Chapter 8

Transfer of Pyrrolizidine Alkaloids into Eggs: Food Safety Implications

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The maximum permitted concentration of pyrrolizidine alkaloids (PAs) in herbal medicines on sale in Germany is 0.1 micrograms per daily dose. These regulations provide a basis for assessing the safety of foods contaminated by PAs. PAs occur widely in agricultural production systems throughout the world and can enter the human food chain as contaminants. Grain, milk and honey are among the products that can sometimes be contaminated by PAs. A natural episode of PA poisoning in chickens caused by a lapse in the quality control of the grain component of their feed, has provided evidence that PAs can also be transferred into eggs. The concentrations of PAs detected in the eggs exceeded the levels deemed tolerable for herbal medicines in Germany. Even higher concentrations of PAs have previously been recorded in milk and honey. While there is no evidence of chronic health problems caused by PAs in these products, they and other products, including grain and meat, warrant monitoring for PAs to ensure safe levels of dietary intake are not being exceeded.

In 1992, following a comprehensive risk assessment study, the German Federal Health Bureau established regulations specifying that 0.1 micrograms was the maximum amount of hepatotoxic pyrrolizidine alkaloids (PAs) and their N-oxides allowed in a daily dose of herbal medicines incorporating PA-containing plants or plant extracts (*1*). One microgram per day is allowed if intake is limited to 6 weeks per year. Prescription of herbal medicines containing PAs is prohibited in the case of pregnant and lactating women.

While the harmful effects of PAs have been recognized for many years (*2*) the establishing of these regulations appears to be the first time PAs have been subjected to a formal risk assessment process leading to the determination of a maximum tolerable daily intake for PAs. As well as providing a measure of the safety

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of herbal preparations, the German drug regulations provide a basis on which to judge the risk to public health and safety from the presence of PA contaminants in food.

The term risk as it applies to the determination of safety of chemicals in food reflects not only the toxicity or hazard of the chemicals but also the likely intake of the chemical in a normal diet (*3*). Thus while the hazardous nature of a chemical may be well recognized the actual risk it poses to public health and safety may be negligible if the quantity in a normal diet is below the threshold of toxicity for that substance. Exceptions include genotoxic carcinogens such as aflatoxins and the pyrrolic metabolites of PAs (*1, 3*). Genotoxic carcinogens theoretically have no threshold of toxicity (*1, 3*). A single molecule of a genotoxic carcinogen has the potential to induce a genetic change that may ultimately be fatal. It has been suggested therefore that genotoxic substances in food should be reduced to as low as reasonably achievable (*3*).

While PAs cause acute poisoning when consumed in relatively large amounts, it is their potential to cause chronic, long-term health effects such as cirrhosis and possibly cancer that the German regulators were most concerned with when they established a maximum permitted intake of PAs in herbal preparations (*1*).

The literature contains many examples of poultry being poisoned by PAs in their feed (*4-9*) but eggs have not previously been investigated for PA contamination. A natural outbreak of PA poisoning in layer birds in a small-scale egg production system provided an opportunity to investigate the transfer of PAs into eggs. It also enabled consideration of the food safety implications of any PA contamination that was found in light of the German maximum permitted concentration for PAs in herbal products.

Transfer of PAs into eggs

The poisoning episode. The likely cause of PA poisoning among three flocks of layer chickens was considered to be *Heliotropium europaeum* seeds present in wheat at an estimated concentration of 0.6% by weight. Also present as contaminants in the grain, but in much smaller quantities, were seeds of yellow Burr weed (*Amsinckia* spp.) and Sheepweed (*Buglossoides arvensis*) which are also possible sources of PAs.

The farmer involved was a small-scale egg producer who had purchased grain from a neighbour. As a result the grain was not subject to normal quality assurance testing or cleaning before being incorporated into the feed.

