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Report of the Scientific Committee of the Spanish Agency for Food Safety and Nutrition (AESAN) concerning the risk associated with the presence of pyrrolizidine alkaloids in pollen intended for human consumption

Section of Food Safety and Nutrition
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Abstract

Pyrrolizidine alkaloids are natural toxins, the product of the secondary metabolism of plants which are produced as a defence mechanism against herbivores. Their chemical structure is based on a pyrrolizidine ring, consisting of two rings fused together by a bridge nitrogen atom. There are approximately 600 known alkaloids, 95 % of which are found in five plant families: Asteraceae, Boraginaceae, Fabaceae, Orchidaceae and Apocynaceae.

The pyrrolizidine alkaloids have a common toxicity profile, the main ones being various degrees of liver damage (centrolobular hepatocellular necrosis) and veno occlusive disease. In addition, the International Agency for Research on Cancer (IARC) has classified them as "possibly carcinogenic to humans" (group 2B). The European Food Safety Authority (EFSA) issued opinions on different aspects of these alkaloids in 2011, 2016 and 2017, and the European Commission has provisionally selected 17 of these as relevant in food.

In the absence of a legal limit which enables the competent inspection authorities to make decisions based on this limit in the event of the detection of these alkaloids in pollen, the Section of Food Safety and Nutrition of the Scientific Committee of the Spanish Agency for Food Safety and Nutrition (AESAN) is asked to conduct an assessment of the risk associated with the presence of pyrrolizidine alkaloids in pollen intended for human consumption to serve as a basis for taking such decisions.

According to currently available data and the estimations made, and considering all the uncertainties identified in the risk assessment process, the intake of pyrrolizidine alkaloids through the consumption of pollen may lead to chronic risk for the Spanish population. However, the appearance of acute risks is not considered likely. In order to conduct a more realistic risk assessment, studies of pollen consumption among the Spanish population are recommended. Similarly, the establishment of legal limits for pyrrolizidine alkaloid content is considered to be appropriate in order to limit the risk of exposure.

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Key words

Alkaloids, pyrrolizidine, pollen.

1. Introduction

Pyrrolizidine alkaloids (PAs) are large group of natural toxins, a secondary metabolism product that plants produce as a defence mechanism against herbivores. It has been estimated that approximately 6 000 plant species across the world could contain PAs (Dusemund et al., 2018). The botanical distribution of the PAs is limited to various non-related Angiospermae families that primarily belong to the Asteraceae (*Senecio*, *Eupatoria*, *Tussilago* genera), Boraginaceae (*Echium*, *Heliotropium*, *Symphytum*, *Trichodesma*), Fabaceae (*Crotalaria*) families, and some Apocynaceae and Orchidaceae genera. Furthermore, they are found in a smaller number of other kinds of families such as Ranunculaceae, Convolvulaceae, Celastraceae, Proteaceae and Poaceae (Ober and Kaltenegger, 2009).

The chemical structure of these alkaloids is based on a pyrrolizidine ring system consisting of two rings fused together by a bridge nitrogen atom (Figure 1) (AECOSAN, 2018). There are two main groups: the 1.2-unsaturated PAs and the 1.2-saturated PAs, depending on whether or not a double link between ring positions 1 and 2 is present. Approximately 600 different PAs are known to date. However, those 1.2-unsaturated PAs are the ones that present toxicity, primarily due to the fact that these compounds, once inside the body, are transformed into highly reactive pyrroles. These are therefore the focus of the risk assessment.

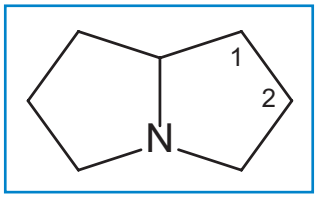

Figure 1. Basic structure of the pyrrolizidine ring system

The pyrrolizidine alkaloids have a common toxicity profile, the main ones being various degrees of liver damage (centrilobular hepatocellular necrosis) and veno-occlusive disease. In addition, the International Agency for Research on Cancer (IARC) has classified them as "possibly carcinogenic to humans" (group 2B) (IARC, 1976).

