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Research Article

# Cereal alkaloids

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## Abstract

Alkaloids are one of the largest and the most studied groups of plant secondary metabolites being present in cereals, cereal-based food and animal feed among other plants and plant derived food. Alkaloids are the most abundant in higher plants being reperesented with 25%. They are known about their toxicity from the ancient times to date causing severe health problems in humans and livestock including several epidemics. In spite of that, due to their strong physiological properties, alkaloids are used as pharmaceuticals. They can act as defence compounds in plants, being efficient against pathogens due to their toxicity. They have also biological significance as active stimulators, inhibitors and terminators of growth being structurally very similar to plant growth hormones.

**Key words:** Ergot alkaloids, Tropane alkaloids, Pyrrolizidine alkaloids, cereals, cereal food, feed, chemistry, biochemistry, pharmacology, regulations.

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## 1.Introduction

Cereals are common food for humans and necessary components in animal feed in many countries all over the world. Alkaloids are toxic compounds exclusively biosinthesised by plants and can infect cereals and cereal-based food and animal feed. They can Currently more than 15 000 different alkaloids are known and they are classified into several subclasses based on the amino acids from which they are derived and according to their chemical structures (1). The best characterized groups of alkaloids are tyrosine-derived isoquinoline alkaloids, the tryptophan-derived indole alkaloids, and ornithine-derived nicotine and tropane alkaloids.

Contrary to other secondary metabolities, e.g. plant phenolics, which are abundant throughout the whole plant kingdom, alkaloids are often restricted to certain plant families or even certain plant species. Advanced analytical methods for research on alkaloids were developed along with increasing the interest on their research, isolation and structure elucidation over the decades (2). The lack of understanding about their synthesis in the plant cells and the functions of various enzymes and their genes in directing and controlling the long multistep biosynthetic pathways, has delayed the development of biotechnological production in cell and tissue cultures. Several laboratories are characterizating now many alkaloid pathways using classical biochemical or new functional genomics approaches (3,4). Further research on the biosynthesis pathway of ergot alkaloids, as well as of other alkaloids is of interest especially because of the broad range of their pharmaceutical uses. The knowledge about the genes and enzymes has increased and the molecular genetic engineering may be used to improve industrial production of medically important alkaloids, including novel forms of drugs with new or improved pharmacological activities (5).

## 2. Types of alkaloids present in cereals, cereal-based food and feed

### 2.1. Ergot alkaloids (EAs)

The term ergot or ergot fungi refers to a group of fungi of the genus *Claviceps***,** that includes about 50 known species (6). *Claviceps purpurea* ("rye ergot fungus") is the best known member of this group that grows on rye and related cereals such as wheat, barley and triticale. The alkaloids produced by this fungus can cause ergotism or St. Anthony's fire (5,7,8) in humans and other mammals who consume grains contaminated with its fruiting structure, called *ergot sclerotium* (Figures.1,2). Ergot kernel germinates in the spring, prior of flowering in grains, or grasses, and gives rise to a stroma (stromata plural), where sexual reproduction is occurred for the production of Ascospores (Figure 3).

Ergotism is one of the oldest known mycotoxicoses that occurred in ancient time. One of the most known events was the human epidemics produced by ergot in the Middle Ages (St. Anthony's fire) with symptoms of gangrene, central nervous and gastrointestinal effects. Animals (livestock) are affected similarly to what has been observed in humans. In swine agalactia has been attributed to ergot alkaloids, while the loss of ears and other appendages is a common effect of ergot in animals.

Two types of ergotism have been described; gangrenous and convulsive. The differences may be due to the different kinds of alkaloids present in the ergot as variations in amount and kinds of alkaloids that can occur in the ergot (sclerotia). *C.purpurea* has caused several epidemics, particularly during the middle ages, due to consumption of rye products contaminated with *C. purpurea* sclerotia (ergots) (5,7). Outbreaks that have occurred in Ethiopia in 1978 where gangrene and loss of limbs, and that occurred in India in 1975 where more of the nervous type symptoms of giddiness, drowsiness, nausea and vomiting. Depending on the amount and kind of alkaloids they consume, patients can show various symptoms such as painful spasms, diarrhea, paresthesia, nausea and vomiting, headache or psychosis are typical convulsive symptoms and gangrenous symptoms, especially for fingers and toes (6,9). Alkaloids of the ergot sclerotium are presented in Table 1.

Ergometrine was the alkaloid found in the Ethiopian sclerotia and in India the clavine alkaloids agroclavine, elymoclavine, chanoclavine, penniclavine and setoclavine were found. The ergots produced in these two outbreaks were caused by different species of *Claviceps*. Because some of the EAs are vasoconstrictive and have other beneficial pharmacological properties, they have been used therapeutically. In the United States, most of the widely grown tall fescue posesses an endophytic fungus called *Neotyphodium coenophialum*. This endophyte produces ergovaline, an ergopeptine, which can, if consumed levels are sufficient, produce ergot-like toxicosis in animals grazed on pastures containing the fescue grass (10).

**Figure 1.** Ergot sclerotia developing on wheat spikes[a]

[a]*Origin:Schnaufer R. et al., Report of the investigative office for food control and animal health, Baden-Wuerttemberg, 2014.*

**Figure 2.** Wheat kernels with developed ergot sclerotia[a]

[a]*Origin: Neef S. et al., Untersuchung von Roggenmehlen im Jahr 2009 auf den Gehalt an Mutterkornalkaloide, Baden-Württemberg,Ministerium, Die Untersuchungsämter für Lebensmittel überwachung und Tiergesundheit, 2009.*

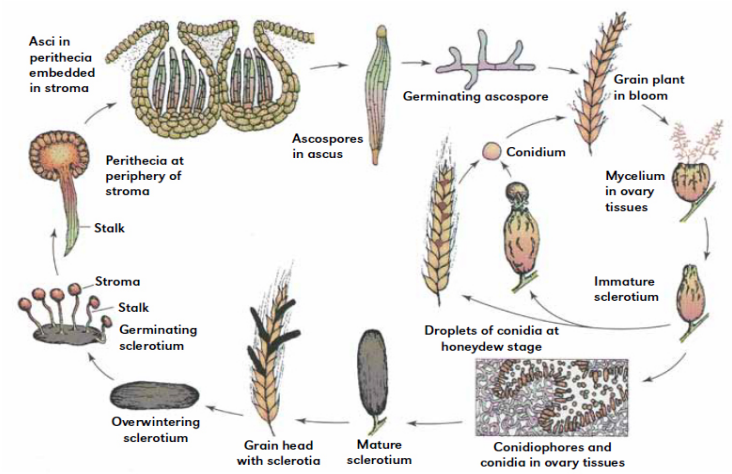


**Figure 3.** Life cycle of ergot sclerotia in small grain cereals and grasses[a] [a]*Origin:* Schumann, G.L. and Uppala, S. Ergot of rye. *The Americam Phyropatologycal Society ,The Plant Health Instructor,* 2000.

*Claviceps africana* causes sorghum ergot in sorghum growing areas in Africa and Asia, where it was first recorded more than 90 years ago. It has been spreading rapidly around the world. By the mid-1990s it reached Brazil, South Africa, and Australia, and by 1997 it reached most South American countries and the Caribbean including Mexico. By 1997 this disease reached Texas in the United States (11).