Rather surprisingly, the oldest flock (750 birds) was the first affected after the contaminated feed was introduced. They were taken out of production within 12 days of being given the contaminated feed when their egg production was badly affected. Egg production in the second flock (620, 12 month-old birds) dropped significantly over 4 -5 weeks after the contaminated grain was incorporated into the feed. The third flock of 1179, 22 week-old birds was also badly affected over the same period of exposure to the contaminated feed. After withdrawal of the contaminated wheat, egg production in both remaining flocks continued to fluctuate for several months.

Samples examined and methods of analysis. The contaminated wheat and two lots of eggs, designated A and B, were analyzed for PAs.

The A eggs are believed to have come from the oldest flock, and were collected soon after the first signs of poisoning had been seen. The B eggs were

collected from the remaining flocks after the contaminated wheat had been withdrawn but when the birds were still badly affected.

Standard procedures for alkaloid isolation were followed, including reduction of PA N-oxides to free bases before analysis (*10*). Alkaloids were characterised and identified by fast atom bombardment-mass spectrometry (FAB-MS) and gas chromatography-electron impact mass spectrometry (GC-EIMS) using standard procedures (*10-12*). Samples for GC-EIMS were run both as acetyl and combined acetyl-methylboronate derivatives (*12*).

The content of individual alkaloids in the total alkaloid fraction from each sample was estimated from GC-EIMS peak areas using a standard curve generated with authentic lasiocarpine. The total PA content was obtained by summation of the concentrations of the individual alkaloids.

Analysis of the grain. The FAB spectrum of the total alkaloid extract from the grain (Figure 1) showed $[M+H]^+$ ions corresponding to *H. europaeum* alkaloids: supinine, heleurine, heliotrine, europine and lasiocarpine. Exact mass measurements of the $[M+H]^+$ ions were in good agreement with the expected elemental compositions for these alkaloids.

The three main alkaloids (heliotrine, europine, and lasiocarpine) were also detected by GC-EIMS. Comparison of EI spectra and GC retention times with those of authentic samples confirmed the identity of the alkaloids. The concentration of these alkaloids in the wheat was estimated to be 26 parts per million (ppm, milligrams per kilogram) made up of 6.7 ppm heliotrine, 9.5 ppm europine and 9.8 ppm lasiocarpine.

Analysis of the eggs. The A and B eggs were each divided into two samples and both sub-samples were separately extracted to give extracts A1 and A2 and B1 and B2.

The FAB spectrum of a total alkaloid extract of the A1 eggs is shown in Figure 2. A very similar result was seen with the A2 egg extract. Alkaloids indicated by $[M+H]^+$ ions were: 7-angelyl retronecine, lycopsamine/intermedine, heliotrine, europine, uplandicine, echiumine, echimidine, lasiocarpine and acetyl-echimidine, or isomers of these. Only heliotrine, europine and lasiocarpine are typical of *H. europaeum*. FAB-MS detected only *H. europaeum* alkaloids in the B eggs.

GC-MS retention times and EI mass spectra confirmed the identifications of the principal alkaloids indicated in the FAB spectra, i.e. heliotrine, europine, uplandicine, angelylretronecine, lasiocarpine and acetylechimidine. The total ion chromatogram obtained for the A1 egg total base fraction is shown in Figure 3. Location of the GC peaks of alkaloids indicated by FAB-MS was greatly facilitated against considerable background of co-extractives by selecting fragment ions of m/z 180 and 220 (Figure 3). An intense fragment ion at m/z 180 is characteristic, after acetylation, of lycopsamine, intermedine, heliotrine, europine and uplandicine and an intense ion at m/z 220 is found in the mass spectra of 7-angelylretronecine, echiumine, echimidine, lasiocarpine and acetyl echimidine.

Quantitation of the total alkaloids present in the egg extracts is shown in Table I. The highest levels of PAs were found in the A eggs collected while birds were still being exposed to the contaminated feed. Consumption of a single A or B egg would have resulted in a PA exposure hundreds of time the tolerable daily intake specified by the German regulations.

