegine B2	1.0	1.3 ± 0.2
	10.0	15.8 ± 1.4
	100.0	138.6 ± 18.2
Calystegine C1	1.0	1.4 ± 0.1
	10.0	13.3 ± 1.7
	100.0	144.0 ± 5.9
Swainsonine	0.1	0.11 ± 0.02
	1.0	1.56 ± 0.29
	10.0	14.1 ± 1.4

Alkaloid	Projected dose (mg/kg bw per day)	Mean delivered dose ± SD (mg/kg bw per day)
Castanospermine	1.0	1.2 ± 0.3
	10.0	12.1 ± 0.4
	100.0	142.1 ± 9.5
Control (saline)	0.0	0.0

bw: body weight; SD: standard deviation.

Bourebaba et al. (2016) studied the possible antidiabetic effects of calystegines in Albino mice. A mixture of calystegines extracted from seeds of *Hyoscyamus albus* was tested in an acute oral study in female mice. The purified extract contained 0.32% total calystegines. Identification and quantification of individual calystegines was carried out, resulting in the identification of seven calystegines (A3, A5 (both as free- and glycosylated forms), B1, B2, B4 and N1[1]), with individual concentrations ranging from 42.91 (A5) to 212.54 (B4) μg/g dry weight seeds. Groups of six mice were orally administered the calystegines extract at single doses of 0, 5, 20, 50, 300 or 2,000 mg total calystegine/kg bw and observed for a 14-day period. No lethality or any other clinical signs of toxicity were observed in the treated mice. Liver histopathology revealed changes in the hepatic tissue architecture at the highest does group only, which was considered of negligible importance by the authors. The authors subsequently administered healthy male mice with the calystegines extract at oral doses of 0, 10 or 20 mg total calystegine/kg bw, 30 min before than the administration of 2 g/kg glucose. Both doses of the extract showed hypoglycaemic activity, decreasing blood glucose levels by approximately 40% and 60%, respectively, compared to the control group.

3.3. Toxicity of plants containing calystegines

Several case reports and studies on the toxicity of calystegines containing plants on livestock or experimental animals were retrieved. Only in a subset of these published articles the chemical identification and quantification of the alkaloids present in the plants were performed.

In particular, most of the cases were related to poisoning of livestock or experiments following consumption of *I. carnea.* Neurological effects, such as ataxia, tremors, muscle atrophy, were the main signs of intoxication by *I. carnea* in particular in goats (e.g. De Balogh et al., 1999; Medeiros et al., 2003; Barbosa et al., 2006; Armién et al., 2007) and guinea pigs (Cholich et al., 2009, 2013). Fetotoxicity and developmental toxicity were also observed in goats, including fetal/post-natal mortality, behavioural alterations, and developmental effects (retrognathia and arthrogryposis) (Armién et al., 2011; Gotardo et al., 2011, 2012, 2016). Histopathological analysis of intoxicated animals revealed cytoplasmic vacuolation in several tissues, including central nervous system, liver, pancreas and kidney (see e.g. De Balogh et al., 1999; Armién et al., 2007; Cholich et al., 2009; Mendonça et al., 2012).

Besides calystegines, *I. carnea* contains the biologically active indolizidine alkaloid swainsonine. Several studies tried to evaluate the relative contribution of individual alkaloids to the toxicity of *I. carnea* in ruminants and rodents (see e.g. Hueza et al., 2005; Armién et al., 2007; Stegelmeier et al., 2008). In all cases, swainsonine showed a similar toxicological profile to that observed following *I. carnea* poisoning (Armién et al., 2007) or following exposure to the alkaloid mixture extracted from the plant (Hueza et al., 2005), albeit it induced effects of lower severity and at higher concentrations compared with those present in the plant/plant extract. Conversely, exposure to isolated calystegines failed to reproduce the toxic effects observed with *I. carnea*. The authors of the aforementioned studies concluded that swainsonine is the main toxic alkaloid of *I. carnea*, but concurrent exposure to calystegines may induce synergistic effects. Considering these main limitations, the available data on *I. carnea* cannot be used to draw conclusions on the toxicity of calystegines and will not be further discussed in this report.