Investigations on rye were conducted that is one of the most commonly affected cereal by EAs (12,13), as well as on the retention of six alkaloids in hard red spring wheat infected by derived from the reduction system (14). The predominant EA found in wheat grown in Western Canada was ergocristine. Cleaning of wheat by the Carter dockage tester did not remove ergot bodies effectively. During milling alkaloids were stable. The greatest levels of EAs were detected in the late reduction flours and in the shorts derived from the reduction system, due to more plastic property of ergot than hard wheat endosperm, what led to its flattening during smooth roll reduction grinding. Relatively low levels were detected in the bran and break flours, while the lowest levels were found in the early reduction flours. Individual alkaloids appeared equally stable during processing into bread, pasta, and Oriental noodles. EAs were very stable during bread processing and baking. Some alkaloids were lost during cooking of Oriental noodles and spaghetti, but total retention in the cooked products consistently exceeded 25% of that in the flour. The broad range of alkaloid concentrations observed in the millfeed and flour streams from ergoty wheat, and the different stability of the EAs

depending on the final end-product produced, underline the complexities of establishing safe tolerance limits for ergot.
Other researchers determined the concentration of EA in samples of oats, rye and wheat (15). Samples were grouped into grains and categories. For barley and wheat there was strong evidence that alkaloid contamination was lower in some categories, while for oats and rye the evidence for similar differences was not statistically significant. Cases of ergotism in livestock and associated ergot alkaloid concentrations in feed were also investigated (16), as well as the impacts of cereal ergot in food animal production (17). Toxic effects, metabolism, and carry-over of ergot alkaloids in laying hens, with a special focus on changes of the alkaloid isomeric ratio in feed caused by hydrothermal treatment examined was the dose-dependent effects of dietary EAs on laying hens (18).

### 2.1.1. Chemistry of EAs

EAs are nitrogen-containing natural products belonging to indole alkaloids. According to their structures, EAs can be divided into three groups: clavines, lysergic acid amides and peptides (ergopeptines) (19). All of them share the first biosynthetic steps, which lead to the formation of the tetracyclic ergoline ring system (except the simplest, tricyclic compound: chanoclavine). Different modifications on the ergoline ring by specific enzymes result in an abundance of bioactive natural products, which are used as pharmaceutical drugs or precursors.

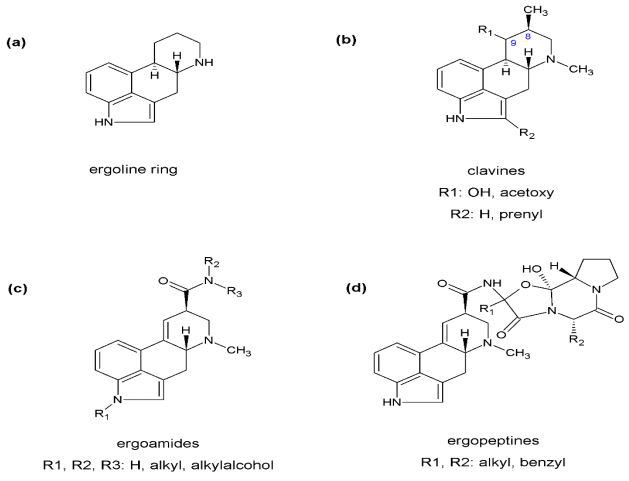


Figure 4. Chemical structures of ergot alkaloids:
(a) ergoline ring (core structure of all ergot alkaloids);
(b) core structure of clavines; (c) core structure of ergoamides; and (d) core structure of ergopeptines.

*Origin: Gerhards N. et al., Toxins (Basel). 2014; 6(12): 3281 3295*

### 2.1.2. Biosynthetic Pathways of EAs

Most of the biosynthetic pathways of EAs have been explained from the 1950s through to recent years. Gene clusters from several ergot alkaloid producers have been identified and the functions of many of those genes have been demonstrated by knock-out experiments or biochemical investigations of the overproduced enzymes (19). The biosynthetic pathway for EAs has been investigated extensively in *Claviceps* species and *A. fumigatus*.

Common steps of formation of the Ergoline ring is shown on Figure 5, starting with the *C*4-prenylation of *L*-tryptophan (1) with dimethylallyl diphosphate (DMAPP) as prenyl donor. This reaction is catalyzed by the prenyltransferase 4-dimethylallyltryptophan synthase (DMATS). It is clearly shown the formation of 4-γ,γ-dimethylallyltryptophan (DMAT (2)) as product.

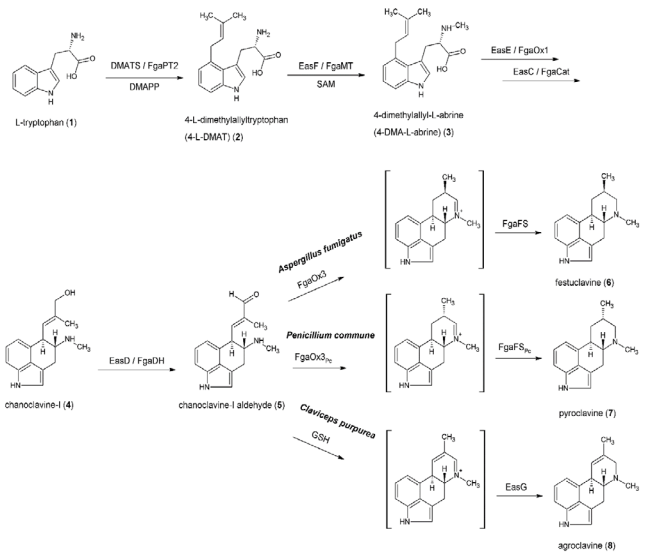


**Figure 5.** Formation of the ergoline scaffold-biosynthetic pathway.

In the biosynthesis of lysergic acid from agroclavine has been postulated to involve cytochrome P-450 monooxygenases. Elymoclavine, paspalic acid and lysergic acid have been identified as intermediates in the biosynthesis of lysergic acid (Figure 6).

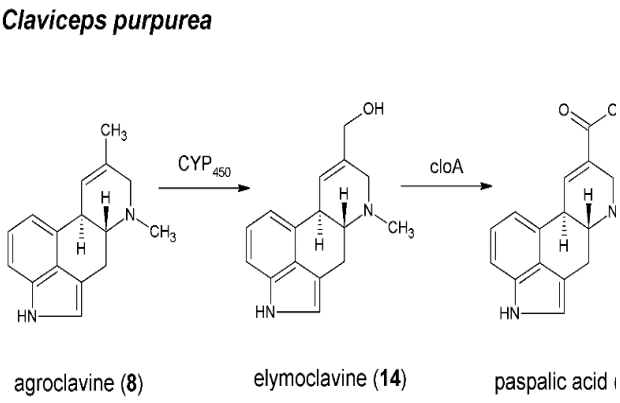


**Figure 6.** Formation of Lysergic acid and Ergotamine from Agroclavine in *C. purpurea*.
*According to Gerhards N. et al., Toxins (Basel). 2014; 6(12): 3281 3295.*