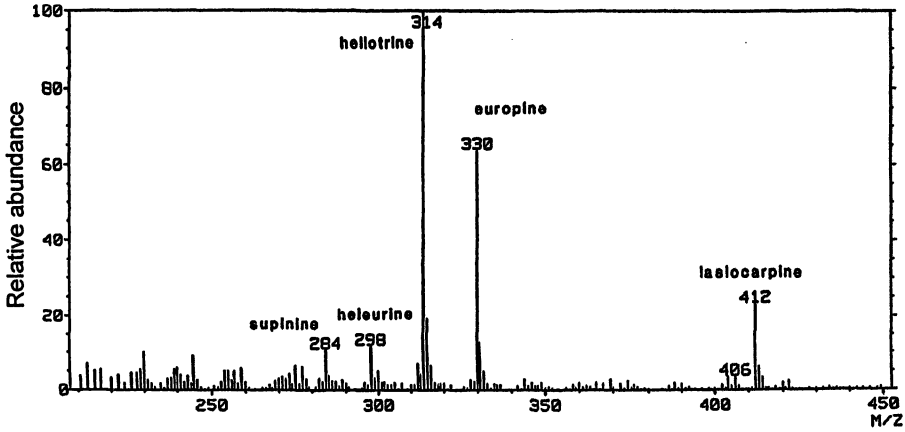

Figure 1. Fast atom bombardment mass spectrum of an alkaloid extract of contaminated wheat considered to be the cause of PA poisoning in chickens, showing the presence of PAs typical of *Heliotropium europaeum viz.* supinine, $[M+H]^+$ m/z 284; heleurine, $[M+H]^+$ m/z 298; heliotrine, $[M+H]^+$ m/z 314; europine, $[M+H]^+$ m/z 330; lasiocarpine, $[M+H]^+$ m/z 412.

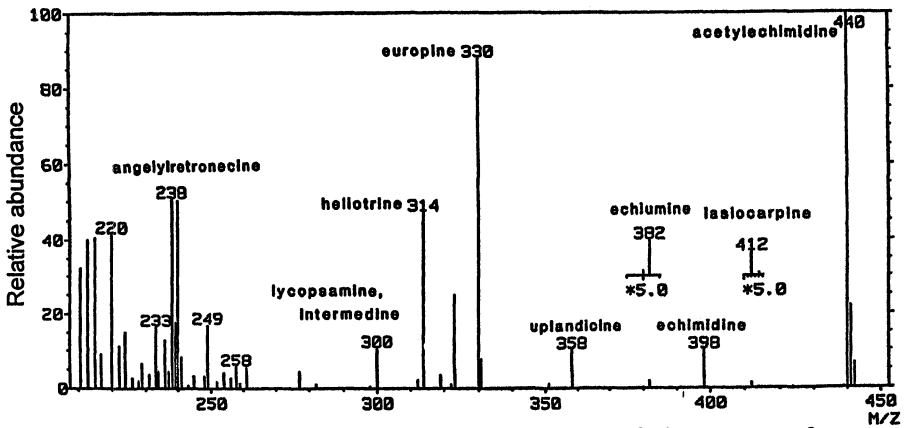

Figure 2. Fast atom bombardment mass spectrum of the extract of eggs collected at an early stage of the poisoning episode. The eggs, designated sub-sample A1, were collected from the oldest birds while they were receiving the suspect feed. PAs indicated by $[M+H]^+$ ions (and confirmed in most cases by GC-EIMS comparison with authentic standards) are: angelylretronecine, $[M+H]^+$ m/z 238; lycopsamine/intermedine, $[M+H]^+$ m/z 300; heliotrine, $[M+H]^+$ m/z 314; europine, $[M+H]^+$ m/z 330; uplandicine, $[M+H]^+$ m/z 358; echiumine, $[M+H]^+$ m/z 382; echimidine, $[M+H]^+$ m/z 398; lasiocarpine, $[M+H]^+$ m/z 412; acetylechimidine, $[M+H]^+$ m/z 440.