The European Food Safety Authority (EFSA) issued opinions on different aspects of these alkaloids in 2011, 2016 and 2017, and the European Commission has provisionally selected 17 of these as relevant in food.

In the absence of a legal limit which enables the competent inspection authorities to make decisions based on said limit when they detect these alkaloids in pollen, the Section of Food Safety and Nutrition of the Scientific Committee of the Spanish Agency for Food Safety and Nutrition (AESAN) has been asked to conduct an assessment of the risks associated with the presence of pyrrolizidine alkaloids in pollen intended for human consumption to serve as a basis for said decision making.

2. Identification and characterisation of the hazard

Just as it was described above, the PAs are natural substances produced by different plant species. In fact, these produce PA mixtures that can be present both as their free base or as N-oxides. The latter, once ingested may rapidly reduce in the gastrointestinal system and, under most conditions, their toxicity *in vivo* is only slightly less than the free base (Stegelmeier et al., 2016).

Regarding their toxicokinetics, they are absorbed rapidly in the gastrointestinal tract. This absorption is very high, as demonstrated by studies done on rats, in which they found that following the administration of an oral dose of riddelliine, 100 % of the administered alkaloid was absorbed in the gastrointestinal tract (EFSA, 2011). Once these compounds are incorporated in the body, they are transformed by three pathways primarily, hydrolysis, N-oxidation, and dehydrogenation of the pyrrolizidine by the cytochrome P450 system, with the latter producing highly reactive pyrroles. The rate of formation of pyrrolic metabolites is influenced by the induction or inhibition of the mixed-function oxidases in the liver, but the relationship between the rate of metabolism and expression of toxicity is uncertain (IPCS INCHEM, 1988). The elimination of this kind of compound is very quick, urine being the primary excretion route (EFSA, 2011).

The toxicity of PAs is due to the pyrrole derivatives formed by the hepatic mixed-function oxidases. These act as alkylating agents and may react with enzymes and nucleic acids, being responsible for acute and chronic hepatoxicity, genotoxicity and carcinogenicity. They damage the endothelial cells of the central tubular veins of the liver, causing a thickening of their walls and a nonthrombotic obstruction of the hepatic veins, which is known as hepatic veno-occlusive disease or hepatic sinusoidal obstruction syndrome (EFSA, 2017) (Letsyo et al., 2017), which may cause cirrhosis and liver failure. Pyrrole derivatives may also be carcinogens (in fact, they are classified in group 2B of the IARC). This is because they are capable of introducing modifications into the DNA, even becoming mutagens (Stegelmeier et al., 2016).

Regarding toxicological guidance values for PAs, the Panel on Contaminants in the Food Chain (CONTAM) of the EFSA was unable to establish an acute reference dose (ARfD). The limited information available on cases of human poisoning allowed for the identification of the lowest known dose, approximately 2 mg/kg b.w./day (1-3 mg/kg b.w./day) associated with acute/short-term effects (EFSA, 2017). This is based on the case of a 6-month old girl who received a daily dose of approximately 0.8-1.7 mg PAs/kg b.w. during 2 weeks and was diagnosed with hepatic veno-occlusive disease, and the case of a 2-month old boy who was administered a dose of approximately 3 mg/kg b.w. during 4 days with a fatal outcome.

Regarding the chronic effects, the CONTAM Panel of EFSA (2017) updated the reference point (RP) in order to carry out the chronic risk assessment. They selected the $BMDL_{10}$ of 237 µg/kg b.w./day derived from the incidence of hepatic hemangiosarcoma in female rats exposed to riddelliine, in order to carry out the risk assessment of the 1.2-unsaturated PAs, assuming the same potency for all of them. Beforehand, they had been using the $BMDL_{10}$ of 70 µg/kg b.w./day for lasiocarpine. Both PAs are classified among the most toxic. The Panel concluded that the RP change maintains the conservative nature of prior risk assessments.