Elymoclavine (14) is formed from agroclavine (8) via a 2-electron oxidation and is further converted to paspalic acid (15) via a 4-electron oxidation. These reactions have all been proposed to be catalyzed by cytochrome P-450 monooxygenases. Conversion of 15 to 16 can be achieved either by catalysis of an isomerase or spontaneously, as already observed *in vitro*. D-lysergic acid is an important link between the clavine pathway and the formation of ergopeptines such as ergotamine (17) and ergoamides.
The terminal pathway in *C. purpurea*, *Balansia obtecta*, and *Epichloë* pecies leading to ergopeptines includes the attachment of a tripeptide chain to activated lysergic acid, the tripeptide forming a bicyclic structure including a lactam ring and an oxazolidinone ring (e.g., ergotamine 18). It was shown that the D-lysergyl tripeptide lactams (23), the precursors of ergopeptines, are formed by an NRPS enzyme complex containing two separable activities, D-Lysergyl peptide synthetases 1 and 2 (LPS1 and LPS2). LPS2 catalyzes the first step, the generation of activated lysergic acid (hence it was predicted to be a monomodular NRPS), which is subsequently transferred to the large (trimodular) LPS1, where the D-lysergyl mono-, di- and tripeptide thioester intermediates are formed, and finally the D-lysergyl tripeptide lactam is released (Figure 7).

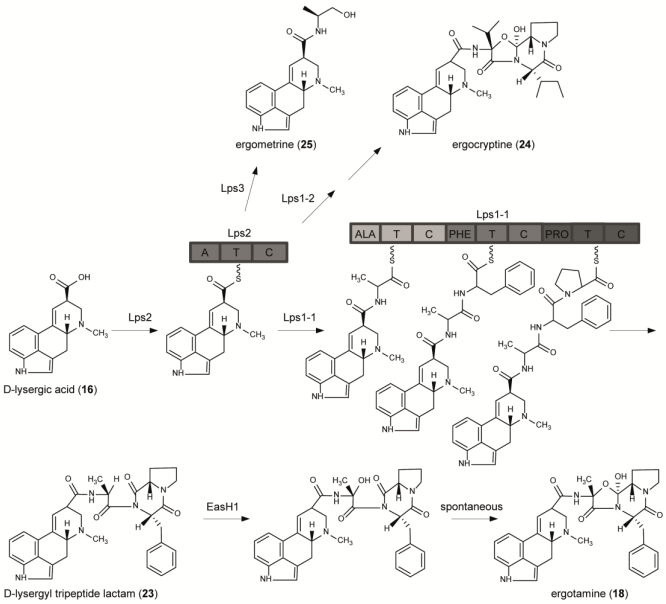


**Figure 7.** Biosynthesys patway from Lysergic Acid to Ergoamides and Ergopeptines.

*According to Gerhards N. et al., Toxins (Basel). 2014; 6(12): 3281 3295.*

### 2.1.3. Determination of EAs

Monitoring of the presence of EAs in 209 cereal grains (wheat, barley, oats and rey) was conducted by UK Food Standard Agency. A useful strategy with silico modeling was undertaken to support hazard identification at an early stage (20). The occurrence of EAs in cereals and cereal products in Europe was investigated in 2014 (21). A total of 1,065 samples of cereals and cereal products intended for human consumption and animal feeding were analysed by liquid chromatography-tandem mass spectrometry for the presence of EAs. The samples included rye, wheat and multigrain-based food as well as rye, wheat and triticale-based feed. The study revealed that 59% of the analysed food and feed samples were contaminated with EAs to some extent. In 55% of the samples, the levels of the -ine isomers were above the limit of quantification (LOQ), while contamination with the -inine isomers was found in 51% of the samples. The mean values for the main EAs (-ine forms) and the epimers (-inine forms) were 1 and 2 µg/kg, respectively. EAs were present in 84% of rye food, 67% of wheat food, 48% of multigrain food, 52% of rye feed, 27% of wheat feed, and 44% of triticale feed at total alkaloid levels ranging from ≤1 (LOQ) to 12,340 µg/kg.
The feed samples, in particular Swiss rye feed, accounted for the highest levels of EAs. The

frequencies and levels of contamination were significantly lower in organic samples compared to conventional samples. Maximum levels of individual ergot alkaloids up to 3,270 µg/kg (for ergotamine) were observed. Overall, ergosine, ergokryptine and ergocristine were the frequently occurring EAs. The co-occurrence of all six EAs was noted in 35% of the positive samples. Occurrence of a single EA was mainly observed for ergometrine.

The European Union Reference Laboratory for Mycotoxins organised a proficiency test on the determination of EAs in cereals. The aim of this study was to evaluate the proficiency of the European National Reference Laboratories (NRLs) and Official Food Control Laboratories (OCLs) on the determination of EAs which were identified as priority by the European Food Safety Authority (EFSA) and for which maximum levels can be expected in the near future. EU Commission one test item planed to provide consisting of a naturally contaminated rye product. Participants would be asked to analyse the 6 EAs (and the corresponding -inine epimers) mentioned in the Commission Recommendation 2012/154/EU (22). EFSA has determined that new validated methods are still required to quantify EAs in feed materials to provide more reliable regulatory limits for each individual alkaloid in food and feed (23).

Ergot can be detected upon visual detection with dark sclerotia bodies being up to 10 times larger than grain kernels. Ergot bodies may range in size from a few millimeters to more than 4 cm depending on the size of the host plant (24). Sclerotia bodies can be smaller increasing the degree of difficulty in detecting them within grain screenings (25). The visible sclerotia method whould help in the development of a method suitable for use by official control laboratories by the EU Commission (22).

Different analytical methods were used for analyzing the EAs. Thin layer chromatography was used as valuable method to identify a compound of interest in a mixture, with particular interest in developing countries (24, 26, 27). Liquid chromatography (LC) is often used in combination with mass spectrometric detection (MS) for analysis of ergot alkaloids (24, 27). This method was used for identidfication and characterization of unknown ergot derivatives (28). The high-performance-liquid chromatography (HPLC) was applied for detection of various alkaloids including ergometrine, ergotamine, ergocornine, ergocryptine, ergocrystine, ergosine and their isomers (29). The concentrations detected with this method were as low as 0.02-1.2 µg/kg with epimerization using multi-analite LC-MS/MS (24), and as low as 0.17-2.78 µg/kg without epimerization (30). Enzyme-linked-immunosorbent assay (ELISA) technique has been applied for ergot screening in crops (31) showing difficulties in standard for identifying contamination with EAs (32). The speed of detection and possibility of analysing both small and large quantities of feeds were the advantages of the method of near infra red spectroscopy (NIR) relative to other methods for analysis of EAs, although it is very dependent on an accurate calibration (27).

### 2.1.4. Pharmacological activities of EAs

EAs can also act as potent drugs due to their strong interactions with dopamine, serotonin and adrenergic receptors of the central nervous system, as well as with adrenergic receptors in blood vessels. Several EAs have pharmaceutical applications such as methylergometrine in gynecology to stop bleeding after childbirth, ergotamine to treat migraines, and the semi-synthetic derivative bromocriptine to treat Parkinson's disease (5, 15, 16, 33). The pharmacological application is based on the structural similarity of EAs alkaloids with the three neuro transmitters.