Stegelmeier et al. (2013) and Hueza et al. (2005) reported other cases of livestock intoxications related to consumption of other plants, such as *Solanum dimidiatum*, *Solanum kwebense,* for which the presence calystegines as predominant alkaloids has been postulated to be the cause of neurological effects in livestock. Among these, van der Lugt et al. (2010) reported a case of poisoning in cattle in South Africa following grazing of *Solanum kwebense*. The condition of the intoxicated

[1] Calystegine N1 has a structure similar to calystegine B2, but with an amino substituent replacing the hydroxyl group on the carbon 1. Calystegine N1 is considered a precursor of calystegine B2 (Andersson, 2002).

livestock, known as *maldronksiekte*, includes neurological effects. The authors studied the neurological effects in three cattle showing clinical signs for 3 months or more. Gross inspection of the brains revealed decreased size of the cerebellum. Histopathological examination revealed cellular loss and changes in Purkinje cells, including swelling and cytoplasmic vacuolation. Lectin histochemistry analyses using *Canavalia ensiformis* (ConA) agglutinin indicated the presence of carbohydrate moieties in the affected Purkinje cells, suggesting lysosomal storage toxicity due to the possible inability of the neurons to metabolise a plant toxin or a cellular substrate. The leaves of *S. kwebense* were subsequently collected from the farm where the poisoning cases had occurred, and analysed for the presence of alkaloids. Low levels of calystegines were quantified in one sample only (concentrations not reported in the article), whereas nor swainsonine neither other alkaloids with known glycosidase inhibiting activity were detected in the collected samples.

Finally, Todd et al. (1995) reported a series of intoxication cases in horses related to grazing of *Convolvulus arvensis*, with weight loss and gastrointestinal effects as the main signs of toxicity. The presence of calystegines was reported in the plant roots but was not confirmed in the aerial part and the toxicity was attributed to the presence of tropane alkaloids, in particular pseudotropine.

3.4. Genotoxicity

No experimental data on genotoxicity of calystegines were retrieved. Computational toxicology software packages were applied to have *in silico* predictions of genotoxicity potential. For this exercise, the six calystegines detected in food by Mulder et al. (2016) were tested, i.e. calystegines A3, A5, B1, B2, B3 and B4. The *in silico* predictions were performed using the software listed in Table 2. It should be noted that in many of the software applied, the chemical descriptors do not consider the stereochemical information. As a result, the diastereoisomers calystegines B2, B3 and B4 are considered as one substance. The obtained results are summarised here below. The output reports from the various software packages are available on the Knowledge Junction community on Zenodo at: https://doi.org/10.5281/zenodo.2536761

Toxtree

Two Toxtree models (carcinogenicity and mutagenicity by ISS and *in vitro* mutagenicity (AMES) by ISS) gave negative predictions for all six structures.

The model on *in vivo* micronucleus in rodent predicted a structural alert for all structures; however, it should be noted that this structural alert is considered to have a low positive predictivity (i.e. high risk of false positive results) (Benigni et al., 2010).

Vega

In the VEGA platform, a few models related with genotoxicity are implemented. For this assessment, a consensus model, which combines the predictions from the individual models taking into account their reliability, was used. Only calystegine A5 was predicted as mutagenic with a consensus score[2] slightly higher than those for non-mutagenic predictions (0.3 vs 0.2). The analysis of the predictions shows that the positive prediction is driven by results with moderate reliability provided by Caesar and SarPy models. Both models are based on different algorithms but the same training set. The inspection of the individual predictions indicated that in both cases the compounds with known experimental values selected in the training set showed only some similarity and the accuracy of predictions for similar molecules found in the training set is not optimal. Therefore, the reliability of these positive predictions is questionable.

TIMES

All predictions provided by TIMES were negative (for all structures and all endpoints), but results were associated to a low reliability. TIMES includes a tissue metabolic simulator predicting *in vitro* rat S9 and *in vivo* rat metabolism. Predicted *in vivo* rat metabolites were in particular examined to identify the presence of possible structural alerts for genotoxicity. The list of predicted metabolites included mainly phase II metabolites (glucoronide- or sulfate conjugates); therefore, no metabolic activation was predicted.

[2] The consensus algorithm uses the applicability domain assessment of each single model's prediction as its weight, so the final assessment is more influenced by the models producing more reliable predictions. A consensus score for each predicted class (i.e. mutagenic or non-mutagenic) is calculated as the sum of the weights for each model normalised over the number of used models, so that it has a theoretical [0–1] range. The predicted class with the highest score is used as the final consensus prediction.

ADMET Predictor

ADMET Predictor classified all structures as 'toxic' (positive) for chromosomal aberrations with confidential scores between 58% and 65%.