3. Exposure assessment

3.1 PA content in pollen

Currently, the Commission Regulation (EC) No. 1881/2006 of 19 December 2006 setting maximum levels for certain contaminants in foodstuffs (EU, 2006) and its subsequent modifications, does not establish a maximum limit for PAs.

In the opinions of the EFSA the available data on the PA content in pollen is limited. Thus, the EFSA (2016) collected the following data obtained after the analysis of 41 pollen samples (Table 1):

Table 1. PA content in pollen

Variable	Mean (µg/kg)	90th percentile (µg/kg)	95th percentile (µg/kg)[1]
Lower bound (LB)	235	967	-
Mean (MB)	244	970	-
Upper bound (UB)	253	974	-

[1] Unable to be calculated as the sample number was <60. **Source:** (EFSA, 2016).

Regarding the PA content in pollen in Spain, there is data from a total of 138 samples obtained in the years 2011 (1 sample), 2012 (14), 2013 (20), 2014 (15), 2015 (26), 2016 (43) and 2017 (19). The basic descriptive statistic data is shown below (Table 2):

Table 2. Basic descriptive statistic of the PAs analysed in samples of Spanish pollen

N	138
Mean (µg/kg)	988.55
Median (µg/kg)	583.50
Confidence interval 95 %	742.28-1 234.80
Minimum	0.00
Maximum	12 536.00
Standard deviation	1 476.01
90th percentile	1 890.70
95th percentile	4 051.35

These values refer to the sum of the total of PAs quantified in each sample, in which the number and/or type of PAs analysed may vary.

3.2 Pollen consumption

Pollen is consumed as a food supplement due to its high nutritional value (Cornara et al., 2017) and because of its attributed antifungal, antimicrobial, antiviral, anti-inflammatory, immunostimulant, etc. properties (Komosinska-Vassev et al., 2015). Nevertheless, their current levels of consumption are unknown. In the "EFSA Comprehensive European Food Consumption Database" there are no specific registered data on pollen consumption, but there are for other types of supplements (vitamin supplements, mineral supplements, mixed supplements, miscellaneous or nutraceutical, etc.), the consumption level of which may differ significantly to that of pollen (EFSA, 2018). According to the EFSA (2016), in the consumption database there are only 32 entries included. On the other hand, the ENALIA2 (National Food Survey in the adult, elderly and pregnant populations) survey did not collect pollen consumption data on the Spanish population (AECOSAN, 2017).

According to Komosinska-Vassev et al. (2015) the recommended daily dose of pollen in adults is 20-40 g and in children 7.5-15 g. The treatment time is normally 1-3 months but may be repeated 2-4 times/year.

3.3 Estimate of the "Exposure Dose" or the "Estimated Daily Intake" (EDI)

In 2016, the ESFA published a scientific report on the dietary exposure to PAs in the European population. In this report, in order to estimate the chronic exposure, they used the upper and lower bounds of PA contents found (LB= 235 and UB= 253 µg/kg), obtaining values between 0.7 and 12 ng/kg b.w./day. In order to estimate the acute exposure, they used the data from the 90th percentile (LB-P90= 967 µg/kg, UB-P90= 974 µg/kg) obtaining values of between 2.8 and 44 ng/kg b.w./day. In both cases they used data from the "consumers only" group, in other words, the group with the highest level of exposure.

Although in the report they did not collect the pollen consumption data used to derive the EDI, assuming an adult of 70 kg b.w., this would oscillate between 0.2-3 g of pollen daily. If, in place of these intake data, they used those indicated by Komosinska-Vassev et al. (2015) the results obtained from adults would be higher (Table 3).

Table 3. EDI estimate in terms of the PA content in pollen indicated in EFSA (2016) and the consumption indicated in Komosinska-Vassev et al. (2015)

Exposure	PA content in pollen (LB-UB µg/kg)	Pollen consumption (g/day)	EDI (ng/kg b.w./day)
Chronic	235-253	20 40	67.14-72.30 134.28-144.60
Acute	967-974	20 40	276.30-278.30 552.60-556.60

Keeping in mind the PA content in Spanish pollen and considering a body weight of 70 kg for adults and 12 kg for toddlers (1-3 years), the calculated EDIs are shown in tables 4 and 5. The EFSA (2012)

considers that the exposure assessment in adults and toddlers (1-3 years) in the framework of a risk assessment is sufficient given that it would include the exposure of older children and adolescents, since there would probably be intermediate data between both population groups.