Due to the biotechnology development of EAs efficient parasitic and submerse production processes have been developed and the biochemistry of the pathway and the physiology of production have been worked out in detail (34). EAs were the first antimigraine drugs available (35). Possible therapeutic potential of these alkaloids as antischistosomals and the action of PZQ as an ergomimetic was also investigated (33). Recent progress in EAs research and pharmacological activities and possible mechanisms of action of some EAs was reviewed (36).

The elucidation of the ergot alkaloid-biosynthesis pathway is of interest especially because of the broad range of pharmaceutical uses. With increased knowledge about the genes and enzymes, molecular genetic application may be used to improve industrial production of medically important EAs, and novel forms that could act as drugs with new or improved pharmacological activities.

### 2.1.5. Regulations of EAs

The occurrence of EAs contamination in North America and Europe has been increase in recent years. These toxins are well known for their effects on the circulatory and nervous systems. The effect of EAs on the liver and on the intestine was investigated using the pig both as a target species and as a non-rodent model for human (37). It was established a lowest observed adverse effect level (LOAEL) of 100 µg/kg body weight (bw) per day, lower than the

benchmark-standard, standard reference a dose limit (BMDL) retained by EFSA to set the tolerable daily intake. It was also suggested that regulatory limit should be revised.
Draft Annex was proposed by Codex Alimentarius Commission on prevention and reduction of contamination by ergot and EAs in cereal grains (Annex to the code of practice for the prevention and reduction of mycotoxin contamination in cereals). Practices based on good agricultural practices (GAP) and good manufacturing practices (GMP) were recommended (38).
As in the EU no regulatory limits appled to EAs in grain or processed products, the EU Scientific Panel on Contaminants in the Food Chain (CONTAM) of the EFSA has reviewed the ergot issue (39). EFSA concluded that the alkaloid concentrations are very variable and a consistent relationship between the amount of sclerotia and the total ergot alkaloid (ergoline) concentration cannot be established. Because of that, it was developed a validated analytical method (HPLC/MS) covering the major EAs found in grains, what would enable further research on the ergot problem (40).
The CONTAM Panel of EFSA adopted an opinion on EAs in food and feed. It established a group acute reference dose of 1 µg/kg bw, and a group tolerable daily intake of 0,6 µg/kg bw. Commission regulation (EU) 2015/1940 of 28 October 2015 amended Regulation (EC) No 1881/2006 as regards maximum levels of ergot sclerotia in certain unprocessed cereals and cereal-based products (41), (Table 2). Scientific committee of the Belgian Federal Agency for the Safety of the Food Chain (FAVV) in 2012 published the proposed action limits for EAs in cereal grains and cereal-based food (Table 3).

## 2.2. Tropane alkaloids (TAs)

TAs are a class of bicyclic alkaloids that occur naturally in many members of the plant family Solanaceae. Some TAs have pharmacological properties and can act as anticholinergics or stimulants. Over 200 compounds in this class are known occurring primarily as metabolites produced by members of the Solanaceae family comprising over 100 genera and 3000 plant species (42, 43). The use of medicinal plants containing TAs dates back to ancient times and notes of *Atropa belladonna* was documented in Papyrus Ebers in 1800 BC. First tropane esters, l-hyoscyamine and its more stable racemate atropine (dl-hyoscyamine) were isolated and structurally characterized in 1833 (2) whereas scopolamine (hyoscine) was identified almost 60 years later. Commercial production of TAs is based entirely on their isolation from plant material. Chemical synthesis has not shown to be economically feasible.
The most studied TAs are (-)-hyoscyamine and (-)-scopolamine. Atropine is the racemic mixture of (-)-hyoscyamine and (+)-hyoscyamine ennantiomer of which only the (-)-hyoscyamine enantiomer exhibits anticholinergic activity. The total alkaloid content determined varies from 0.01-3% dry weight depending on the species and plant organs, whereas by plant breeding was obtained up to 5%. Hyoscyamine is a major alkaloid in most of the species and scopolamine is the predominant alkaloid only in *Duboisia*. Some *Duboisia* species produce besides tropanes also nicotine alkaloids which share part of the same biosynthetic pathway.

### 2.2.1. Chemistry of TAs

TAs contain a tropane ring in their chemical structure where nitrogen bridge is between C-1 and C-5; and there are two asymmetric carbons, but tropane is optically inactive due to symmetry (Figure 8). TAs are commonly substituted at $R_3$ with an ether bridge as seen in scopolamine, atropine, hyoscyamine, and cocaine. The basic chemical structure of TAs is presented on Figure 8.
Chemically TAs are esters of hydroxytropanes (α-tropanol, α-tropane diol, or α-tropane triol) with short chain acids such as acetic acid, propanonic acid, isobutyric acid, isovaleric acid, 2-methylbutyric acid, tiglic acid, (+)-α-hydroxy-β-phenylpropionic acid, tropic acid and atropic acid (44, 45).
TAs are commonly substituted at $R_3$ with an ether bridge as seen in scopolamine, atropine, hyoscyamine, and cocaine (not presenr here), (Figures 9-11).

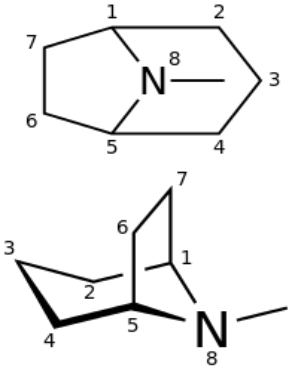


**Figure.8.** Basic chemical structure of tropane alkaloids
Tropane $C_8H_{15}N$

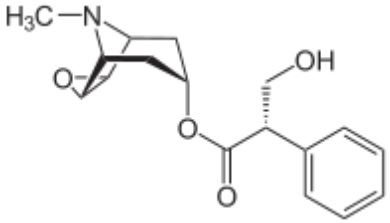


**Figure.9.** Chemical structure of Hyoscine-Scopoloscine, $C_{17}H_{23}NO_3$

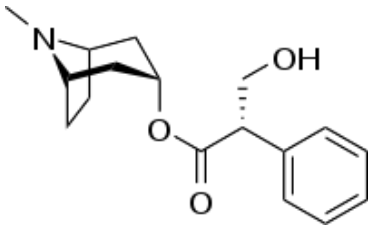


**Figure.10.** Chemical structure of Hyoscyamine, $C_{17}H_{21}NO_4$

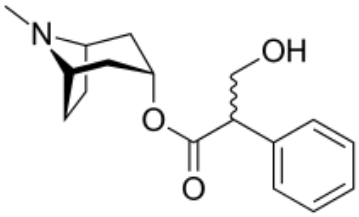


**Figure.11**. Chemical structure of Atropine, $C_{17}H_{23}NO_3$

### 2.2.2. Determination of TAs

TAs can contaminate cereals and cereal-based foods. A total of 1709 samples of plant-derived food products mainly produced in Europe were analysed for TAs in the frame of the project GP/EFSA/CONTAM/2014/01, which was carried out in accordance with the Article 36 of Regulation (EC) No 178/2002, designed to obtain data on the occurance of TAs in Europe, using validated state-of-the-art-analyrical methods. Liquid chromatograpgy coupled to tandem mass spectrofotometry (LC/MS/MS) was applied for analyse of the samples. The products were from organic and conventional production. (44). The limits of quantification for the cereal-based products were 0.5-5 µg/kg. One or more TAs were detected in 21.3% of single component flours, 20.0% of cereal-based foods for young children (age 6-36 months), 6.8% of breakfast cereals, 14.6% of biscuits and pastry and 15.8% of bread. No TAs were detected in pasta. The highest mean TA concentration was detected in cereal-based meals for children of 130.7 µg/kg.