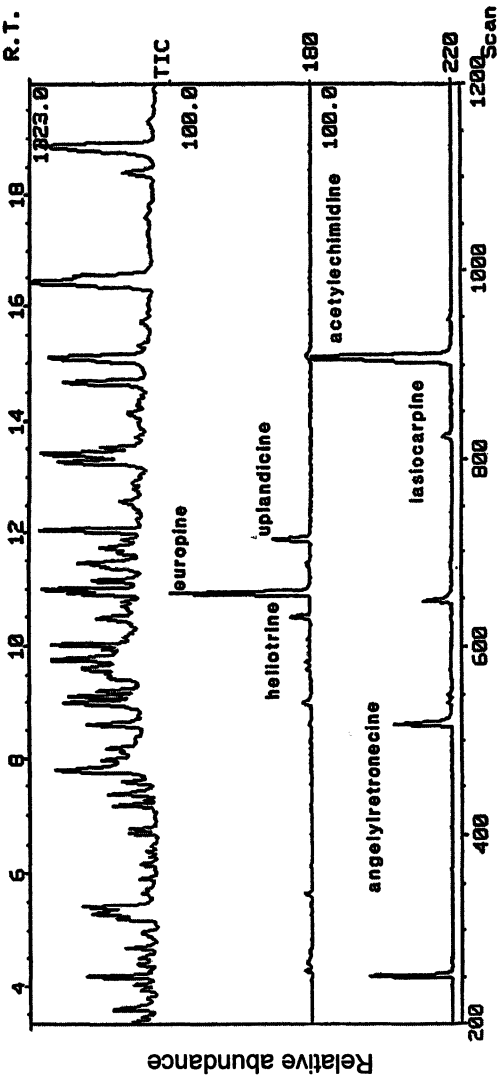

Figure 3. Total (top), m/z 180 (middle) and m/z 220 (bottom) ion chromatograms obtained on GC-EIMS of the extract from sub-sample A1 eggs after acetylation and methylboranate derivatization. The selected ion chromatograms indicate the locations of the principal PAs present in the total ion chromatogram. Comparison of retention times and EI mass spectra with those of authentic standards confirmed the identity of the PAs indicated.

Table I. Quantitation of pyrrolizidine alkaloids (PAs) present in the egg extracts.

Egg sample	*No. of eggs Extracted*	*Wt. of eggs* (g)	*Total PAs* (μg)	*PAs per egg* (μg)[a]	*ppb* (μg/kg)
A1	4	230	38.75	9.7	168
A2	4	227	33.0	8.2	145
B1	4	235	4.6	1.15	19
B2	4	267	13.2	3.3	49

German regulations for herbal preparations permit a maximum daily intake of PAs of 0.1 microgram (0.1μg).

While the expected *H. europaeum* alkaloids are present in both A and B eggs the A eggs also contained PAs normally found, albeit in different proportions, in *Echium plantagineum* (Paterson's curse, Salvation Jane) (*12, 13*) and similar to those in related Boraginaceae such as *Amsinckia* spp. (*14*) and *Symphytum* spp. (Comfrey) (*10*).

The FAB-MS spectrum shown in Figure 4 is typical of that produced by a total alkaloid extract of *E. plantagineum*, the seeds of which are known to occasionally contaminate grain, and this is considered the most likely source of the additional alkaloids seen in the A eggs. Similar alkaloids may occur in *Buglossoides arvensis*, seeds of which were found as contaminants in the wheat. *Amsinckia* spp., the seeds of which were also present, have been shown to contain intermedine, lycopsamine, sincamidine and echiumine but no echimidine (*14*). These two PA-containing plants cannot therefore be ruled out as possible sources of some of the unexpected PAs detected in the A eggs.

The high proportion of acetylechimidine, the very low level of lycopsamine and intermedine and the absence of their acetyl derivatives, distinguish A egg alkaloids from the typical mixture of alkaloids found in *E. plantagineum*. Despite these differences, *E. plantagineum* seems to be the most likely source of the alkaloids found in the A eggs. The atypical mixture of *Echium* alkaloids found in the eggs may reflect a selective transfer of alkaloids from feed into the eggs. This is to be expected on the basis of significant variation in lipid solubility of the alkaloids concerned and the ease with which 7-acetyllycopsamine and 7-acetylintermedine would be expected to be hydrolysed *in vivo*.