For the mutagenicity endpoint, the software predicted for all calystegines B a risk of 1.5 (the threshold value to be considered as a mutagen is 1) based on the combination of the predictions for the individual *Salmonella* Typhimurium strains (with and without rat liver microsomal activation). The further analysis showed that the predicted risk is due to the positive predictions for *S.* Typhimurium strain TA 100 without metabolic activation, with a relatively low confidential score (50%), and for *S.* Typhimurium strain TA102 or *Escherichia coli* strain WP2 uvrA after rat metabolic activation with a relatively high confidential score (79%). The negative predictions for the other strains, as well as for all strains in the case of calystegines A3 and A5 were with relatively high confidential scores (73–97%).

ACDPercepta

All predictions provided by ACDPercepta were negative, but results were associated to a low reliability.

Derek Nexus

Derek Nexus indicated no alerts for all endpoints, with the exclusion of bacterial mutagenicity. For this latter endpoint, although also no alerts were found, some of the structural features of calystegines were observed in mutagenic compounds in the software reference set, which could be consider as an uncertainty in the 'no alert' predictions.

Danish (Q)SAR database

The search performed in the database gave results only for calystegine B3 (and respective diastereoisomers B2 and B4). The results of the battery model (combination of the predictions from the individual models) showed negative predictions with a high reliability for Ames test in S. Typhimurium and for four out of six others *in vitro* genotoxicity endpoints. Negative prediction but with low reliability was reported for chromosome aberrations in CHL cells and a positive prediction again with low reliability for UDS in Rat Hepatocytes.

Three out of five *in vivo* genotoxic endpoints were predicted as negative with high reliability, whereas sister chromatid exchange in mouse bone marrow cells was predicted as negative but with low reliability and the SLRL test in *Drosophila melanogaster* as inconclusive. The software evaluated a high similarity between calystegines B3, B1, A3 and A5; therefore, the results from the Danish (Q)SAR database could be used with additional uncertainties to the other calystegines.

4. Discussion and conclusions

Calystegines are polyhydroxylated nortropane alkaloids that have been found in various solanaceous foods, in particular in potatoes and aubergines. The biological activity and potential toxicity of calystegines are associated with their capacity to inhibit glycosidases and block carbohydrate metabolism inducing lysosomal storage toxicity. The present report summarises the retrieved information on the possible toxicity of calystegines.

No data were retrieved on the toxicokinetics of calystegines. Their chemical structures including several hydrophilic moieties suggest a low potential for bioaccumulation following systemic absorption. *In silico* simulations of rat metabolism were performed via the application of the software TIMES used to predict the genotoxic potential of calystegines A3, A5, B1, B2, B3 and B4. The simulation indicated the formation of phase II metabolites (glucuronide- and sulfate conjugates) of the parent compounds as the main metabolic pathway.

Only few *in vivo* rodent studies on individual calystegines or mixtures of calystegines were retrieved. The study from Bourebaba et al. (2016) suggests that calystegines are not acutely toxic in mice. In this study, no mortality was observed upon a single oral administration of an extract of *Hyoscyamus albus* containing seven calystegines up to 2,000 mg total calystegines/kg bw. Liver histopathology revealed changes in the liver architecture at doses > 300 mg/kg bw, which were considered as non-adverse by the authors. Hypoglycaemic activity was observed in mice dosed with 10 or 20 mg/kg bw. Two-week repeated administration by gavage of four individual calystegines (B1, B2, B3 and C1) at singles doses ranging between 1.2 and 3.0 mg/kg bw caused no adverse effects in rats (Hueza et al., 2005). In the same study, rats administered 15 g/kg bw of a plant extract containing the aforementioned calystegines at concentrations reflecting the individual dosages (i.e. containing a total

calystegine dose of approximately 8.4 mg/kg bw) for 14 days showed adverse effects in several organs. However, these effects were similar to those exerted by administration of 1.8 mg/kg bw swainsonine, also present at this level in the plant extract. Finally, 28-day parenteral administration of individual calystegines (A3, B2 and C1) in mice induced no mortality or clinical signs of toxicity (Stegelmeier et al., 2008). Histopathological changes compatible with lysosomal storage toxicity were observed only for calystegine A3 at the highest administered dose (140 mg/kg bw). Overall, these studies are insufficient to conclude on the possible chronic toxicity effects of calystegines in humans, in particular considering the short duration of the studies and potential lower sensitivity of rats and mice to glycosidase inhibitors, compared to other species such as goats and guinea pigs.