Interlaboratory comparison on determination of TAs (atropine and scopolamine) in cereals, cereal products, herbal infusions and tea was also perfomed. The European Union Reference Laboratory for Mycotoxins organises a proficiency test on the determination of tropane alkaloids (atropine and scopolamine) in cereals and cereal products as well as in herbal infusions and tea (46). For risk assessment purposes, EFSA derived an acute reference dose (ARfD) of 0,016 µg/kg bw for the sum of (-)-hyosciamine and (-)- scopolamine (group ARfD) (47). In risk assessment, the determined levels of TAs in food are considered in relation to the acute reference dose. The CONTAM Panel concluded that, based on the limited information available, the dietary exposure of toddlers could exceed significantly the group ARfD.

EFSA's CONTAM Panel assessed the risk for public and animal health related to TAs in food and feed in 2013 (45). Commission Regulation (EU) 2016/239) set a maximum level (ML) for presence of TAs in certain cereal-based foods for infants and young children (48). The regulation mentions those foods containing millet, sorghum, buckwheat or their derived products. The ML is set at 1 µg/kg for atropine and 1 µg/kg for scopolamine (Table 4 in 2.2.5.). An ML for the sum of atropine and scopolamine was not given.

Spectroscopic methods (UV) have shown that are of limited value since the TAs considered in this opinion show rather low absorption values at rather unspecific wavelengths (49).

The scientific committee of the Belgian Federal Agency for the Safety of the Food Chain (FAVV) has published in 2017 action limits for TAs in certain foodstuffs, animal products and feed besides for ochratoxin A and EAs (50), (Table 5 in 2.2.5.).

### 2.2.3. Biosynthetic Pathways of TAs

As with other secondary products, the biosynthesis of TAs in plants has been extensively investigated by the use of labelled precursors (2). Advances in the techniques of alkaloid analysis and enzyme purification, have made it possible to isolate and characterize biosynthetic enzymes in the pathway (51, 52).

The researchers considered that the important characteristics of alkaloid biosynthetic enzymes should be catalytic properties, regulation, intracellular localization, and tissue distribution. Much progress in the understanding of the nature of the enzymes that synthesize alkaloids in plants has been made after 1979. The available tools for

studying the regulation of enzyme biosynthesis as well as the cellular and subcellular localization have been sophisticated with application of molecular genetic methods what makes it possible to identify biosynthetic enzymes. Successful crystallization of enzymes of alkaloid biosynthesis was reported (53). Especially, the first alkaloid biosynthetic enzyme for which a crystal structure has been determined was also the first one for which a cDNA was isolated, strictosidine synthase from *R. serpentine* (54). Regulation of the metabolic alkaloid pathways at the gene and enzyme level is complex, and there is still much to be achieved about metabolite levels and pathway interconnections (55).
Biosynthetic patways of TAs are presented on Figures 12-14.

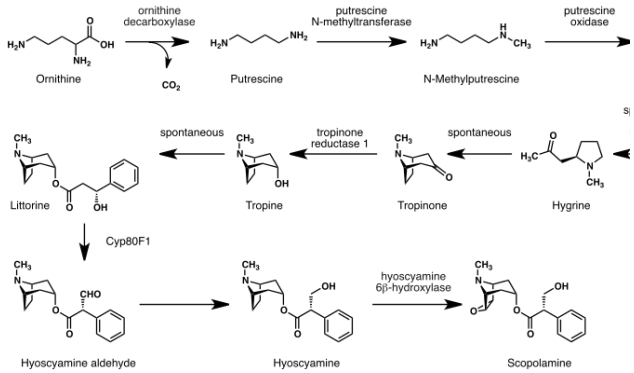


**Figure 12.** Biosynthesis of scopolamine.
*Adapted from Ziegler J, Facchini PJ, Annual Review of Plant Biology 2008;59 (1):735-69.*

The biosynthesis of scopolamine (hyoscine) (Figure 12) begins with the decarboxylation of L-ornithine to putrescine by ornithine decarboxylase. Putrescine is methylated to N-methylputrescine by putrescine N-methyltransferase. A putrescine oxidase that specifically recognizes methylated putrescine catalyzes the deamination of this compound to 4-methylaminobutanal, which then undergoes a spontaneous ring formation to N-methyl-pyrrolium cation. In the next step, the pyrrolium cation condenses with acetoacetic acid yielding hygrine. No enzymatic activity could be demonstrated to catalyze this reaction. Hygrine further rearranges to tropinone. Subsequently, tropinone reductase I converts tropinone to tropine which condenses with phenylalanine-derived phenyllactate to littorine. A cytochrome P450 classified as Cyp80F1 oxidizes and rearranges littorine to hyoscyamine aldehyde. In the final step hyoscyamine undergoes epoxidation catalyzed by 6 beta-hydroxyhyoscyamine epoxidaseyielding hyoscine.
Biosynthetic patway leading from L-arginine to nicotine, scopolamine, calistegins and cocaine is presented on the Figure 13.

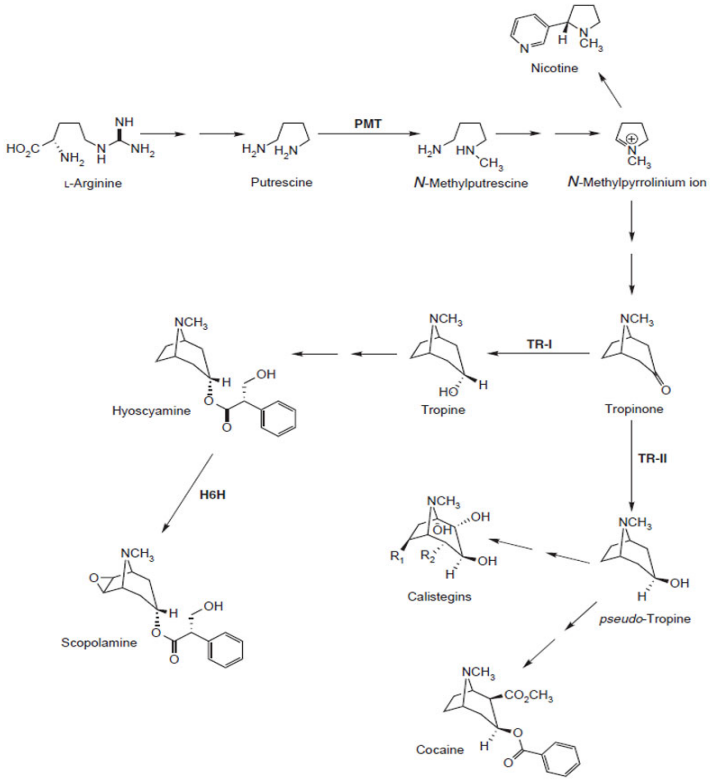


**Figure 13.** Biosynthetic patway leading from L-arginine to nicotine, scopolamine, calistegins and cocaine.
*According to Biocyclopedia: Tropane Alkaloid Biosynthesis, Molecular Biology of Plant Pathways Engineering Plant Alkaloid Biosynthetic Pathways.*