Whatever the source of the unexpected alkaloids, it seems that they were present in the feed in substantial amounts at an early stage of the poisoning episode, probably in feed pre-dating introduction of the *H. europaeum*-contaminated wheat. This would help to explain why the older birds were the first to be affected even though the younger birds could be expected to be more susceptible to poisoning. *Echium* seeds were not found in the wheat sample provided for analysis so that some other component of the diet or an earlier grain source is implicated.

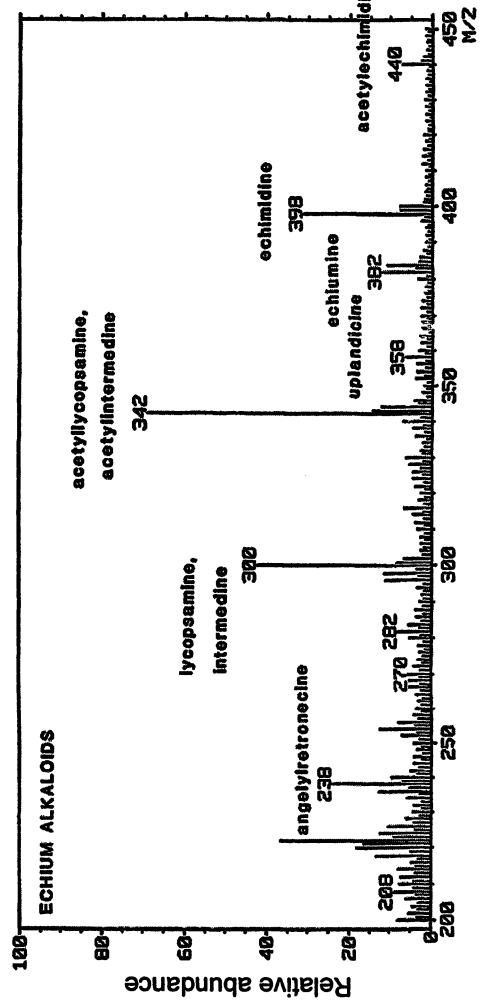

Figure 4. A typical FAB-MS spectrum obtained from a total alkaloid extract of *Echium plantaginaeum*. $[M+H]^+$ ions corresponding to the PAs present in *E. plantaginaeum* are: angelylretronecine, $[M+H]^+$ m/z 238; lycopsamine, $[M+H]^+$ m/z 300; intermedine, $[M+H]^+$ m/z 300; acetyllycopsamine, $[M+H]^+$ m/z 342; acetylintermedine, $[M+H]^+$ m/z 342; uplandicine, $[M+H]^+$ m/z 358; echiumine, $[M+H]^+$ m/z 382; echimidine, $[M+H]^+$ m/z 398; acetylechimidine, $[M+H]^+$ m/z 440.

Food safety and pyrrolizidine alkaloids

It is unusual for PA-containing plants to be intentionally used as foods. Examples of such limited use include Comfrey (*Symphytum* spp.) that is sometimes eaten in salads (*10*) and Borage (*Borago officinalis*) which is occasionally used as a cucumber-flavored garnish (*15*).

The principal route of human exposure to PAs in food is via contaminated staple foods (*2*). There have, for example, been a number of cases in which very large numbers of people have been acutely poisoned by PA contamination of grain (*16-26*).

It should be noted however that most of the recorded cases of acute PA poisoning from contaminated grain have involved people in economically disadvantaged countries and under socially stressful circumstances or where a local source of a contaminated grain, not subject to quality assurance, has been consumed.

Most economically advanced countries in which PA-containing plants are known to be present in agricultural systems have regulations or marketing standards for grain contamination that are likely to prevent acute PA poisoning (*27*). In addition grain for human consumption is normally freed of visible contamination by PA-containing seeds before milling. However the adequacy of the existing quality assurance measures and grain cleaning to prevent chronic effects of PAs is open to debate in light of the German risk assessment outcome and the genotoxic nature of PA metabolites.