Several studies and case reports were retrieved on the toxic effects induced in livestock or experimental animals following consumption or administration of plants containing calystegines. Severe toxicity effects, including mortality, neurological disorders, developmental toxicity and organ toxicity are reported in these studies. However, several limitations were noted. In particular, the chemical characterisation of the alkaloids present in the plants was not undertaken in all the studies. In addition, most of the data are related to the consumption of *Ipomoea* species, whose toxicity is mainly driven by the presence of swainsonine. However, an exacerbation of the effects of *I. carnea* in comparison to those observed with swainsonine only was in several instances attributed to the presence of calystegines. Overall, these studies could not be used to draw conclusions on the toxicity of calystegines.

No experimental genotoxicity studies on calystegines were retrieved. The genotoxic potential of calystegines A3, A5, B1, B2, B3 and B4 was assessed via *in silico* predictions using six software packages and the information retrieved from Danish (Q)SAR database. Overall, in most of the cases, the outcome of the computational predictions indicated no alerts for genotoxicity; however, some indications for possible genotoxicity were also observed. It should be noted that all positive predictions as well as a several negative predictions were scored with low reliability by the software, which prevents a firm conclusion on the genotoxicity of the substances.

Overall, the available data do not allow drawing conclusions on the possible toxic effects of calystegines in humans or in livestock, and more data in relevant experimental models would be necessary to characterise the toxic profile of this group of substances.

References

Andersson C, 2002. Calystegine alkaloids in Solanaceous food plants. TemaNord 2002:513. Nordic Council of Ministers, Copenhagen 2002.

Armién AG, Tokarnia CH, Peixoto PV and Frese K, 2007. Spontaneous and experimental glycoprotein storage disease of goats induced by *Ipomoea carnea* subsp *fistulosa* (Convolvulaceae). Veterinary Pathology, 44, 170–184.

Armién AG, Tokarnia CH, Peixoto PV, Barbosa JD and Frese K, 2011. Clinical and morphologic changes in ewes and fetuses poisoned by *Ipomoea carnea* subspecies *fistulosa*. Journal of Veterinary Diagnostic Investigation, 23, 221–232.

Asano N, Kato A, Matsui K, Watson AA, Nash RJ, Molyneux RJ, Hackett L, Topping J and Winchester B, 1997. The effects of calystegines isolated from edible fruits and vegetables on mammalian liver glycosidases. Glycobiology, 7, 1085–1088.

Barbosa RC, Riet-Correa F, Medeiros RM, Lima EF, Barros SS, Gimeno EJ, Molyneux RJ and Gardner DR, 2006. Intoxication by *Ipomoea sericophylla* and *Ipomoea riedelii* in goats in the state of Paraiba, Northeastern Brazil. Toxicon, 47, 371–379.

Benigni R, Bossa C and Worth A. 2010. Structural analysis and predictive value of the rodent *in vivo* micronucleus assay results. Mutagenesis, 25, 335–341.

Bourebaba L, Saci S, Touguit D, Gali L, Terkmane S, Oukil N and Bedjou F, 2016. Evaluation of antidiabetic effect of total calystegines extracted from *Hyoscyamus albus*. Biomedicine and Pharmacotherapy, 82, 337–344.

Cholich LA, Gimeno EJ, Teibler PG, Jorge NL and Acosta de Perez OC, 2009. The guinea pig as an animal model for *Ipomoea carnea* induced alpha-mannosidosis. Toxicon, 54, 276–282.

Cholich LA, Márquez M, Pumarola i Batlle M, Gimeno EJ, Teibler GP, Rios EE and Acosta OC, 2013. Experimental intoxication of guinea pigs with *Ipomoea carnea*: behavioural and neuropathological alterations. Toxicon, 76, 28–36.

De Balogh KKIM, Dimande AP, Van Der Lugt JJ, Molyneux RJ, Naudé TW and Welman WG, 1999. A lysosomal storage disease induced by *Ipomoea carnea* in goats in Mozambique. Journal of Veterinary Diagnostic Investigation, 11, 266–273.

Friedman M, Roitman JN and Kozukue N, 2003. Glycoalkaloid and calystegine contents of eight potato cultivars. Journal of Agricultural and Food Chemistry, 51, 2964–2973.

Gotardo AT, Pfister JA, Ferreira MB and Górniak SL, 2011. Effects of prepartum ingestion of *Ipomoea carnea* on postpartum maternal and neonate behavior in goats. Birth Defects Research Part B - Developmental and Reproductive Toxicology, 92, 131–138.

Gotardo AT, Schumaher BH, Pfister JA, Traldi AS, Maiorka PC, Spinosa HS and Górniak SL, 2012. The use of ultrasonography to study teratogenicity in ruminants: evaluation of *Ipomoea carnea* in goats. Birth Defects Research Part B - Developmental and Reproductive Toxicology, 95, 289–295.