### 2.2.4. Pharmacological activities of TAs

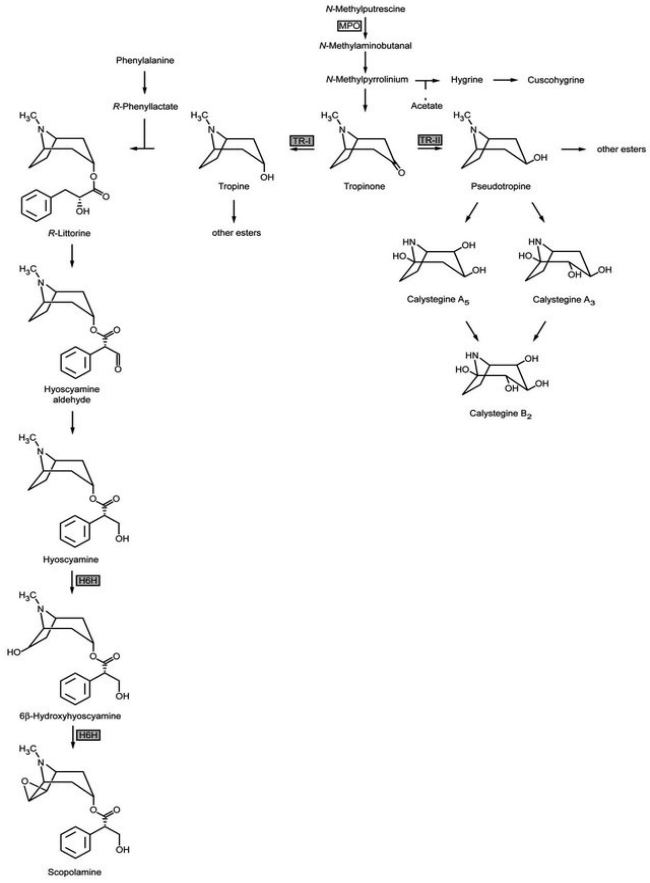


**Figure 14.** Biosynthetic pathway of tropane alkaloids. *According to Oksman-Caldentey K.M ,Current pharmaceutical biotechnology 2007;8(4):203-10.*

TAs are plant toxins (56). Atropine and scopolamine, the best known TAs, are strong antimuscarinic agents. Toxic effects of other TAs are largely unknown. Some of them are used in medicinal products. They affect the heart rate and the central nervous system even at low doses. Among them there are stimulants such as cocaine and cocaine related alkaloids. Thypical symptoms are drowsiness, headeaches and nausea (47). Many plants belonging to the Solanaceae family have been used as a source of pharmaceuticals for centuries because of tropane and nicotine alkaloids. TAs, atropine, hyoscyamine and scopolamine are among the oldest drugs in medicine. The lack of understanding how these compounds are synthesized in a plant cell it does not let their bio-technological production utilizing plant cells as hosts (2). Atropine, hyoscyamine and scopolamine affect the parasympathetic nervous system. They competitively inhibit muscarinic receptors for acetylcholine and act as nonselective muscarinic antagonists, producing peripheral antimuscarinic and central sedative, antiemetic, and amnestic effects. Atropine is applied as antidote for the anticholinesterases, such as organophosphates. The parasympatholytic scopolamine, being structurally very similar to atropine, has a stronger effect on the central nervous system at low therapeutic doses than atropine and hyoscyamine. Therefore scopolamine is specifically used in conditions requiring decreased parasympathetic activity, primarily for its effect on the eye, gastrointestinal tract, heart, and salivary and bronchial secretion glands, and in special circumstances for a CNS action. Scopolamine is known to be the most effective single agent to prevent motion sickness, and it was the first drug to be made commercially available in a transdermal therapeutic system (TTSpatch) delivering this alkaloid. Although the market volume for TAs is not very high, there are no other classes of compounds that can be substituted for these plant derived drugs and therefore the demand for them will continue. Two effective bronchodilators, the semi-synthetic quaternary muscarinic antagonists ipratropium and tiotropium have been developed and are used extensively in the treatment of chronically obstructed pulmonary disorder (2).

### 2.2.5. Regulations of TAs

The EFSA CONTAM Panel in 2013 adopted an opinion on TAs in food and feed and assessed the risk for public and animal health related to TAs in food and feed (45). Commission Regulation (EC) No 1881/2006 (57) set the maximum levels for certain contaminants in foodstuffs (Text with EEA relevance) (OJL 364, 20.12.2006, p.5 amended by Office Journal). Torelable levels for TAs in processed cereal-based foods and baby foods according to Commission regulation (EU) 2015/1940 (41) are presented in Table 4, and proposed action limits for TAs in cereal grains and cereal food according to Scientific committee of the Belgian Federal Agency for the Safety of the Food Chain (FAVV) from 2012 are presented in Table 5.

Commission regulation (EU) 2015/1940 of 28 October 2015 (58) stated: The TAs refered to are atropine and scopolamine. Atropine is the racemic mixture of (-) - hyoscyamine and (+) - hyoscyamine of which only the (- ) - hyoscyamine enantoomere exhibits anticholinergic activity. As for analytical reasonds it is not always possible to distinguish between the enantiomers of hyoscyamine, the maximum levels are established for atropine and scopolamine.

TAs can contaminate cereal-based foods. For risk assessment purposes, EFSA derived an acute reference dose (ARfD) of 0,016 μg/kg body weight (bw) for the sum of (-)- hyosciamine and (-)-scopolamine (group ARfD) (47). In risk assessment, the determined levels of TAs in food are considered in relation to the acute reference dose.

### 2.3. Pyrrolizidine Alkaloids (PAs)

PAs are a group of secondary compounds that are produced by plants all over the world. Over 660 PAs and their corresponding N-oxide derivatives have been identified from more than 6 000 plant species. PAs are most widely distributed natural toxins. Cases of human toxicity caused by the used of toxic plant species as herbal teas or traditional medicines, as well as consumtion of cereal and cereal products (flour and bread) contaminated with PA-containing seeds have been reported (59). The toxicity of PAs is largly documented and is almost associated to their metabolites (60-62).

In animal studies, certain PAs shows hepatoxic, carcinogenic and genotoxic effects. This applies to 1,2 unsaturated PA that is further esterified with at least one branched C5-carboxilic acid. Due to their health-damaging potential, 1,2 unsaturated PAs are undesired in food and feed. Cases of poisoning with these alkaloids are known with animals under the names “walking disease”, “dunziekte”, “Winton disease”, “Schweinsberger disease” and “Zdar disease”. Liver cirrhosis occurred frequently among other effects in slauthering animals (cattle) fed with hay and silage contaminated with alpine ragwort. Cases of illness have also been described in the medical literature in humans who ingested high doses of 1,2 unsaturated PAs, but there are only a few well documented cases. Liver is the most affected organ by PAs. In Pakistan, India and Afghanistan, people were infected after eating cereals that had been contaminated with seeds of the *Heliotropium* and *Crotalaria* spacies. Risk assesment studies were conducted to determine the total sum of 1,2 unsaturated PAs in selected food to estimate the dietary exposure to PAs and to assess the health risk (60).