Table 4. EDI estimate for adults (70 kg b.w.) keeping in mind the PA content in Spanish pollen and the consumption calculated from the EFSA (2016)

Exposure	PA content in pollen (µg/kg)	Pollen consumption (g/day)	EDI (ng/kg b.w./day)
Chronic	Mean: 988.55	0.2	2.82
		3.2	45.20
	Median: 583.50	0.2	1.67
		3.2	26.70
Acute	90th percentile: 1 890.70	0.2	5.40
		3.2	86.43
	95th percentile: 4 051.35	0.2	11.60
		3.2	185.20

Table 5. EDI estimate for toddlers (1-3 years) and adults keeping in mind the PA content in Spanish pollen and the consumption noted in Komosinska-Vassev et al. (2015)

Exposure	PA content in pollen (µg/kg)	Pollen consumption (g/day)	EDI (ng/kg b.w./day)
Chronic	Mean: 988.55	7.5 (toddlers)	617.84
		15 (toddlers)	1 235.70
		20 (adults)	282.44
		40 (adults)	564.88
	Median: 583.50	7.5	364.70
		15	729.40
		20	166.71
		40	333.42

Table 5. EDI estimate for toddlers (1-3 years) and adults keeping in mind the PA content in Spanish pollen and the consumption noted in Komosinska-Vassev et al. (2015)

Exposure	PA content in pollen (µg/kg)	Pollen consumption (g/day)	EDI (ng/kg b.w./day)
Acute	90th percentile: 1 890.70	7.5	1 181.70
		15	2 363.40
		20	540.20
		40	1 080.40
	95th percentile: 4 051.35	7.5	2 532.09
		15	5 064.18
		20	1 157.53
		40	2 315.06

4. Risk characterisation

4.1 Acute effects

According to the EFSA (2017), based on the uncertainty of the PA levels and the severity of its effects, the exposure to PA levels 100 times smaller than the acute reference dose (1-3 mg/kg b.w./day) may be associated with acute/short-term risk. Given the estimated EDIs (2,8-44 ng/kg b.w./day), the EFSA considered that the consumption of pollen-based supplements does not pose acute risks to human health. The same could be said even for a consumption of 40 g/day of pollen.

If the data on PA content in Spanish pollen, the corresponding EDIs of tables 4 and 5, and the ARfD of 1-3 mg/kg b.w./day are taken into account, the risk characterisation of acute effects would be the following (Table 6):

Table 6. Characterisation of acute risks for consumption of Spanish pollen with PAs

PA content in pollen (µg/kg)	Pollen consumption (g/day)	EDI (ng/kg b.w./day)	% ARfD (1 mg/kg b.w./day)	% ARfD/100 (10 µg/kg b.w./day)
90th percentile: 1 890.70	0.2	5.40 (adults)	0.00054	0.054
	3.2	86.43 (adults)	0.00864	0.86
	7.5	1 181.70 (children)	0.12	11.82
	15.0	2 363.40 (children)	0.24	23.63
	20.0	540.20 (adults)	0.05	5.40
	40.0	1 080.40 (adults)	0.11	10.80

Table 6. Characterisation of acute risks for consumption of Spanish pollen with PAs

PA content in pollen (µg/kg)	Pollen consumption (g/day)	EDI (ng/kg b.w./day)	% ARfD (1 mg/kg b.w./day)	% ARfD/100 (10 µg/kg b.w./day)
95th percentile: 4 051.35	0.2	11.60 (adults)	0.00116	0.12
	3.2	185.20 (adults)	0.01852	1.85
	7.5	2 532.09 (children)	0.25	25.32
	15.0	5 064.18 (children)	0.50	50.64
	20.0	1 157.53 (adults)	0.11	11.57
	40.0	2 315.06 (adults)	0.23	23.15

In no case does the EDI surpass the ARfD, nor the safety margin considered, therefore the acute exposure to PAs by means of pollen consumption does not constitute a probable risk for acute toxic effects. These results coincide with the prediction of the EFSA (2017).