Gotardo AT, Pfister JA, Raspantini PCF and Górniak SL, 2016. Maternal ingestion of *Ipomoea carnea*: effects on goat-kid bonding and behavior. Toxins (Basel), 8, pii: E74. https://doi.org/10.3390/toxins803007

Hueza IM, Guerra JL, Haraguchi M, Naoki A and Gorniak SL, 2005. The role of alkaloids in *Ipomoea carnea* toxicosis: a study in rats. Experimental and Toxicologic Pathology, 57, 53–58.

Huxtable CR, Dorling PR and Walkley US, 1982. Onset and regression of neuroaxonal lesions in sheep with mannosidosis induced experimentally with swainsonine. Acta Neuropathologica (Berl.), 58, 27–33.

Ikeda K, Kato A, Adachi I, Haraguchi M and Asano N, 2003. Alkaloids from the poisonous plant *Ipomoea carnea*: effects on intracellular lysosomal glycosidase activities in human lymphoblast cultures. Journal of Agricultural and Food Chemistry, 51, 7642–7646.

van der Lugt JJ, Bastianello SS, van Ederen AM and van Wilpe E, 2010. Cerebellar cortical degeneration in cattle caused by *Solanum kwebense*. Veterinary Journal, 185, 225–227.

Medeiros RMT, Barbosa RC, Riet-Correa F, Lima EF, Tabosa IM, De Barros SS, Gardner DR and Molyneux RJ, 2003. Tremorgenic syndrome in goats caused by *Ipomoea asarifolia* in Northeastern Brazil. Toxicon, 41, 933–935.

Mendonça FS, Albuquerque RF, Evêncio-Neto J, Freitas SH, Dória RGS, Boabaid FM, Driemeier D, Gardner DR, Riet-Correa F and Colodel EM, 2012. Alpha-mannosidosis in goats caused by the swainsonine-containing plant *Ipomoea verbascoidea*. Journal of Veterinary Diagnostic Investigation, 24, 90–95.

Molyneux RJ, James LF, Ralphs MH, Pfister JA, Panter KE and Nash RJ, 1994. Polyhydroxy alkaloid glycosidase inhibitors from poisonous plants of global distribution; analysis and identification. In: Colegate SM (ed.). *Plant-Associated Toxins: Agricultural, Phytochemical and Ecological Aspects*. Wallingford Oxon, UK, Cab International. pp. 107–12.

Molyneux RJ, McKenzie RA, O'Sullivan BM and Elbein AD, 1995. Identification of the glycosidase inhibitors swainsonine and calystegine B2 in Weir vine (*Ipomoea* sp. Q6 [aff. calobra]) and correlation with toxicity. Journal of Natural Products, 58, 878–886.

Mulder PPJ, De Nijs M, Castellari M, Hortos M, MacDonald S, Crews C, Hajslova J and Stranska M, 2016. Occurrence of tropane alkaloids in food. EFSA supporting publication 2016:13(12):EN-1140, 200 pp. https://doi.org/10.2903/sp.efsa.2016.EN-1140

Saul R, Molyneux RJ and Elbein AD, 1984. Studies on the mechanism of castanospermine inhibition of alpha- and beta-glucosidases. Archives of Biochemistry and Biophysics 230, 668–75.

Stegelmeier BL, Molyneux RJ, Asano N, Watson AA and Nash RJ, 2008. The comparative pathology of the glycosidase inhibitors swainsonine, castanospermine, and calystegines A3, B2, and C1 in mice. Toxicologic Pathology, 36, 651–659.

Stegelmeier BL, Field R, Panter KE, Hall JO, Welch KD, Pfister JA, Gardner DR, Lee ST, Colegate S, Davis TZ, Green BT and Cook D, 2013. Selected poisonous plants affecting animal and human health. In: Haschek WM, Rousseaux CG and Walling MA (eds.). Haschek and Rousseaux's Handbook of Toxicologic Pathology. Academic Press, New York, USA. pp. 1259–1314.

Todd FG, Stermitz FR, Schultheis P, Knight AP and Traub-Dargatz J, 1995. Tropane alkaloids and toxicity of *Convolvulus arvensis*. Phytochemistry, 39, 301–303.

Abbreviations

bw	body weight
CHL	Chinese hamster lung
CHO	Chinese hamster ovary
CONTAM Panel	EFSA Panel on Contaminants in the Food Chain
MS	Member State
(Q)SAR	quantitative structure–activity relationship
SD	standard deviation
SHE	Syrian hamster embryo
SLRL	sex-Linked recessive lethal
UDS	unscheduled DNA synthesis