In the case of grain, quality assurance usually takes the form of a tolerance for a certain number of PA-containing seeds per volume or weight of grain (*27, 4*). While counting seeds as a measure of contamination may prevent acute PA poisoning it is inadequate for preventing levels of contamination that could, according to the German risk assessment, lead to chronic effects such as cirrhosis and possibly cancer. As an example it has been calculated that the 0.1% tolerance for *H. lasiocarpum* seeds in stored grain allowed in the former USSR could result in 1820 micrograms of PAs per kg of grain (*27*). The regulations, guidelines and marketing standards set by other countries, if based on tolerance of a certain number of PA-containing seeds rather than an actual concentration of PAs, are also likely to result in levels of PAs much higher than would be deemed tolerable according to the German standards for herbal preparations. Indeed the presence of the seeds of PA-containing plants in grain is only a visible sign that such plants were growing in the crop at the time of harvest. The dust from these plants also adheres to the grain and can be another source of PAs (data not shown here). Grain cleaning that normally precedes milling is likely to remove foreign seeds but not PA-containing dust.

PA contamination of other foods. Chronic and acute PA poisoning of food-producing livestock occurs in many countries (*28, 29*), indicating considerable exposure to PAs. There is therefore potential for even low level retention to result in animal products contaminated by PAs in excess of the German standard. PAs have for example been shown to pass into the milk of cows, goats and rats (*30-37*). In the case of cows, experimental exposure to a PA-containing plant, *Senecio jacobaea*, has been reported to lead to levels of PAs between 470 and 835 micrograms per liter of milk (*32*). Honey has also been found to contain PAs (*38-41*). Honey from *Senecio jacobaea* has been shown to contain between 300 and 3200 micrograms of PAs per kilogram (*38, 39*). *Senecio vernalis* honey is reported to contain between 500 and 1000

microgram of PAs per kilogram (*40*) while *E. plantagineum* honey has been shown to contain between 270 and 950 micrograms per kilogram (*41*).

As expected from a consideration of the levels of PAs present in these products relative to the amount required to cause acute toxicity, there have been no confirmed cases of acute human poisoning from these sources. There is however an unpublished report of infants in Egypt showing typical PA liver damage attributed to the consumption of contaminated goats milk (cited in *42*). Consumption of milk and honey, eggs and grain will sometimes result in the ingestion of PAs in excess of the level of 0.1 micrograms PAs per day deemed the maximum permitted by the German regulations and thus could expose consumers to the risk of long-term chronic health effects.

Conclusions

The investigation described here shows that PAs can enter the human food chain in eggs. It adds to previous knowledge that grain, milk and honey are items of food that can be contaminated by PAs at levels sometimes considerably in excess of that specified as tolerable in German regulations for herbal medicines.

In countries where effective quality assurance standards are in place, the level of PAs in food is likely to be very low and may not pose an unacceptable risk to health. However to fully assess the risk that PA-contaminated food poses for public health requires, among other things, determination of the level of these natural toxicants in a normal diet and consensus on what constitutes an acceptable level of exposure.

Given the unpredictable occurrence of low-level dietary exposure to PAs and the likelihood that exposure will differ from country to country and individual to individual, there is a need to develop biochemical methods for quantifying individual human exposure to PAs. Methods for quantifying PA pyrrolic metabolite-DNA or protein adducts in tissues could provide such a measure. This approach has been used to measure exposure to other genotoxic carcinogens in the environment such as polycyclic aromatic hydrocarbons and aflatoxins, the metabolites of which also produce DNA adducts *in vivo* (*43, 44*).

Long-term monitoring of populations that have been acutely poisoned by PAs in food, such as the victims of the recent Tadjikistan poisoning (*25, 26*) and their contemporaries, could also contribute to defining the risk to human health from dietary exposure to PAs.

It should be possible, using these approaches, to establish normal levels of PA exposure in various populations and to confirm or cast doubt on the appropriateness of the maximum tolerable daily intake for PAs specified in the German regulations.

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