4.2 Chronic effects

The Scientific Committee of the EFSA concluded that for substances that are both genotoxic and carcinogenic, a margin of exposure (MOE) ≥10 000, based on a $BMDL_{10}$ obtained from an animal study, and taking into account the uncertainties in the interpretation, would be of little concern from a public health perspective (EFSA, 2005). Using the new RP ($BMDLD_{10}$ of 237 µg/kg b.w./day for riddelliine) and calculating the MOE, they concluded that there is a possible concern for human health regarding PA exposure, particularly for frequent and large consumers of herbal teas and infusions. For the consumption of pollen supplements, no conclusions were drawn.

If the value of chronic EDI established in the EFSA (2016) of 0.7-12 ng/kg b.w./day were taken as data, an MOE would be obtained between 338 571.43 and 19 750.00, both >10 000, therefore the presence of pollen would not be a reason for concern. If the calculated EDI is used, taking into account an intake of 20 g/day of pollen, the MOE would oscillate between 3 529.93 and 3 278.00 (i.e., <10 000) and, consequently, there would be health concerns.

However, it must be noted that the EFSA, as has already been mentioned, is carrying out a conservative risk evaluation assuming the $BMDL_{10}$ of riddelliine for all of the PAs present in pollen, which are a mix that may have a lower toxic potency and may or may not contain riddelliine. In this respect, Chen et al. (2017) proposed refining the EDI and MOE data taking into account a relative potency factor applied to PAs. The calculated factors were 1, 0.9, 0.05, 0.23, 0.03 and 0.02 for lasiocarpine, riddelliine, monocrotaline, clivorine, senkirkine and symphytine, respectively. Nevertheless, the EFSA (2017) did not consider it appropriate to use the approach of Chen et al. (2017) and reaffirmed the conservative nature of their risk assessment.

If the data on PA content in Spanish pollen, the corresponding EDIs of tables 4 and 5, and the $BMDL_{10}$ of 237 µg/kg b.w./day for riddelliine are taken into account, the risk characterisation of chronic effects based on the MOE (MOE= $BMDL_{10}$/EDI) would be the following (Table 7):

Table 7. Characterisation of chronic hazards due to consumption of Spanish pollen with PAs

PA content in pollen (µg/kg)	Pollen consumption (g/day)	EDI (ng/kg b.w./day)	Risk? MOE≥ 10 000 No MOE <10 000 Yes
Mean: 988.55	0.2 (adults)	2.82	84 042.53 No
	3.2 (adults)	45.20	5 243.36 Yes
	7.5 (children)	617.84	383.60 Yes
	15 (children)	1 235.70	191.80 Yes
	20 (adults)	282.44	839.12 Yes
	40 (adults)	564.88	419.56 Yes
Median: 583.50	0.2	1.67	141 916.17 No
	3.2	26.70	8 876.40 Yes
	7.5	364.70	649.85 Yes
	15	729.40	324.92 Yes
	20	166.71	1 421.63 Yes
	40	333.42	710.81 Yes

In this case, both for adults and children and considering the possible exposure scenarios raised, in the majority of circumstances there is a probability of chronic risks occurring. Only when an intake of 0.2 g/day of pollen is considered (the lesser value calculated from EFSA (2016) data) is there no probability of chronic risks.

Given that the $BMDL_{10}$ used as RP in assessments of chronic effects is known, and that a value of MOE≥ 10 000 is considered a small health risk, the estimated maximum EDI that would be expected to not cause risks is:

237 µg/kg b.w./day / EDI= 10 000

EDI= 0.0237 µg/kg b.w./day

Assuming an individual of 70 kg b.w., the EDI would be 0.0237 x 70= 1.659 µg PAs/day. In other words, the maximum PA intake that would not prompt chronic risks would be 1.659 µg/day (initially, considering all of the sources of PAs). Considering that PAs exclusively come from pollen, the amount that could be consumed without risk is going to depend on its degree of contamination.