#### 2.3.1. Chemistry of PAs

PAs are esters composed of necine base and one or more necic acids. The necine base can be either saturated or unsaturated (i.e. contain a double bond in the 1,2 position). Toxic PAs are those which contain unsaturated necine bases whereas the ones with saturated necine bases are considered to be non-toxic (63).

The chemical structure of PAs enables toxification through oxidation to form dehydro pyrrolizidine, which possesses alkylating properties and is therefore potentially genotoxic and carcinogenic. On the Figures 15-18 is presented the structure of PAs.

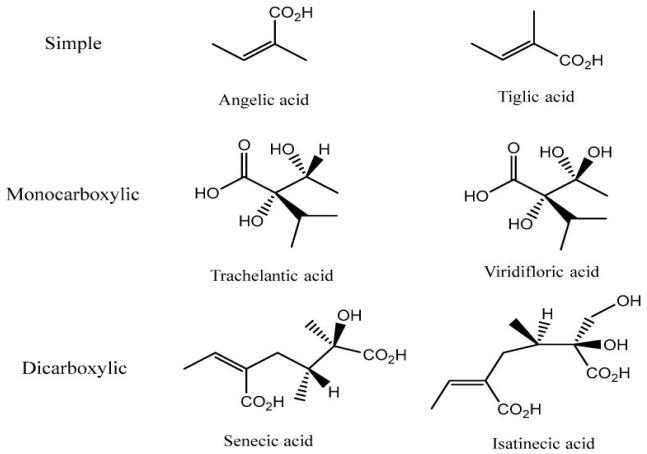


**Figure 15.** Chemical structures of necic acids.
*According to Moreira et al., Int. J. Mol. Sci. 2018;19:1668.*

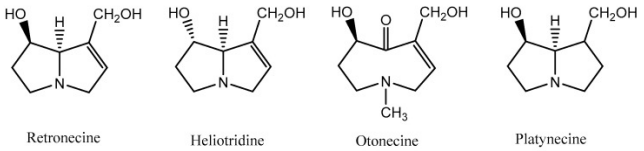


**Figure 16.** Groups of PA according to the necine base.
*According to Moreira et al., Int. J. Mol. Sci. 2018;19:1668.*

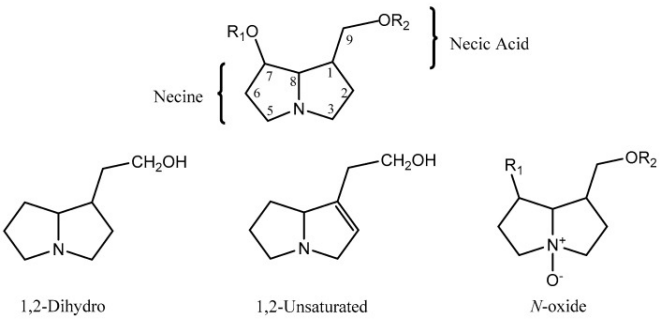


**Figure. 17.** Structure of pyrrolizidine alkaloids and its different forms.R1 and R2 corespond to different necic acids.
*According to Moreira et al., Int. J. Mol. Sci. 2018;19:1668.*

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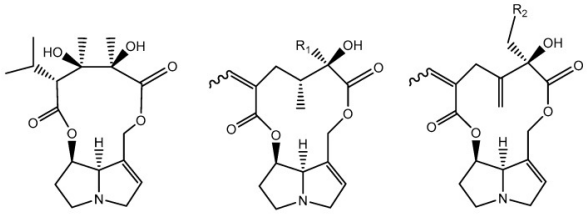

**Figure 18.** Chemical structure of tricodesmine, senecionine (R1= H)/retrorsine (R1= OH) and seneciphylline (R2=H)/riddeline (R2= OH), macrocylic diesters. *According to Moreira et al., Int. J. Mol. Sci. 2018;19:1668.*

### 2.3.2. Determination of PAs

According to the risk assessmet studies, PAs are a class of undesirable compounds in food. Because there is no consensus for in the PAs daily intake limit, quality control of foodstuffs is important and can be also important for establishing legally binding limits. For that purpose, on the request of European Medicines Agency (EMA) to the European Pharmacopeia (64), an appropriate and universal analytical method for determination of PAs was chosen (65).

Analytical detection of 1,2 unsaturated PAs is related to their structural diversity, low concentrations and their presence in a wide range of foodstuffs. Special detection methods were developed by BrF in recent years and validated them in ring trials, which can be used in food and feed monitoring programmes of federal states and by the industry. Additional methods were also developed at the BrF in order the total concentration of 1,2 unsaturated PAs to be estimated, because only a limited number of the occurring 1,2 unsaturated PAs are currently available as a reference standard. This study comprehensively investigated the occurrence of PAs in animal-derived food as well as (herbal) teas and (herbal) food supplements from six European countries. The results revealed that in animal products, PAs were only detectable in trace amounts and only in a few cases. Contamination of different food products like milk, eggs, and meat products with significant levels of PAs seems to be rare in the European Union. This is likely due to a combination of the situation that animal feed is rarely highly contaminated and the fact that metabolic processes in the animals lead to an efficient reduction of the ingested PAs (66).

Many analytical techniques have been applied to detect PAs so far. X-ray analysis and NMR methods are essential and prevalently used in the structural elucidation of purified PAs, while Spectrophotometry (UV) and Thin Layer Chromatography (TLC) are improved for detection of PAs. Quantitative determinations of 1,2-unsaturated PAs was achieved with Nuclear Magnetic Resonance (NMR). Immunological methods are widespread and important laboratory methods for a rapid, selective and sensitive quantitative detection of a complex mixture of different PAs and PA-N oxides (PANOs), including also (HP)LC-MS. Enzyme-linked Immunosorbent Assay (ELISA), Gas Chromatography (GC) and Gas Chromatography-Mass Spectrometry (GC-MS) have been widely used in the determination of PAs. Liquid chromatography (LC), High Performance Liquid Chromatography (HPLC) and multiple variants of Liquid Chromatography-(tandem) Mass Spectrometry (LC-MS/(MS)) are available for separation of PAs and of a simultaneous detection of PAs and PANOs (65).

### 2.3.3. Biosynthetic Pathways of PAs

PAs are typical plant secondary products produced by the plant as a defense against herbivores. PAs were useful for studying the evolution of a biosynthetic pathway in plant secondary metabolism. The molecular, kinetic, and expression data of this system are discussed with respect to current models of gene and pathway evolution (63, 67).

biosynthesis of the Pas. According to the most widely accepted one, biosynthesis patway begins with a $NAD^+$-dependent condensation of two molecules of putrescine. The both theories agree that bacterial homospermidine synthase is able to accept either putrescine and spermidine as a substrate. The reaction is catalyzed by homospermidine synthase and the result is the symmetrical intermediate homospermidine. In continuing homospermidine is cyclized to the corresponding iminium ion, which is reduced and cyclized to trachelanthamidine and isoretronecanole (Figure 19A), (63).

As regard to senecic acid, cyclization of the open-chain monocarboxylic acid diesters takes place. The biosynthesis takes place in the roots, where these compounds are formed as PANO. In continuing they can be easily transported to the aerial parts and be stored in cell vacuoles which is due to their high solubility in water (Figure 19B).