5. Uncertainties associated with the risk assessment process

5.1 In the hazard characterisation

- The EFSA (2017) indicated the need for having more toxicological data regarding the PAs most commonly found in food. In particular, information about the toxicokinetics, metabolic activa-

tion and carcinogenic potency of the PAs which would allow for a substantial refining in the risk assessment.

5.2 In the exposure assessment

With respect to the quality of PA content data:

- The number of pollen samples analysed by the EFSA (2016) is limited (41).
- The variability of PA content data in the samples of Spanish pollen is very high (5.8 % 0-10 µg/kg; 8 % 11-100; 14.5 % 101-200; 18.8 % 201-500; 18.8 % 501-1 000; 25.4 % 1 001-2 000; 6.5 % 2 001-5 000; 2.2 %> 5 001), with values as low as 0 and one sample containing as much as 12 536 µg/kg. All of the data were used.
- The CONTAM Panel of the EFSA identified a list of 17 PAs of importance to be monitored both in food and animal feed (EFSA, 2017). Those PAs are the following: intermedine/licopsamine, intermedine-N-oxide/licopsamine-N-oxide, senecionine/senecivernine, senecionine-N-oxide/senecivernine-N-oxide, seneciphiline, seneciphiline-N-oxide, retrorsine, retrorsine-N-oxide, equimidine, equimidine-N-oxide, lasiocarpine, lasiocarpine-N-oxide and senkirkine. Not all of them were analysed in the samples. What stands out is that among the 17 identified PAs by the EFSA as important, riddelliine was not included, taking into account that the characterisation of chronic risk is based on the $BMDL_{10}$ of this particular PA.
- The detection limit (LOD) of the method used (LC-MS/MS) was not provided, just the quantification limits (LOQ) and these vary for a same PA according to the year the analysis is done.
- Some LOQs are considered unsuitable according to the EFSA, although they correspond to a PA not considered to be among those 17 of special interest.
- The EFSA (2017) recommended the development of more sensitive and selective analytical methods in order to value the presence of PAs in foods and animal feed, thus decreasing uncertainty in the assessment of the exposure.
- Regarding the PA content in Spanish pollen samples, a more conservative approach could be followed (worst case scenario) in which instead of using a value of 0 µg/kg when it cannot be quantified, the LOD of the applied method for that PA in question is used as content (a PA content <LOD does not imply that there none). However, there are no LOD data available.

Regarding the consumption of pollen (EDI estimate):

- Although the PA concentration data in the samples used by the EFSA (2016) to derive the EDI is known, the consumption data used was not specified. In doing the calculations, unrealistic intake data was obtained, based on the recommendations of the manufacturers and the scientific bibliography.
- Real data on pollen consumption in the Spanish population is unavailable, therefore in order to make the calculations the data indicated in the scientific bibliography were used.

5.3 In the risk characterisation

- It is done based on the $BMDL_{10}$ of riddelliine specifically, which does not have to be included in the pollen samples as it is considered to be one of the most toxic PAs, thus overestimating

the risk. In fact, according to the EFSA (2017) for food supplements (plant extracts and pollen-based supplements), the primary contributions to the total PAs generally comes from licopsamine, intermedine and their N-oxides.

- The calculation of the MOE assumes a daily, lifelong consumption of the food in question (pollen), which may overestimate the risk if this is not the consumption pattern followed.

Conclusions of the Scientific Committee

According to the data currently available and the estimate made taking into account all the uncertainties identified in the process of risk assessment, the intake of PAs through pollen consumption could give rise to chronic risks in the Spanish population, unless very low consumption was considered. However, the appearance of acute risks is considered unlikely. In order to conduct a more realistic risk assessment, it would be necessary to have additional data on the toxicological characterisation of the individual PAs, in addition to carrying out studies on the Spanish population's pollen consumption. Similarly, the establishment of legal limits for PA content is considered to be appropriate in order to limit exposure.

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