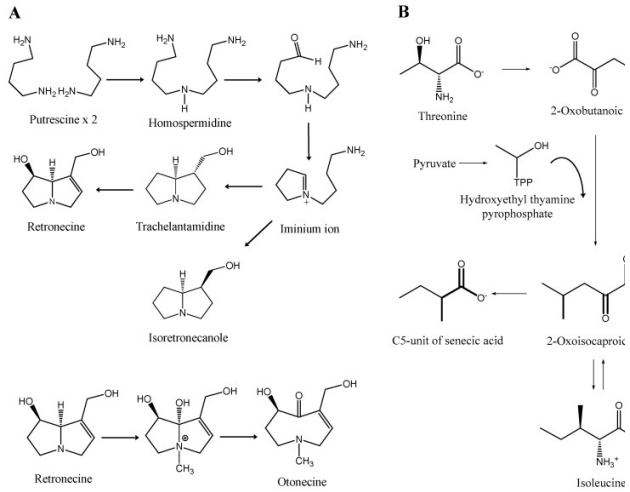


**Figure 19.** Biosynthesis of necines (A) and of senecicacid (B). *According to Moreira et al., Int. J. Mol. Sci. 2018;19:1668.*

### 2.3.4. Pharmacological activities of PAs

Despite the toxicity, PAs exibit biological properties, which can be used in drug application (63). Many alkaloids have been described as effective anti-microbials (64). Anti-microbial activity of usaramine, monocrotaline and azido-retronecine against some bacteria has been demonstrated (68). PAs have also shown to possess anti-inflammatory activity (69, 70), anti-cancer activity (71, 72), and anti-HIV activity (73). Polyhydroxylated PAs have been described as capable of interacting with human immunodeficiency virus (HIV) activity. They also showed enzyme inhibiting activity (acetylcholinesterase inhibitors) (74). A dose of 12.5 mg/kg of some PAs extract was shown to ameliorate nonsteroidal anti-inflammatory drugs-induced gastric ulcer (75, 76).

### 2.3.5 Regulations of PAs

Some countries established regulations about PAs in foodstuff. So, in the USA, the Food and Drug Administration ordered the ban of all PAs containing comfrey preparations from the market (77). EFSA determined that the ingestion of toxic PAs induces hepatic veno-occlusive disease (VOD) and that they have carcinogenic effects in rodents (78). In 2011 EFSA concluded that no tolerable daily intake could be established. It was followed the margin of exposure (MOE) approach, a "ratio of two factors, which essesses for a given population the dose at which a small but measurable adverse effect is first observed and the level of exposure to the substance considered". The MOE defined was of 1:10,000 for an exposure of 7 ng/kg of bw per day (65). The European Medicines Agency, based on toxicological considerations and the available guidelines for assessment/management of genotoxic carcinogens, also showed concern about the hazards of PAs, recommending a maximum daily intake of 0.35 μg/kg PA/day for a person with a body weight of 50 kg and life-long exposure (79). Austria excluded all products with PAs from the market, and in the Netherlands, all foodstuff, herbal preparations, and extracts of plants known to have PAs were limited to 1 μg/kg or 1 μg/L in the final product (80).

The presence of PAs in cereals, wheat grains, as well as in other plants such as herbal teas and medicines, food supplements, vegetable, honey and pollen were reported (81-85). Cases of intoxications by contamination with cereals, teas and salads have been also extensively reported (86, 87).

There are still not legal limit values for 1,2 unsaturated PAs in foods and feeds, but the Codex Alimentarius Commission in 2014 has prepared recommendations for prevent and reduce PA contamination in food and feed in a Code of Practice on "Management of the presence of PA-containing plants" and "Control of plant release and spread" (88). Within the European Union, the general recommendation applies that exposure to genotoxic and carcinogenic substances should be minimized to the lowest value achievable by reasonable means (ALARA principle:as low as reasonably achievable), as even low intake quantities can result in an increased health risk, especially if consumed regularly. The International Program on Chemical Safety (IPCS), the agency of the World Health Organization and Food and Agriculture Organization in the published "Pyrrolizidine Alkaloids Health and Safety Guide" gave statements about the hazards for humans and animals and the conformation that contaminated grain, foodstuff, herbal medicines, beverages and grazing with PAs could cause acute or chronic illness (89).

EFSA delivered scientific opinion on PAs in food and feed (65). The CONTAM Panel concluded that 1,2 unsaturated PAs may act as genotoxic carcinogens in humans and applied the Margin of exposure (MOE) approach. A bench mark dose lower confidence limit for a 10% exess canser risk ($BMDL_{10}$) of 70 μg/kg bw per day for induction of liver haemangiosarcomas by lasiocarpine in male rats was calculated as the reference for comparison with the estimated dietary exposure. The CONTAM Panel concluded that there is a possible health concern for those toddlers and children who are high consumers of honey. Generally there is a low risk of PA poisoning in livestock and companion animals in the EU, due to accidental exposure as PA poisonings reported recently.

Joint FAO/WHO expert Committee on Food Additives and contaminants (JECFA) in 2014 has published a list of substances scheduled for evaluation and request for data based on recommendations of the Codex Committee on Contaminants in Foods (CCCF) (90), previous Expert Committee and direct requests from governments. In Annex 1 about toxicological evaluation and exposure assessment of PAs information required was pointed out: "to identify most relevant PAs for human health-both in terms of occurrence and toxicity, to define the extent to which consumtion of PAs contaminated feed by food producing animal contributes risk to human health, and to identify critical data gaps".

## 3. Conclusion

Alkaloids such as EAs, TAs and PAs are a wide spread group of plant secondary metabolites that can contaminate cereal grains, cereal-based foods and animal feed. These compounds have became known for their toxicity, as per several outbreaks being registered first in developing countries, although localized outbreaks still occur. They are probably the most common natural toxic compounds being a health threat to humans and animals, because of what they are undesirable substances in human food and animal feed. Ergot is the oldest recorded plant disease, and the toxigenic effects of ergot alkaloids have been recognized for many years. These mycotoxins produce symptoms of ergotism in humans and mycotoxicoses in animals. 1,2-unsaturated PAs may act as genotoxic carcinogens in humans. Therefore, it is important to determine the sources of human exposure, assess human health risk posed by these compounds, as well as to reduce exposure to these compounds.

Despite of their toxic effects, many alkaloid containing plants belonging to the Solanaceae family have been used as a source of pharmaceuticals for centuries because of their bioactive compounds, tropane alkaloids. TAs atropine, hyoscyamine and scopolamine, are among the oldest drugs in medicine. Ergot alkaloids were the first antimigraine drugs available. PAs are most widely distributed natural toxins and cases of human toxicity caused by the used of toxic plant species as herbal teas or traditional medicines are documented. Most of these alkaloids have commercial production that is based entirely on their isolation from plant material, and some of them are produced by chemical synthesis.

Several countries worldwide have regulatory restriction on the use of these alkaloid-containing cereal grains and cereal products. Effective intervention strategies on prevention should be developed, in what the understanding of the biotransformations by which alkaloids exert toxic activities would much contribute. Thereby, for the protection of human and animal health from risks of harmful effects of consuming cereal alkaloids, it is of great importance further to develop the chemistry, pharmacology and toxicology of these alkaloids.

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